Investigation of prey ingestion and digestion in the ciliate predator *Tetrahymena pyriformis* and the impact on growth

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Declaration

This dissertation is entirely my own work and has not been submitted in full or in part for the award of a higher degree at any other educational institution.

No sections of this thesis have been published.

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Abstract

This study evaluated the effect of four prey states on ingestion, digestive vacuole (DV) formation, digestion and growth in the ciliate *Tetrahymena pyriformis*. The prey included several strains of live RFP-expressing heterotrophic bacteria (Live), heat-killed-DTAF-stained heterotrophic bacteria (Dead), live autotrophic *Synechococcus* species (Pico) together with fluorescently-labelled microspheres (FLMs). Prey biovolume did not affect ingestion rates over the 5 minute period tested, therefore the effect of the different prey states could be directly compared. Live heterotrophic bacteria were able to quickly upregulate DV formation rate; something not demonstrated by any other prey. All prey, except FLMs, showed controlled vacuole filling over a 20 minute period whereby new DVs contained an equivalent number of prey to those in older vacuoles. Live heterotrophic and autotrophic prey yielded a significantly lower maximum number of prey/vacuole compared to Dead cells, and whereas significant differences in prey/vacuole between bacterial species were discerned for Dead species, no such differences were evident for Live species. It was hypothesised that ingestion of prey is receptor-mediated and that the predominant pathway into the ciliate for Live prey is the lectin-receptor-based route, and that for inert prey is the non-specific-receptor-mediated route, with a yet unidentified route for Dead prey.

Using pulse-chase experiments, the digestion of Live and Dead heterotrophic bacteria were found to be equivalent, i.e. *ca.* 50% of ingested cells within a DV were digested. There was however a very strong relationship (for both prey states) between biovolume of prey in the vacuole and the total biovolume of prey digested in that vacuole implying that larger prey cells might lead to higher ciliate specific growth rates. This was not found to be the case. No relationship between ciliate specific growth rate was discerned with prey biovolume, or even digestion rates, as % prey/vacuole/min over the digestion period (where there is a linear decline in prey due to digestion). The

digestion period (DP) was considerably shorted with Dead cells and it was hypothesised that, due to an incomplete suite of ligands, DVs did not go through the complete sequence of fusion events with lysosomes.

Digestion of prey had little effect on ciliate growth rates. However there was no obvious relationship between growth rate and any parameter tested. This suggests that digestion and growth are too far removed with regards to cellular processes.

In conclusion, the findings suggest that there are two prey recognition systems in *T. pyriformis*, one which recognised prey particles and leads to ingestion, and a second, present in the DV, which recognises the type of prey enclosed within it and leads to digestion behavioural changes.

Chapter 1. Introduction

1.1. Protists

Protists are a group of unicellular eukaryotic organisms that are diverse, both morphologically and physiologically, with cell sizes that are extremely variable and can range from 1 to 5000μm. Some have 'animal-like' behaviours such as motility and predation (heterotrophs) playing an important role in food-web dynamics and nutrient recycling, while others exhibit the fundamental plant characteristic of autotrophism. Protists are ubiquitous worldwide and can be found in even the most physically or chemically extreme habitats, for example the low temperatures of lakes in Antarctica (Vincent and James, 1996, Laybourn-Parry et al., 1997) and in the extremely high temperatures of hot springs (Uyemura, 1936, Dombrowski, 1961).

The classification of protists has previously been problematic due to identification errors, rigid classification criteria and ambiguous characteristics amongst other issues (Adl et al., 2005, 2007). Modern methodologies such as the use of molecular data and phylogenomic approaches have vastly improved the knowledge of the relationships among lineages within the protists (Restrepo et al., 2016). This has greatly improved the classification and provided phylogenetic trees detailing the evolution history of protists (Restrepo et al., 2016).

There are more than 60,000 known protist species, although the estimated potential number of species is much higher (Adl et al., 2007, Hogg, 2013), many of which have been of interest to researchers and have long served as model organisms in laboratories. This is because, amongst other reasons, they are small and inexpensive to cultivate in large numbers, have short generation times, are easily manipulated in experiments and are genetically stable over many generations (Montagnes et al., 2012). In addition, many research interests focus on disease-causing protists that are exclusively parasitic. For example, trypanosomiasis (sleeping sickness) causing

parasitic flagellates *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* (Welburn et al., 2001); amoebic dysentery causing *Entamoeba histolytica* (Gonzalez-Ruiz et al., 1994); and the balantidiasis causing ciliate *Balantidium coli* (Poudyal et al., 2011).

Free-living heterotrophic protists, for ease, can however be divided into three groups based on their mode of motility; amoebae, flagellates and ciliates.

1.1.1 Amoebae

Amoebae have the most diverse cell size and can range from 40µm (e.g. *Vahlkampfia caledonica*) to 5000 µm (e.g. *Pelomyxa palustris*) (Anderson et al., 1997, Chistyakova and Frolov, 2011). They are known for having pseudopodia which are used for locomotion and for feeding by forming digestive vacuoles (DVs) around their prey (Laybourn-Parry, 1984). Even so, pseudopodia of different amoebae can be highly diverse. Depending on whether they have a skeletal structure or not, amoebae are crudely divided into two groups: the naked amoebae and the testate amoebae. Naked amoebae lack any form of exoskeleton whereas testate amoebae retain a shell-like exoskeleton with a single chamber and a single opening. It may comprise many different materials, including calcium, chitin, silicon dioxide and agglutinated materials like small particles of sand (Anderson, 1987).

Amoebae are major grazers of bacterial biofilms due to their limited ability to feed when not attached to a surface (Pickup et al., 2007a). Furthermore, amoebae are able to encourage the formation of biofilms by attaching to surfaces and secreting organic nutrients that bacteria can utilise (Khan and Panjwani, 2000), in turn, creating a perfect feeding ground for themselves. If attached to large biofilm aggregates that have broken off the surface, they would then be able to translocate and feed in more different environments including drinking water (Hoffmann and Michel, 2001), swimming pools (Rivera et al., 1993) and hospital water networks (Rohr et al., 1998). Biofilms are notorious for harbouring pathogenic bacteria (e.g. *Pseudomonas aeruginosa, Vibrio*

cholera and *Legionella pneumophila*) (Hall-Stoodley and Stoodley, 2005, Gómez-Lus et al., 2013) and the presence of amoebae in such water sources suggests the presence of biofilms and therefore a potential risk to human health.

1.1.2 Flagellates

In older classifications, flagellated protozoa were grouped into the plant-like Phytomastigophorea and animal-like Zoomastigophorea. However these groups are known to be highly polyphyletic and members are placed in many different eukaryotic groups. This is because flagellates are a morphologically and physiologically diverse group with a cell size generally ranging from 5 to 20µm (Arndt and Mathes, 1991, Patterson, 1996). They are uninucleate and can be autotrophic (e.g. *Dunaliella* sp.), parasitic (e.g. *Trypanosoma*), symbiotic (e.g. termite gut flagellates) or free-living (e.g. *Cercomonas* sp.) (Schuster and Pollak, 1978, Biddanda and Pomeroy, 1988, Brune, 2014).

Flagellates have at least one, but can have up to eight, flagella for locomotion and food capturing (Sleigh, 1989, Patterson, 1996). The different modes of feeding include filter feeding (e.g. *Monosiga ovata*), direct interception (e.g. *Ochromonas* sp.) and raptorial feeding (e.g. *Rhynchomonas nasuta*) (Boenigk and Arndt, 2000a, 2000b). Flagellates employ different modes of locomotion including gliding and free-swimming forms, although some can be temporarily or permanently attached to a substrate which is a behaviour that has been shown to be advantageous in the feeding process (Christensen-Dalsgaard and Fenchel, 2003).

They appear in great numbers, often exceeding 1000 cells per ml at the surfaces of water columns, and they are major grazers of planktonic bacteria (Patterson et al., 1993), responsible for transferring significant amounts of nutrients from bacteria to higher trophic levels in the food web (Arndt et al., 2000).

1.1.3 Ciliates

Ciliates are the largest (over 7,000 identified species) and most complex group of protists with a large size range of 20-4000µm. Some species (e.g. *Stentor, Spirostomum* and *Bursaria truncatella*) that exceed 1 mm in length can be seen without a microscope (Applewhite and Morowitz, 1966, Corliss, 2013). As the name suggests, ciliates are characterised by their complex cortex covered by either simple cilia or compound ciliary structures. This orderly matrix of cilia is functionally similar to flagella and is involved in locomotion (by beating in coordination) as well as feeding. Ciliate are divided into two feeding groups, filter feeders (e.g. *Tetrahymena, Vorticella*) and diffusion feeders (*Podophrya)*. In the former, cilia and ciliary structures near the cytostome (‘mouth’) are able to create feeding currents that direct prey towards the cytostome where prey can be ingested into DVs (Laybourn-Parry, 1984, Patterson, 1996, Hogg, 2013). Diffusion feeders rely on prey to swim towards them and stick to their feeding tentacles (Finlay and Esteban, 1998). Finally, both types of feeders have a cytoproct where undigested food is egested.

Ciliates show nuclear dimorphism where they possess one macronucleus that governs normal cell function and one or more micronuclei involved in replication of DNA during asexual reproduction (Herrick, 1994). Ciliates typically reproduce asexually by homothetogenic binary fission where the parent cell gives rise to two daughter cells by a fission plane that is transverse to the cell's long axis (i.e. anterior–posterior axis) (Foissner, 2010). However, they also have the ability to reproduce sexually by conjugation or autogamy (i.e. self-fertilization) (Lynn, 2008).

Ciliates have been showed to graze bacteria at quicker rates than flagellates (Sherr et al., 1991). In addition to this, and due to their small size, they (and flagellates) can have similar generation times to their prey. This allows them to adequately control bacterial populations in a range of environments and they are a very important

component of the food web (Berninger et al., 1991, Hobbie, 1994, Sherr and Sherr, 1994, Finlay and Esteban, 1998, Lischke et al., 2016).

1.2 Ecological significance

Protists are ubiquitous in aqueous and terrestrial environments, colonising natural and man-made habitats worldwide (Fenchel, 2013). Compared to multicellular organisms, these single cell organisms can readily evolve physiological and behavioural adaptations to the changing environments and cope with extreme physical, chemical and biological conditions, e.g. in highly polluted water treatment plants (Curds and Cockburn, 1970a, Curds and Cockburn, 1970b, Curds, 1973). They are also suggested to play a role in the decomposition of polluting agents of organic origin, given their role in bacterial grazing and the decomposition process (Ribblett et al., 2005).

The importance of protists in natural environments is due to their role as a link between bacteria and higher predators in the food chain, channelling large amounts of nutrients such as carbon, nitrogen and phosphorus to higher trophic levels through the microbial loop (Azam et al., 1983) (discussed further in Section 1.2.1).

Finally, the parasitic nature of some protists, as well as the ability to allow pathogenic bacteria to harbour within them, has caused significant interest in terms of the health of humans and indeed animals (discussed further in Section 1.2.2).

1.2.1 Microbial loop

Bacterial-grazing heterotrophic protists play a very important role in the microbial loop (Figure 1.1) where 10-50% of carbon is fixed by photosynthetic plankton being utilised by bacteria (Fuhrman and Azam, 1982). Protists prevent nutrients from being trapped at the microbial level and bridge the transfer of energy from prokaryotes up the food web (Azam et al., 1983). In addition, protists contribute to the microbial loop by promoting organic matter decomposition and increased rates of nutrient turnover. Due to a loss of CO_2 during respiration, they actively pump nitrogen and phosphorus out of the cells in the form of NH_4 and PO_4 to maintain a cellular C:N:P ratio of 50:10:1 (Sherr et al., 1983, Fagerbakke et al., 1996, Dolan, 1997). Along with the egestion of carbon

rich spent DVs, protists actively recycle nutrients as dissolved organic matter to the bacterial and phytoplankton communities (Johannes, 1964, Nagata and Kirchman, 1992, Dolan, 1997, Twining and Fisher, 2004).

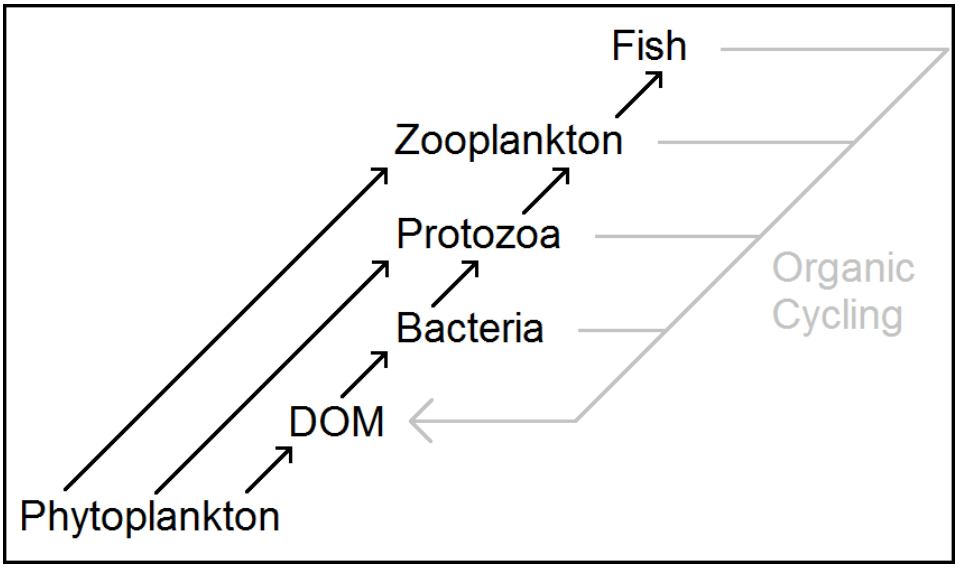


Figure 1.1 An interpretation of the 'microbial loop' described by Azam et al. (1983). Black arrows represent the flow of energy up the food chain. Grey arrows represent the recycling of materials into dissolved organic matter (DOM), which is also secreted by phytoplankton, to be used by bacteria (Azam et al., 1983).

Protists are able to consume large amounts of bacteria, with reports of up to 80% bacterial production clearance (i.e. 80% of bacterial population growth is grazed) (Sherr et al., 1989). Grazing of bacteria and algae keeps them in a sustained growing state with a cell density below their optimum population concentration (carrying capacity). However, there is a lower prey threshold density below which protists cannot feed and this prevents these predators from completely eliminating their prey (Taylor, 1977, Sieburth, 1984, Iriberri et al., 1995).

Selective grazing of bacteria has been observed, where protists graze on preferred prey over others when presented with both (Andersson et al., 1986, Gonzalez et al., 1990b, Epstein and Shiaris, 1992, Šimek and Chrzanowski, 1992, Strom and Loukos, 1998, Thurman et al., 2010b). Even though the reasons as to why they present such

behaviour is unknown, selective grazing can cause profound effects on bacterial communities (Sherr et al., 1989, Strom and Loukos, 1998, Fu et al., 2003, Thurman et al., 2010b, Dopheide et al., 2011a) by changing bacterial morphology (Jürgens and Matz, 2002) and community composition (Hahn and Höfle, 2001, Matz and Jürgens, 2003).

Ciliates are estimated to be 20% of plankton biomass which allows high rates of bacteria grazing and also the potential to be an important food source for larger organisms (Berk et al., 1977). Indeed, in aquatic environments protists (in particular ciliates) are grazed on by rotifers (Arndt, 1993, Gilbert and Jack, 1993) and crustaceans, and are a significant element of their diet (Strachan, 1980). It has also been suggested that some copepods actively select ciliates as their food source and they can comprise *ca.* 30% of their daily carbon intake (Berk et al., 1977, Calbet and Saiz, 2005). In soil, protists are exposed to a number of other predators, including earthworms (Mieen, 1963, Bonkowski and Schaefer, 1997) and nematodes (Anderson et al., 1977, Dash et al., 1980).

1.2.2 Role in pathogenesis

Protists can directly play a significant role in human pathogenesis. For example, the parasitic protist *Plasmodium* causes malaria which is responsible for 438,000 deaths per year, most being children (WHO, 2015). *Trypanosoma brucei* causes sleeping sickness and is one of the most neglected infectious diseases with approximately 70 million people at risk of infection (Simarro et al., 2012).

On the other hand, the indirect role of protists in pathogenesis is also very significant. As mentioned in Section 1.2.1, grazing keeps the bacterial population under control, however some pathogenic bacteria are able survive the protist's digestion process (e.g. *Legionella*, *Mycobacterium*) (Meena, 2010, Medie et al., 2011, Escoll et al., 2013). Because some bacteria have been found to associate with protists (Winiecka-Krusnell and Linder, 2001, Molmeret et al., 2005, Matsuo et al., 2010, Smith et al., 2012)

protists have been termed the ‘Trojan horses’ of the microbial world (Barker and Brown, 1994). Bacteria have been seen to be free-living within the cytoplasm with no surrounding membranes (i.e. not inside DVs), or surviving and dividing within DVs and eventually egested unharmed (Newsome et al., 1985, Abu Kwaik, 1996a, Brieland et al., 1997, Abu Kwaik et al., 1998, Lau and Ashbolt, 2009, Matsuo et al., 2010, Smith et al., 2012, Raghu Nadhanan and Thomas, 2014).

Not only can pathogens such as *Legionella pneumophila* (Brieland et al., 1997, Gao et al., 1997, Escoll et al., 2013) and *Salmonella enterica* (Brandl et al., 2005) exploit protists as hosts, it has been shown that *L. pneumophila* cells gain greater infectivity (Cirillo et al., 1994) and are more resistant to certain biocides and disinfectants after being though the protist’s DVs (Barker et al., 1992). The protists intracellular environment is therefore thought to be a training ground for pathogenic bacteria, i.e. if a bacterium is able to avoid digestion in protists and exploit them as a host, there is a good likelihood of doing the same to macrophages in the immune system. There are similarities in the intracellular infections of macrophages and protists by *L. pneumophila* (Horwitz, 1983, Swanson and Isberg, 1993, Bozue and Johnson, 1996, Abu Kwaik, 1996b, Gao et al., 1997) and therefore the tools necessary for macrophage infection by *L. pneumophila* are thought to have been obtained while they co-evolved with protists (Gao et al., 1997, Molmeret et al., 2005, Escoll et al., 2013). Moreover, protists have the potential to act as a vector by introducing large numbers of *L. pneumophila* into the human body (Molmeret et al., 2005).

On the other hand, the mechanisms of uptake of *L. pneumophila* by macrophages and protists are different. The uptake of *L. pneumophila* by macrophages is triggered by binding to the complement receptors and is mediated by microfilament-dependent phagocytosis (Payne and Horwitz, 1987). The uptake by amoebae is mediated by a microfilament-independent mechanism and is sensitive to methylamine, an inhibitor of receptor-mediated endocytosis (King et al., 1991). The receptors involved seem to vary

between different protists. For example, Harb et al. (1998) showed the ability of *L. pneumophila* to attach and invade different protozoan hosts by adapting different mechanisms, whereby the uptake by *Vermamoeba* (*Hartmannella) vermiformis,* but not by *Acanthamoeba polyphaga,* was dramatically blocked by galactose and N-acetyl-D-galactosamine, and mutants that could not invade *A. polyphaga* could still invade *V. vermiformis*.

1.3 Phagocytosis

Ciliates and other phagocytes ingest prey via a process known as phagocytosis. Phagocytosis is the clathrin-independent and actin-dependent process by which a cell engulfs large extracellular particles to form an intercellular vesicle known as a digestive vacuole (DV) (Cardelli, 2001). This membrane bound compartment has an isolated microenvironment separate to the surrounding cytosol, allowing the contents to be digested by a series of biochemical changes. The phagosome then fuses with the plasma membrane and any undigested material is egested from the cell via exocytosis (Desjardins et al., 1994b).

1.3.1 Phagocytes in the immune system

The ability to internalise particles is a key mechanism of the innate immunity. "Professional phagocytes" (coined by Rabinovitch [1995]), which include macrophages, monocytes and polymorphonuclear granulocytes, are involved with the uptake and degradation of microorganisms, damaged or apoptotic cells, cell debris and pollutants. They are therefore important in fighting infectious agents, inflammation and tissue homeostasis (Rabinovitch, 1995, Aderem and Underhill, 1999, Zhang et al., 2002, Johnsen and Horvitz, 2016).

There has been ongoing interest in how phagocytes are able to identify the nature and location of the pathogen and trigger a targeted immune response. The large combinations of different phagocytes and 'prey' means the process can differ greatly. However, it is possible to present a general sequence of events that are involved.

Initially, contact of the particle with the plasma membrane of the phagocyte provides an opportunity for a ligand-receptor interaction and for the phagocyte to assess the chemical profile/identity of the particle (Rabinovitch, 1995). Next, the dynamic movement of the plasma membrane interacts with the whole surface of the particle,

assessing the physical size and shape. Finally, the particle is engulfed by the phagocyte to form an internal phagosome (Underhill and Goodridge, 2012).

There are two ways professional phagocytes engulf particles: opsonised phagocytoses (see Section 1.3.1.1) and non-opsonised phagocytosis (see Section 1.3.1.2). They also have a variety of receptors, for example opsonic receptors (e.g. Fc receptors) and non-opsonic receptors (e.g. lectin-like recognition molecules such as CD169) (Klaas and Crocker, 2012, Flannagan et al., 2015) (Table 1.1).

Type of phagocytosis	Receptors	Targets
Opsonic phagocytosis	Fc receptors	Antibody-opsonised particles
	Complement receptors	Complement-opsonized particles
	α5β1 integrin	Fibronectin
Non-opsonic phagocytosis	Dectin 1*	β-glucan
	Scavenger receptor A	Bacteria (diverse charged molecules)
	αVβ5 integrin	Apoptotic cells
	Mannose receptors*	Mannose/Mannan

*Table 1.1 Receptors involved in opsonic and non-opsonic phagocytosis in immune cells. Adapted from Underhill and Goodridge (2012). * indicates lectin receptors.*

1.3.1.1 Opsonised phagocytosis

In opsonised phagocytosis, particles are surrounded by and bound to naturally occurring soluble opsonins. The opsonin coating facilitates the attachment and internalization of the particle by 'marking' them, allowing professional phagocytes to indirectly recognise them and initiate phagocytosis (Czop et al., 1978, Hart et al., 2004, van Kessel et al., 2014).

The mechanisms of opsonised internalization of particles are complex and can vary depending on the target and its location. In general, opsonisation refers to coating with either immunoglobulin molecules (i.e. antibodies) that are specific for the antigen present on the surface of the particle, or complement proteins (e.g. C3b) (van Kessel et al., 2014). These two opsonins trigger the Fcγ receptor IIA (FcR) mediated uptake and Complement receptor (CR) mediated uptake, respectively (Table 1.1). FcR

initiates ‘reaching’ phagocytosis while CR initiates ‘sinking’ phagocytosis (Figure 1.2) (Underhill and Goodridge, 2012, Wolf and Underhill, 2014).

FcR-mediated phagocytosis is initiated when a particle coated with antibodies comes into contact with a phagocyte. The close proximity allows the Fc domain of the antibody to bind to the transmembrane surface receptors (i.e. FcR) on the phagocyte plasma membrane, causing FcR cross linking, and triggers a signaling cascade necessary for phagocytosis (Greenberg and Grinstein, 2002, Swanson and Hoppe, 2004). This includes the phosphorylation of tyrosines within the immunoreceptor tyrosine based activation motif of FcR, leading to recruitment of proteins and lipid kinases (Swanson and Hoppe, 2004). After a series of downstream signals, actin filament polymerization extends the membrane outwards (i.e. ‘reaches’) and around the particle to form a phagosome (Caron et al., 2000, May and Machesky, 2001) (Figure 1.2).

Similarly, CR-mediated phagocytosis is initiated when a particle coated with complement comes into contact with a phagocyte. First, the phagocyte forms an actin-rich protrusion pedestal that first pushes the particle away (Lee et al., 2011). Endosomes containing Complement Receptor of the Immunoglobulin superfamily (CRig) are transported to and fuse with the plasma membrane at the site of contact (Stuart and Ezekowitz, 2005). These receptors ensure a strong binding with the particle. After a cascade of activation events (including RhoA GTPase), a different form of phagocytosis from Fc mediated phagocytosis occurs in which the particle ‘sinks’ into the cell (Brown, 1992, Richards and Endres, 2014) (Figure 1.2). Again, the actin cytoskeletal system is vital for the formation of the phagosome (Patel and Harrison, 2008).

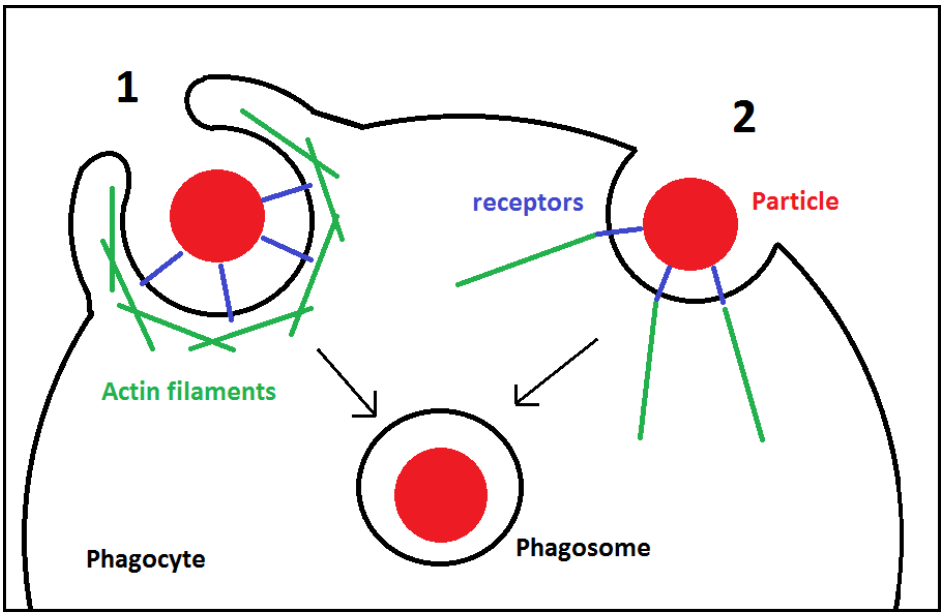


Figure 1.2 Two types of phagocytosis. 1) "Reaching" phagocytosis initiated by Fc receptors, plasma membrane extends outward to engulf the particle. 2) "Sinking" phagocytosis initiated by complement receptors. The particle depresses into the cell membrane. Both types of phagocytosis require an actin cytoskeleton and actin polymerization. Diagram adapted from Underhill and Goodridge (2012).

1.3.1.2 Non-opsonised phagocytosis

The mechanisms involved in non-opsonised phagocytosis are much less understood. As the name suggests, it is the uptake of particles that does not require them to first be coated with certain proteins for recognition. This type of phagocytosis is more akin to that carried out by protists.

Currently there has been no definitive answer as to whether non-opsonised phagocytosis employs "reaching" or "sinking" phagocytosis (Figure 1.2) and indeed there have been observations of both occurring. Gilberti and Knecht (2015) showed that when a macrophage takes in silica particles by non-opsonised phagocytosis, the actin rich protrusions do not extend and surround the particles. Using scanning electron microscope images, Lu et al. (2016) showed that the phagocytosis of non-opsonised latex beads by macrophages did not require the formation of extension of phagocytic cup. Both suggest "sinking" phagocytosis. On the other hand, Gilberti et al.

(2008) showed that macrophages extend pseudopods to capture non-opsonised crystalline silica particles in a “reaching” style phagocytosis.

A few receptors have been suggested to be involved in this non-specific binding phagocytic process (Table 1.1). Firstly, the Dectin-1 receptor is expressed on myeloid phagocytes (including macrophages) and has an important role in defending against fungal infections (Ferwerda et al., 2009, Saijo et al., 2007). It detects β-glucans in fungal cell walls and is thus not involved in the phagocytosis of bacteria (Goodridge et al., 2009, Kerrigan and Brown, 2010). However, another lectin (the mannose receptor) which is expressed abundantly on macrophages (Lasky et al., 1989, Stahl, 1992, Martinez-Pomares, 2012) can and does recognise a variety of bacteria (Shepherd et al., 1984, Saraiva et al., 1987, Ezekowitz et al., 1991, Allavena et al., 2004, Taylor et al., 2005a, 2005b, Zhao et al., 2015).

The scavenger receptor (SR) family, in particular Scavenger Receptor-A (SR-A) (Taylor et al., 2005b, Platt et al., 1998), have been shown to be involved in the phagocytosis of non-opsonised and non-biological particles (Kobzik, 1995, Palecanda et al., 1999, Arredouani et al., 2005, Hamilton et al., 2006). However, the exact mechanism by which these receptors bind particles that are devoid of surface receptors is unclear but it has been suggested that hydrophobicity may play an important role (Kobzik, 1995, Gilberti et al., 2008). Even so, macrophages are still able to ingest particles regardless of the inhibition of SR-A (Kobzik, 1995).

Certain ligands may be hidden or masked within the inner membrane, protected by the outer cell wall and require membrane disruption in order to be recognised and bound to. For example, Chung and Kocks (2011) using the scavenger receptor Eater (expressed on phagocytes in *Drosophila melanogaster*) showed that the receptor was only able to attach to Gram-negative bacterial cells (*Escherichia coli, Serratia marcescens* and *Pseudomonas aeruginosa*) that had undergone membrane disruption treatment (e.g. heat or ethanol inactivation). The attachment to Gram-positive cells did

not require membrane disruption, suggesting that the Eater ligand is peptidoglycan (or ligands within it). The peptidoglycan layer is a distinguishing feature between Gram-positive and Gram-negative bacteria. Gram-negative bacteria have a thin peptidoglycan layer over which exists an extensive lipopolysaccharide (LPS) outer membrane whereas Gram-positive bacteria have a thick peptidoglycan layer and do not possess an outer membrane (Silhavy et al., 2010). This means that peptidoglycan is 'available' to Eater in live Gram-positive cells but it is masked in live Gram-negative bacteria. Another difference between these two bacterial groups is that because Gram-positive bacteria do not have an outer membrane, all the surface proteins and ligands are either anchored into the inner membrane (e.g. using membrane-spanning helices) or are covalently bound to the peptidoglycan (Scott and Barnett, 2006).

Once the particles are bound to the phagocyte, they activate generic phagocytosis machinery, involving receptor clustering at the binding site (Gilberti and Knecht, 2015). Unlike FcR and CR mediated uptake, non-opsonised phagocytosis activates both Rac1 and RhoA GTPase. In addition, non-opsonised uptake depends on both the actin and microtubule cytoskeleton. Microtubules are shown to be involved in an early stage of phagocytosis as well as required for the activation of RhoA, Rac1 GTPase and PI3Kinase (Gilberti and Knecht, 2015).

1.3.2 Phagocytosis in protists

In filter feeding ciliates such as *Paramecium* and *Tetrahymena*, phagocytosis is used as a feeding mechanism where extracellular food particles are ingested via the oral apparatus into the cell to form a DV. This oral apparatus is well studied (Smith, 1982). However, literature on the molecular mechanism of phagocytosis is much more limited.

Tetrahymena has one oral apparatus at the anterior end of the cell. This oral apparatus mainly consists of three membranelles, an undulating membrane and oral ribs that are between the previous two, all of which consist of ciliated and non-ciliated basal bodies (Figure 1.3) (Elliott and Clemmons, 1966, Frankel, 1999). The oral ribs create a

crescent shaped funnel into the cell and at the end of the funnel is a cytostome, a cytopharynx and a cytosomal lip, which is a projection supported by microtubules and is able to close the opening of the cytostome. Finally, a deep fibre extends from the cell cortex into the cytoplasm (Elliott and Clemmons, 1966, Elliott and Kennedy, 1973).

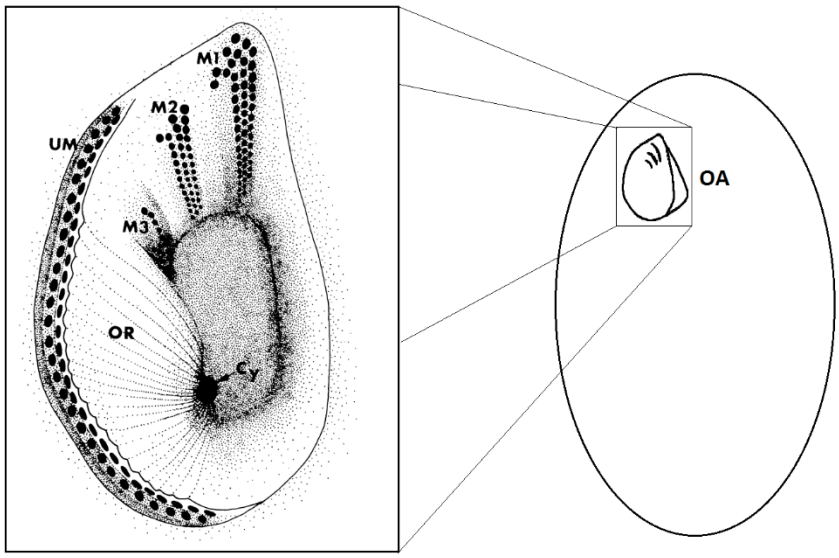


Figure 1.3 Cross section of the oral apparatus (OA) structure in Tetrahymena adapted from Smith (1982). The close up figure shows the right surface of the oral apparatus lined with the oral ribs (OR). This curves into the cavity and funnels down towards the cytosome (Cy). The cytostome then connects to the cytopharynx and receiving vacuole. The oral ribs are also separate by the undulating membrane (UM) and the three membranelles (M1, M2 and M3). M1 is located next to the left surface. M2 is positioned in the centre and is in line with the cytostome. M3 is positioned by the right wall (Smith, 1982).

By using the cilia on the oral membranes to create water currents, *Tetrahymena* cells are able to funnel water-borne food particles towards the cytostome (Elliott and Kennedy, 1973, Nilsson, 1979). Ingestion is initiated by the contact of the collected food particles in the oral apparatus and cytostome. The exact mechanism by which phagocytosis is initiate is unknown. However, this step is actin-dependent (Williams et al., 2006) and some proteins have been suggested to be involved. First, lectins (i.e. proteins that bind carbohydrates) play a role in prey recognition and attachment (Harb et al., 1998, Wilks and Sleigh, 2004, Roberts et al., 2006, Wootton et al., 2007). Also,

cytoplasmic dynein (Dyh1) (Lee et al., 1999) and Ca^{2+}/Calmodulin (CaM) signalling (Gonda et al., 2000, Ladenburger and Plattner, 2011) have also been suggested to act as regulators. Actin and Ca^{2+}/CaM signalling are also important for ciliary movement in food collection (Nilsson, 1979, Suzuki et al., 1982, Kink et al., 1990, Ladenburger and Plattner, 2011, Plattner, 2015).

Jacobs et al. (2006) carried out an analysis of the *Tetrahymena* phagosome proteome and compared it to other phagocytes (*Entamoeba histolytica* and mouse macrophages). Twenty-eight of the 453 proteins identified have been implicated in the other two phagocytes, for example Rab7 GTPase, vacuolar ATPase and lysosomal acid lipase/gastric lipase. This indicates that key aspects of phagocytosis have been conserved during evolution and that the mechanism in *Tetrayhymena* could closely resemble that of other phagocytes. Non-opsonised phagocytosis in professional phagocytes seems to most closely matched to that of protists and similarities may be drawn between the two. Firstly, protists do not require the particles to first be opsonised before recognition and phagocytosis, and secondly, lectins (e.g. mannose receptor) have been shown to be involved in the attachment and phagocytosis of particles in both phagocyte types (Wootton et al., 2007, Klaas and Crocker, 2012, Flannagan et al., 2015).

1.3.3 Factors that affect phagocytosis

A number of factors affect the ingestion of prey by protists. The most obvious one is prey concentration (Jürgens and DeMott, 1995, Boenigk and Arndt, 2000a, Thurman et al., 2010a, Yoo et al., 2010, Li et al., 2011), whereby higher prey concentration induces a higher chance of prey-predator contact and thus increases ingestion. This behaviour has been reported in ciliates (e.g. *T. pyriformis* and *Oxyrrhis marina*) (Thurman et al., 2010a, Li et al., 2011), choanoflagellates (e.g. *Monosiga ovata),* kinetoplastids (e.g. *Rhynchomonas nasuta*) (Boenigk and Arndt, 2000a) and flagellates (e.g. *Bodo saltans* and *Spumella* sp.) (Jürgens and DeMott, 1995).

The physiological state and feeding/growth history of the protist can also affect feeding. For example, Hatzis et al. (1994) demonstrated feeding heterogeneity in *T. pyriformis*, showing that stationary phase ciliate populations exhibit more uniform feeding behaviour than exponential cells and that starved cells feed at higher rates than well-fed cells.

Next, the characteristics of the prey themselves can also affect feeding, specifically their size and nature. Size-selective grazing in protists has been reported to occur (Šimek and Chrzanowski, 1992, Jürgens and Šimek, 2000, Jürgens and Matz, 2002, Jürgens et al., 2008), but the most convincing studies are those using inert particles (which do not possess any ligands) as only size is being measured in these cases. In such studies, ciliates have been shown to ingest larger particles over smaller particles cells (Šimek and Chrzanowski, 1992, Jürgens and Šimek, 2000, Jürgens and Matz, 2002, Jürgens et al., 2008). Macrophages on the other hand have a distinct preference for *ca.* 1µm rod shaped particles, compared to spheres and ellipsoid particles with equal volumes Doshi and Mitragotri (2010); a size and shaper similar to live bacterial cells.

Reports on selective feeding in protists based on prey strain have been mixed but in general, evidence is stronger for it occurring in flagellates compared to ciliates (Jezbera et al., 2005, Zwirglmaier et al., 2009, Thurman et al., 2010a). Selectivity has been accredited to receptors (Wootton et al., 2007), prey motility (Matz and Jürgens, 2005), prey hydrophobicity (Monger et al., 1999, Matz and Jürgens, 2001) and prey food quality (C:N:P ratio) (John and Davidson, 2001, Shannon et al., 2007).

Lastly, ingestion can also be affected by the type of prey particle, i.e. whether the prey are inert, heat-killed or live etc. and although many experiments have utilised heat-killed-5-(4,6-Dichlorotriazinyl) Aminofluorescein (DTAF)-stained bacteria (Sherr et al., 1988, Gonzalez et al., 1990a, Gonzalez et al., 1993, Starink et al., 1994, Parry et al., 2001, Pickup et al., 2007c, Thurman et al., 2010a, Bochdansky and Clouse, 2015),

results might not truly reflect the ingestion of their live counterpart (Peruń et al., 2016). This is because the preparation of DTAF-stained prey involves subjecting the bacterial cells to high heat (60ºC) which not only kills the cells but also irreversibly changes the physical and biochemical characteristics of the bacteria (Katsui et al., 1981). Therefore the state of the prey cells must also be considered in feeding experiments.

1.4 Digestion

1.4.1 Vacuole passage time

Prey digestion in protists has mainly been studied with pulse-chase experiments (Mueller et al., 1965, Fok and Shockley, 1985, Sherr et al., 1988, Dolan and Simek, 1998, Jürgens and Šimek, 2000, Jacobs et al., 2006, Dixon, 2010, Thurman et al., 2010a), focusing on the macro level (i.e. interactions between bacterial prey and protistan predators) as opposed to the micro level (i.e. biochemical and molecular interactions). A pulse-chase experiment begins by adding fluorescent prey to a protistan culture so that feeding occurs ('the pulse') and fluorescently DVs are formed. Then follows the 'chase', whereby the mixture is diluted to stop ingestion of the fluorescent prey and this halts the formation of fluorescent DVs. The content of the fluorescent DVs can then be monitored over time.

Prey such as fluorescently-labelled microspheres cannot be digested and remain in the DVs until they are egested. This allows the identification of the life span of the vacuoles inside the cells, known as the vacuole passage time (VPT). Digestion of (potentially-digestible) prey is only monitored within this VPT as this ensures that any loss of prey in the DV is solely due to digestion and not egestion. Beyond the VPT, it is difficult to distinguish whether the loss of prey is due to digestion, egestion or both (Thurman et al., 2010a).

The VPT can be affected by feeding history, protist species and vacuole queuing (Thurman et al., 2010a). Vacuoles are processed in the order they are formed and because egestion can only occur at the cytoproct, vacuoles must queue and wait to be egested (Ricketts and Rappitt, 1976, Thurman et al., 2010a). This queuing behaviour is thought to be responsible for varying VPTs within a given species (Fok et al., 1982, Fok and Shockley, 1985, Dixon, 2010, Thurman et al., 2010a).

1.4.2 Digestive vacuole (phagosome) maturation

The surface composition of newly formed DVs in macrophages are identical to that of the cell's surface membrane (Muller et al., 1980a, 1980b). This means that DVs inherently lack the microbicidal and degradative capacity required for prey digestion. However, these capabilities are acquired subsequently by a process called 'phagosome maturation' which is a sequence of membrane fusion and fission events with components of the endocytic compartment (Pauwels et al., 2017). Phagosome membranes are selectively fusogenic and acquired surface proteins which allows the phagosome to selectively interact with the next endosome in the maturation sequence (Desjardins et al., 1997, Maniak, 2003). This is achieved party due to the molecular differences in surface proteins and the fact that fusion mechanisms vary with regards to the proteins or conditions involved (Mayorga et al., 1991, Jahraus et al., 1998). DV maturation particularly includes fusion events between the DV and acidosomes (to reduce the DV pH) and lysosomes (to provide the enzymes for digestion).

There is only one model, for the ciliate *Paramecium multimicronucleatum*, which describes the events within a DV during one VPT (Table 1.2) (Fok et al., 1982). This four stage model suggests that after a ciliate DV is formed (in Phase-I) it goes through rapid acidification and condensation (in Phase-II) to kill its contents and prepare the DV for membrane fusion with lysosomes. Thereafter it fuses with lysosomes and begins digestion (in Phase-III). After digestion has ended the vacuoles are thought to be inert whilst awaiting defecation (Phase-IV) (Fok et al., 1982).

Phase	DV pH	DV size	Acid phosphatase activity
I – DV formation	Neutral	-	No
II – Acidification & Condensation	Rapid acidification	Rapid decrease	No
III – Lysosomal fusion & digestion	Neutral	Increase	Yes
IV – Defecation competent	Neutral	Slight decrease	No

Table 1.2 The four phases of Paramecium multimicronucleatum digestive vacuole (DV) processing suggested by Fok et al. (1982).

1.4.2.1 Acidification and condensation (Phase-II)

Immediately after the formation of a DV the size of the DV reduces and numerous acidosomes can be found attached to its membrane (Ishida et al., 1997). The attached acidosomes are able to move with the DV and only fuse with it when it is released from the oral region and begins to move towards the posterior of the cell. Microfilaments, which are ubiquitous in the spaces between acidosomes and DV membranes (Allen and Fok, 1983b), appear to be vital to this fusion event (Allen and Fok, 1983a). After fusion, acidosomes release their vacuolar content (H+) into the DV and also transfer the ability to acquire H+ to the DVs (Allen and Fok, 1983a, 1983b, Ishida et al., 1997). Acidification is achieved partly by the action of vacuolar ATP-ase (V-ATPase) (Pitt et al., 1992, Fok et al., 1993, Clarke et al., 2002, Yates et al., 2005), a protein complex that is able to translocate protons across membranes to create a potential gradient (Nishi and Forgac, 2002).

A clear understanding of how the pH changes inside the DVs would be useful but only a few studies have attempted this. The general method used in studies with polymorphonuclear leukocytes (Jensen and Bainton, 1973), macrophages (Geisow et al., 1981), *Tetrahymena* (Nilsson, 1977) and *Paramecium* (Muller and Törö, 1962, Fok et al., 1982) has been to feed the cells with yeast cells stained with sulfonphathalein indicator dyes (neutral red, bromocresol purple, bromocresol green and bromocresol blue) and exploit their pH sensitive colour changing properties (Table 1.3).

The reduction in DV pH, and timings, vary greatly between the aforementioned studies. In macrophages and leukocytes DVs took 15 to 20 minutes to reach their lowest pH level (pH 5.4 and pH 4.0, respectively) (Jensen and Bainton, 1973, Geisow et al., 1981). In *Tetrahymena pyriformis*, Nilsson (1977) showed that the lowest pH value (pH 3.5-4.0) was reached at 1 hour. This was much longer than that recorded for *Paramecium multimicronucleatum* (Muller and Törö, 1962, Fok et al., 1982) where the pH of DVs reduced to pH 3.0 within 5 minutes of formation (Figure 1.4). Even faster

was *Paramecium bursaria* where maximum acidity (pH 2.4-3.0) was achieved at 30 seconds (Kodama and Fujishima, 2005).

pH	Neutral red	Bromocresol purple	Bromocresol green	Bromocresol blue
7.0	Red amber	Blue	Blue	
6.5	Red	Blue green	Blue green	
6.0	Red	Green	Blue green	
5.5		Yellow green	Green	
5.0		Yellow	Green	Blue
4.5			Light green	Blue green
4.0			Yellow green	Green
3.5			Yellow	Yellow green
3.0				Yellow

Table 1.3 Colour of stained yeast cells at different pH. The observations from Fok et al. (1982), Jensen and Bainton (1973) were combined to produce this table.

The use of pH-sensitive dyes may be informative but these methods are not without issues. Qualitative approaches were used to deduce the DV pH, i.e. comparing photographs of the DV colour to those of isolated stained yeast cells at different pH values. This cannot provide accurate pH values and indeed the mentioned studies did not even agree on a colour profile for pH change. Newer techniques such as the use of dextran-linked pH-sensitive fluorescent dyes (e.g. fluorescein and Oregon green 514) are now available and have been used to investigate the pH within DVs in *Plasmodium,* where a lowest DV pH of 3.7 (with a large range of pH 3.7-6.5) was recorded (Saliba et al., 2003, Hayward et al., 2006). Using the same dye, Klonis et al. (2007) recorded a pH of *ca.* 5.5 for the same parasite. It therefore seems that even though methodologies in measuring the pH of DVs have advanced, the accuracy of these measurements are still problematic..

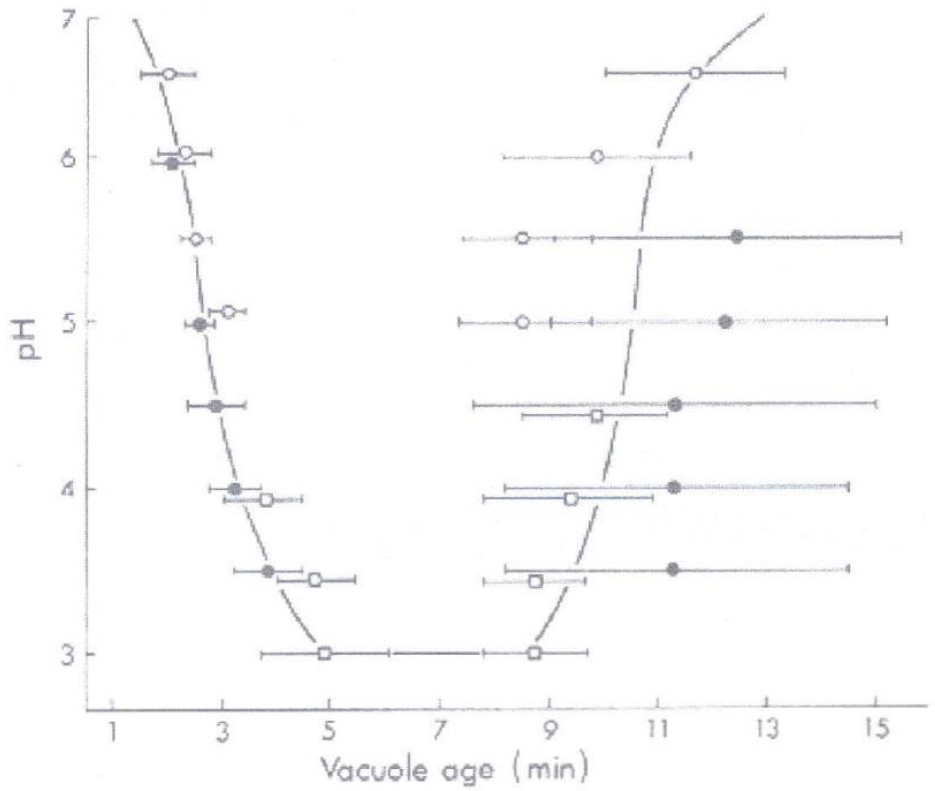


Figure 1.4 Feeding the ciliate Paramecium caudatum with yeast cells stained with different dyes. Data showed that newly formed DVs undergo rapid acidification from pH 6-7 to pH 3-4, this lasts for 2 minutes before pH increases back to the original pH. Figure taken directly from Fok et al. (1982)

1.4.2.2 Lysosomal fusion and digestion (Phase-III)

Acidification of the DV in Phase-II changes the membrane surface markers and this allows lysosomes to fuse with the DV in Phase-III (Fok et al., 1982, Fok and Shockley, 1985). For example, lamp proteins which are a family of glycosylated membrane proteins that are required for a functioning endocytic pathway (Huynh et al., 2007) are not found on the plasma membrane. However, they are shown to be acquired to the DV's membrane surface soon after formation and are incrementally transferred to the DV surface (Pitt et al., 1992, Desjardins et al., 1994b). Also, Rab5 and Rab7 are proteins that play an important role in the DV maturation process (Gorvel et al., 1991, Desjardins et al., 1994b, Duclos et al., 2000, Roberts et al., 2000). Rab5 and Rab7

have been shown to be associated with early endosomes and late endosomes respectively (Chavrier et al., 1990) and are acquired by the DVs rapidly after formation (Vieira et al., 2003). Indeed, the modification of DV membrane markers plays a cruicial role in regulating the orderly interactions and fusion events between the endolytic organelles (Maniak, 2003). However, quantitative proteomic approaches have suggested that a linear pathway of maturation is an oversimplification and that there is either significant cross-talk between endocytic organelles or that DV maturation occurs over many parallel pathways (Rogers and Foster, 2008).

There are two types of fusion described between DVs and lysosomes, 'complete fusion' and 'kiss and run fusion' (Desjardins, 1995). Complete fusion occurs when the lysosome completely fuses with the DV so the membranes and cytosolic contents of both vesicles are combined. Contrarily, 'kiss and run' fusion is an incomplete transfer of membrane and cytosolic content as lysosomes only fuse to the DV temporarily and then is pinched off. However, materials are able to be transferred in this short period of time (Desjardins, 1995). The fact that vacuole size increases during Phase-III (Table 1.2) suggests complete fusion between DVs and the endolytic vesicles. However, there have been studies showing 'kiss and run fusion' events between DVs and lysosomes in macrophages (Wang and Goren, 1987, Duclos et al., 2000) and indeed other protists (e.g. *Leishmania*) (Desjardins and Descoteaux, 1997). It is therefore likely that the fusion events in ciliates are also a mixture of both fusion methods.

Fusion events are achieved with the support of the cell's cytoskeletal system. The polymerization of actin allows the locomotion of endolytic vesicles in the form of gliding or crawling. Indeed, many actin-binding proteins are found to associate with the DV after its formation (Desjardins et al., 1994a) and interact with actin filaments and microtubules that protrude from the DV membrane surface to help guide its movement (Mitchison and Cramer, 1996). This also facilitates the aggregation of such endosomal machinery and DVs for membrane fusion (Defacque et al., 2000, Jahraus et al., 2001).

DV fusion with lysosomes ultimately results in the release of enzymes into DVs. There is a large library of enzymes involved, including proteases, lipases, phosphatases and glycosidases including nucleotide transferase, acid phosphatases, phosphoric monoester, glycoside, peptidyl amino acid and sulphuric ester hydrolases (Fok and Paeste, 1982, Fok et al., 1984, Fok and Shockley, 1985). Of these, acid phosphatase activity has been used as a marker to determine whether there are active enzymes within DVs in ciliates (Muller and Törö, 1962, Elliott and Clemmons, 1966, Fok et al., 1982, Fok and Shockley, 1985). Using this marker, enzymes have only been recorded in Phase-III (Table 1.2) during which the pH steadily rises. Optimum conditions (e.g. pH) for each of the enzymes are often different so it is highly possible that enzyme delivery into DVs occurs in a sequential fashion by fusion with lysosomes containing different enzymes. This is something that has been observed in macrophage phagosome maturation (Tjelle et al., 2000, Vieira et al., 2002).

While VPT denotes the total life span of DVs inside the cells, the digestive period (DP) denotes the period within the VPT where prey are actively being killed and then digested inside the DV. The DP includes Phases I to III of the DV processing model suggested by Fok et al. (1982). And even though VPT can be variable for a given species (due to vacuole queuing), at a given temperature and for a given prey, the length of the DP has been reported to be constant. Capriulo and Degnan (1991) showed that the DP of the ciliate *Fibrea salina* was consistently *ca*. 63-73 min when feeding *Rhodomonas lens* at 23ºC. Fok and Shockley (1985) reported a *ca.* 45 min DP for *T. thermophila* when fed at 24ºC. *Paramecium caudatum* showed a DP of 21 min for at an unreported temperature (Fok et al., 1982), while the ciliate *Uronema marina* was reported to have a DP of 25 minutes when fed at 22ºC (Sherr et al., 1988). Lastly, Thurman et al. (2010a) showed that the DP for *T. pyriformis* feeding on heat-killed DTAF-stained *Mesorhizobium* sp., at 23ºC, was consistently 20 min.

1.4.2.3 Defecation competent (Phase-IV)

The last phase (Phase IV) of DV processing is the defecation competent phase (Table 1.2). Once the DVs have completed digestion they enter Phase-IV and become 'inert' and of neutral pH. They lack the presence of acid phosphatase and their membrane is absent of any V-ATPase (see Section 1.3.1) (Fok et al., 1982, Ishida et al., 1997). The length of this Phase seems to vary greatly (Fok et al., 1982, Fok et al., 1984, Verni and Gualtieri, 1997, Thurman et al., 2010a). For example, Fok et al. (1982) reported defecation of *P. caudatum* started at 20-60 minutes after DV formation while Thurman et al. (2010a) reported that *T. pyriformis* has a defecation competent phase of *ca.* 35-50 minutes. Eventually the DVs fuse with the plasma membrane and egest its contents at the cytoproct (Allen and Wolf, 1979).

During Phase-IV, bacteria that have survived the digestive process are no longer in a hostile environment and may be able to recover, replicate and even undergo conjugation with other surviving cells (Schlimme et al., 1997, Matsuo et al., 2010, Smith et al., 2012). One study showed that when fed to *T. pyriformis*, as much as 90% of ingested *E. coli* K12 could be egested unharmed within faecal pellets (Schlimme et al., 1997). In order to gain enough energy and nutrients from these prey, it seems that protists may be required to compensate for this by ingesting and processing the egested prey (coprotrophy). This re-ingestion and re-digestion process would assure that the prey were fully digested and absorbed. Indeed this has been suggested to occur in *T. pyriformis.* When fed heat-killed-DTAF-stained prey the faecal pellets observed were too large (*ca.* 20µm diameter) to be re-ingested, resulting in a low cell yield. However, when fed with the live counterpart, there was little aggregation of any egested prey and subsequently low yield was not observed (Parry et al., 2001).

1.4.2.4 Membrane recycling

Membrane is a limited resource to the cell, and therefore it is important that the cell is able to recycle such materials back to the cytostome for further DV formation (Allen, 1974, Allen and Fok, 1980). In ciliates, the size of DVs changes dramatically during the VPT. Soon after formation, the DV shrinks to *ca.* 50% its original size and the pH rapidly decreases (Phase II in Table 1.2). The shrinkage is achieved by removal of large amounts of vacuole membrane material by endocytic-like processes (Fok et al., 1982, Laybourn-Parry et al., 1997). Several mechanisms of membrane recycling have been proposed, including (i) retrieval at the cytoproct by endocytosis (Allen and Fok, 1980); (ii) fusing with lysosomes (Allen and Fok, 1984); (iii) directly (i.e. where an endosomes are incorporated into other organelle's surface membrane by exocytosi) (Allen, 1974, Allen and Wolf, 1979). Lastly, along with vacuole membrane, other materials (e.g. acid phosphatases, lysosomal membranes and surface V-ATPase) are also recycled from the DVs (Allen and Fok, 1984).

1.5 The result of phagocytosis

Prey consumption is tightly linked to growth and it is known that prey concentration positively correlates with growth rate up to a maximum (Fenchel, 1980, Jonsson, 1986, Weisse et al., 2002, Kimmance et al., 2006, Pickup et al., 2007b, Li et al., 2011). For filter feeders in particular, assuming that they have a maximum water processing rate (volume of water it can process per unit time [clearance rate]), increasing the number of prey within that volume of water would logically increase the prey consumption rate, therefore increasing growth (Fenchel, 1980).

Temperature also affects growth rate. It is commonly known that growth rates increase with temperature up to a maximum (Müller and Geller, 1993, Montagnes and Franklin, 2001, Weisse et al., 2001, Weisse et al., 2002, Atkinson et al., 2003, Kimmance et al., 2006, Rose and Caron, 2007, Rose et al., 2009, Li et al., 2011). Studies have shown both a linear (Cossins and Bowler, 1987, Montagnes and Franklin, 2001, Weisse et al., 2001) and an exponential (Choi and Peters, 1992, Lomas and Glibert, 1999, Rose and Caron, 2007) growth response to temperature change. However, in a review that compared 36 available studies, Montagnes et al. (2003) suggested that growth rate usually responds linearly to temperature. It was cited that the errors produced from the simplification of exponential and assumptions made when applying the exponential function to limited data was the reason for results that seem to be exponential.

The type and quality of prey also affects growth (Hansen, 1992, Thurman et al., 2010b). For example, Thurman et al. (2010b) showed that even though *T. pyriformis* ingested different prey at the same rate, the yield (in terms of ciliate-cell/prey-cell) was significantly higher when fed *E. coli* when compared to *K. aerogenes* and *K. ozaenae.* Even though growth rates and yield vary based on prey, growth is actually an end result of the whole feeding process and it is not possible to know at which stage of the feeding process (i.e. ingestion, digestion or assimilation) the prey are affecting growth (Montagnes et al., 2008).

1.6 Aims

The overall aim of this study was to investigate the effect of different prey types on their ingestion and digestion by the ciliate *Tetrahymena pyriformis* and how this affected ciliate specific growth rate. Four prey states were employed, i.e. live heterotrophic bacteria, heat-killed-DTAF-stained heterotrophic bacteria, live autotrophic bacteria (*Synechococcus*) and inert fluorescently-labelled microspheres, together with different prey genera/species/strains within each of the bacterial states.

The specific objectives of this study were to:

- Transform wild type bacteria to express the red fluorescent protein (RFP) so they can be used as a fluorescent live heterotrophic bacterial prey.
- Determine the effects of different prey states (live, dead and inert) on ingestion, vacuole formation and prey deposition into those vacuoles.
- Evaluate whether differential ingestion and digestion occurs with different bacterial species/strains and whether Gram status plays a role.
- Determine how prey ingestion and digestion affect the specific growth rate *T. pyriformis.*

Chapter 2. Materials and methods

2.1. Preparation and maintenance of organisms/FLMs

Experiments employed the ciliate *Tetrahymena pyriformis* feeding on four prey types: Fluorescently-labelled microspheres ('FLMs'), live heterotrophic bacteria transformed to express the Red Fluorescent Protein (RFP) ('Live'), heat-killed and 5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein (DTAF)-stained heterotrophic bacteria ('Dead') and live *Synechococcus* cells ('Pico').

2.1.1 Fluorescently-labelled microspheres (FLMs)

Suspensions of inert fluorescently-labelled yellow/green microspheres (Fluoresbrite™ Polyscience Inc.) of various diameters (Table 2.1) were prepared in sterile water and stored at 4°C. FLM suspensions were sonicated for 2 min before use.

Colour	Diameter (µm)	Biovolume (μm^3)	Stock Concentration (FLM/mL)
Yellow/Green	0.49	0.065	8.27×10^9
Yellow/Green	0.75	0.220	1.08×10^9
Yellow/Green	0.92	0.407	5.19×10^8

Table 2.1 Fluorescently-labelled microspheres (Fluoresbrite™ Polyscience Inc.) used in this study. Biovolume and concentration calculations can be found in Sections 2.3.1 and 2.2.2 respectively.

2.1.2 Routine maintenance of heterotrophic bacteria

Heterotrophic bacteria (Table 2.2) were routinely maintained as streak plates and either stored at room temperature (the RFP-expressing strains) or 4°C. For experiments each was grown for 3 days at their preferred temperature (Table 2.2) then suspensions were prepared in sterile water; these were used immediately.

Bacterium	Source	Strain	Medium	Temperature	Biovolume (μm^3)
Aeromonas hydrophila (RFP)	NCIMB	9240	LB + G60	35°C	1.016±0.061
**Citrobacter freundii*	MAST labs		LB	30°C	ND
**Citrobacter koseri*	MAST labs		LB	30°C	ND
**Enterobacter cloacae* (RFP)	MAST labs		LB + G60	30°C	1.334±0.074
Escherichia coli RFP (Donor)	P Hill	AKN132	LB + Amp100	30°C	0.511±0.025
Escherichia coli (Helper)	P Hill	S17	LB + Amp50	30°C	ND
Escherichia coli (Mobiliser)	P Hill	RK2013	LB + Km50	30°C	ND
Klebsiella aerogenes (RFP)	NCTC	9528	LB + G60	25°C	0.918±0.043
Klebsiella pneumoniae (RFP)	NCTC	5055	LB + G60	35°C	1.701±0.101
**Mesorhizobium* sp.	J Parry	B1	LB	25°C	ND
**Morganella morganii*	MAST labs		LB	30°C	ND
Pseudomonas aeruginosa (RFP)	NCIMB	10412	LB + G60	25°C	0.655±0.031
**Pseudomonas aeruginosa* m-	H Curt-Flemming		LB	30°C	ND
**Pseudomonas aeruginosa* m+	H Curt-Flemming		LB	30°C	ND
Pseudomonas fluorescens (RFP)	R Pickup	FH1	LB + G60	30°C	0.785±0.047
Salmonella enterica (RFP)	NCTC	74	LB + G60	35°C	0.759±0.033
Salmonella enterica (RFP)	NCTC	12694	LB + G60	25°C	0.512±0.028
Serratia liquefaciens (RFP)	MAST labs		LB + G60	25°C	0.641±0.031
Serratia marcescens (RFP)	NCIMB	1377	LB + G60	25°C	0.444±0.022
**Shigella sonnei*	MAST labs		LB	30°C	ND
Staphylococcus aureus	NCTC	6571	LB	30°C	ND
Staphylococcus aureus RFP	P Hill		LB + C6	35°C	0.272±0.022

*Table 2.2 Heterotrophic bacterial strains used in this study together with their source, incubation temperature and any antibiotic additions to the Lysogeny Bertani agar (LB) (see Appendix A). Cell biovolumes are presented for RFP-expressing strains (see Section 2.3.2). National Collection of Industrial Marine and Food Bacteria (NCIMB), National Collection of Type Culture (NCTC), Red-fluorescing protein (RFP), mucoid (m+), non-mucoid (m-), Gentamicin (G), Ampicillin (Amp), Kanamycin (Km), Chloramphenicol (C), 6 µg/ml (6), 50 µg/ml (50), 60 µg/ml (60), 100 µg/ml (100). ND, not determined. *The eight strains used in RFP-transformation experiments (Section 2.1.3).*

2.1.3 Transforming bacteria to express the Red Fluorescing Protein (RFP)

All RFP-expressing bacteria (other than *E. coli*, *Enterobacter cloacae* and *Staphylococcus aureus*) (Table 2.2) were transformed by J Parry prior to this study but *E. cloacae* was successfully transformed in this study using method described below. All RFP-expressing bacteria (n=12) are referred to as 'Live' prey in experiments involving *T. pyriformis*.

The Tn7 tagging system (Lambertsen et al., 2004) relies on four-parental mating conjugation, i.e., the recipient bacterium is mixed with a donor (*E. coli* RFP [carrying resistance to Gentamycin]), a helper (*E. coli* S17) and a mobiliser (*E. coli* RK2013) (Table 2.2). Transformations were attempted on eight bacterial strains (* Table 2.2).

Each of the four-parental strains was first grown separately overnight in Lysogeny Bertani (LB) broth (+/- antibiotics) (Appendix A) at their preferred temperature (see Table 2.2). Cells were washed 4 times with LB broth to remove any antibiotics then 0.5 ml of each participant was combined and the 2 ml mixture filtered onto a 0.2 µm filter (Whatman, Millipore). The filter was placed (cells side down) onto the surface of an LB agar plate (no antibiotics) (Appendix A) and incubated overnight at the recipient's preferred temperature (Table 2.2). The filter was then placed in 1 ml of LB broth (no antibiotics) and the tube was vortexed and sonicated for 10 min to dislodge the cells into suspension.

In order to separate the transformant from all other cells, i.e., untransformed recipient, donor, helper and mobiliser, a selective medium was required. In most cases* this was Vogel-Bonner Minimum Medium agar (VBMM) containing Gentamycin at 60 µg/ml (GM60) (Appendix A) because *E. coli* cannot grow on this medium and the eight recipients tested here were sensitive to Gentamycin (unlike the transformant). **Three*

bacterial recipient strains could not grow on VBMM so an alternative selective medium was required (see Section 3.1).

The cell mixture was streaked onto VBMM + GM60. The recipient, donor, mobiliser and helper were also streaked on this medium to act as negative controls (no growth). If presumptive transformant colonies emerged after incubation, they were again streaked onto VBMM + GM60 and then onto LB agar + GM60 for routine maintenance. The antibiotic susceptibility profile of the transformant was compared to the control to ensure the transformant only had additional resistance to Gentamycin. This was performed by spreading 0.2 ml of suspension onto the surface of Diagnostic Sensitivity Test agar (DST) plates (Appendix A), applying MAST-rings M13, M14 and M43, and examining zones of inhibition after incubation.

The optimum incubation temperature for fluorescence was determined by incubating the transformant at 20, 25, 30 and 35^{o}C, after which suspensions were prepared. Cells were stained with 4',6 Dimamidino-2-phenylindole (DAPI) (Porter and Feig, 1980) and a total cell count (using UV excitation) (see Section 2.2.1.) and a fluorescing cell count (using green excitation) (see Section 2.2.2.) allowed the calculation of % cells that were fluorescing. The temperature which yielded the highest % fluorescence (normally 98-100%) was selected as the routine incubation temperature (Table 2.2).

2.1.4 Heat-killed-DTAF-stained heterotrophic bacteria ('Dead')

Previously prepared heat-killed/DTAF-stained cells were available (stored at -20^{o}C) for *Mesorhizobium* sp. (Lab strain), *Lactobacillus fermentum* and *Listeria monocytogenes*; prepared by C Dixon (2010). Neither had an RFP-expressing counterpart, so four strains (*Table 2.3) were stained here following the method of Sherr et al. (1987). Briefly, bacterial suspensions were prepared and centrifuged at 3500 rpm for 10 min to form a pellet. The supernatant was removed and the pellet was re-suspended in 10 ml of solution 1 (Appendix B). This suspension was vortexed then sonicated for 10 min to break up any aggregates. Approximately 5 mg of 5-([4,6-Dichlorotriazin-2-yl]amino)

fluorescein (DTAF, Sigma) was added, mixed and the tube was placed in a 60°C water bath for 2 h. The cells were washed three times with solution 1 (Appendix B). After the third wash the cells were suspended in Solution 2 (Appendix B) and left overnight at 4°C. The cells were then washed three times in Chalkley's medium (Appendix A) before determining the cell concentration (see Section 2.2.2). The suspension was aliquoted into eppendorf tubes (0.5 ml) and stored at -20ºC. Prior to experiments, aliquots were thawed at room temperature and sonicated for 15 min.

Bacterium	Source	Strain	Biovolume (μm^3)
**Klebsiella aerogenes*	NCTC	9528	0.300±0.011
**Klebsiella pneumoniae*	NCTC	5055	0.270±0.012
Lactobacillus fermentum	NCIMB	11849	0.440±0.015
Listeria monocytogenes	NCIMB	13726	0.470±0.020
Mesorhizobium sp.	J Parry	B1	0.170±0.005
**Salmonella enterica*	NCTC	74	0.120±0.009
**Staphylococcus aureus*	NCTC	6571	0.125+0.006

*Table 2.3 Heterotrophic bacterial strains heat-killed and DTAF-stained together with their source and cell biovolumes after staining (see Section 2.3.2). National Collection of Industrial Marine and Food Bacteria (NCIMB), National Collection of Type Culture (NCTC), *The four DTAF-strains produced in this study; others were prepared by C Dixon.*

2.1.5 Live autotrophic bacteria ('Pico')

All *Synechococcus* strains (Table 2.4) were maintained in BG11 broth (Appendix A) on a rotary shaker in a 16:8 light:dark cycle at room temperature (*ca.* 23°C). Strains were sub-cultured 6 d prior to experimentation.

Bacterium	Source	Strain	Lab Code	Biovolume (μm^3)
Synechococcus sp.	K Harper	S-KH1	Pico 1	1.085±0.047
Synechococcus sp.	K Harper	S-KH2	Pico 2	0.919±0.040
Synechococcus sp.	K Harper	S-KH3	Pico 3	0.588±0.031
Synechococcus sp.	K Harper	S-KH4	Pico 4	0.934±0.044
Synechococcus sp.	K Harper	S-KH5	Pico 5	1.137±0.044
**Synechococcus* sp.	K Harper	S-KH6	Pico 6	1.171±0.043
Synechococcus sp.	K Harper	S-KH7	Pico 7	1.253±0.050
**Synechococcus* sp.	K Harper	S-KH8	Pico 8	0.837±0.036
Synechococcus sp.	K Harper	S-KH9	Pico 9	0.943±0.038
Synechococcus sp.	K Harper	S-KH10	Pico 10	1.062±0.039
Synechococcus sp.	K Harper	S-KH11	Pico 11	0.838±0.030
Synechococcus sp.	K Harper	S-KH12	Pico 12	1.039±0.031
Synechococcus sp.	K Harper	S-KH13	Pico 13	1.055±0.035
Synechococcus sp.	K Harper	S-KH14	Pico 14	0.756±0.026
Synechococcus sp.	K Harper	S-KH15	Pico 15	0.979±0.037
Synechococcus sp.	CCAP	1479/12	Pico 16	0.948±0.038
**Synechococcus leopoliensis*	CCAP	1405/1	Pico 17	2.326±0.080
Synechococcus elongatus	CCAP	1479/1A	Pico 18	0.850±0.036
Synechococcus sp.	CCAP	1479/10	Pico 19	0.978±0.034
Synechococcus sp.	CCAP	1479/11	Pico 20	2.342±0.075

*Table 2.4 Synechococcus strains used in this study together with their source, strain, laboratory code and cell biovolume (see Section 2.3.2). Culture Collection of Algae and Protozoa (CCAP). *denotes non-lysogenic strains.*

2.1.6 The ciliate *Tetrahymena pyriformis*

Tetrahymena pyriformis (CCAP 1630/1W) was grown in Chalkley's medium (Appendix A) supplemented with live, non-RFP-expressing *K. aerogenes* at room temperature (*ca.* 23°C). Ciliate cells used in experiments were grown for 3 d after which they were twice-concentrated by centrifugation at 1500 rpm for 15 min, resulting in a *T. pyriformis* concentration of *ca.* 3 – 5x10^4 cells/ml.

2.2. Counting cells/FLMs

Prior to cell counts, samples were fixed with glutaraldehyde (0.5% v/v final conc.).

2.2.1 Counting non-fluorescent bacteria

Fixed samples containing non-fluorescent prey were stained with 4,6-Diamidine-2-phenylindole dihydrochloride (DAPI) for 30 min at room temperature following Porter and Feig (1980). Depending on the sample being tested, the procedure may have included diluting the suspension 1000-fold prior to staining. A known volume of the stained sample was filtered onto a 0.2 µm filter (Whatman, Millipore) above a vacuum pump. The filter was placed onto a slide with immersion oil above and underneath. After applying a coverslip and a further drop of immersion oil, the filter was observed with UV excitation using an epifluorescence microscope (final magnification x1600). At least 400 cells were counted in numerous Whipple Grids (held within the eye piece). The area of the Whipple Grid is equal to 1/23068th of the filter so the average number of cells per grid was multiplied by 23068 to give the number of cells on the filter. This was then converted to cells/ml in the original undiluted suspension; knowing what volume and dilution had been filtered.

2.2.2 Counting fluorescent prey

Fixed samples containing fluorescing prey were counted as described in Section 2.2.1 without the need for DAPI staining. Green excitation was used for RFP-bacteria while blue excitation was used for DTAF-stained bacteria and FLMs.

2.2.3 Counting *Tetrahymena pyriformis*

Fixed samples containing *T. pyriformis* were counted with a haemocytometer. Cells were counted in each of the nine large squares on the haemocytometer and repeated 8 times to produce 72 (9x8) cell counts. The volume under each of the nine large squares is equal to 1x10^{-4} ml, therefore using the average number of cells (x) under one of the 9 large squares, the cell concentration could be calculated with *Equation 1*:

$$Concentration\ (cells/ml) = \frac{x}{1\times10^{-4}} \qquad \textit{Equation 1}$$

Where (x) is the average number of cells under one of the 9 large squares on the haemocytometer.

2.2.4 Counting prey within *Tetrahymena pyriformis* digestive vacuoles

Fixed samples (750 µl) were filtered onto 1.2 µm filters (Whatman, Millipore) above a vacuum pump. The filter was placed onto a slide with immersion oil above and underneath. After applying a coverslip and a further drop of immersion oil, the filter was observed with appropriate excitation for the given prey using an epifluorescence microscope (final magnification x1600) (Section 2.2.2). A total of 10 ciliate cells were located and the number of fluorescent vacuoles/cell, and number of fluorescent prey within each of those vacuoles, was determined. These data yielded the parameters, average vacuoles/cell, prey/cell and prey/vacuole.

2.3. Particle sizing

2.3.1 FLM volumes

Equation 2 was used to calculate the volumes of spherical FLMs (*V*).

$$V = \frac{4}{3}\pi r^3 \qquad \textit{Equation 2}$$

Where (r) is the radius of the FLM and which was provided by the manufacturer (Table 2.1).

2.3.2 Bacterial biovolumes (Pico, RFP-bacteria and DTAF-stained bacteria)

Cell measurements were made on DAPI-stained cells filtered onto 0.2 µm filters (see Section 2.2.1) and viewed with UV excitation using an epifluorescence microscope (final magnification x1600). It was important to view, take photos (with Zen lite [Zeiss]) and size the cells under UV excitation as excitation of DTAF and RFP can cause a large halo effect and an overestimation of size. For each prey type, the length and diameter of 100 bacterial cells were measured using a point-to-point function in the ImageJ FIJI software.

For rod shaped bacteria, the *Equation 3* was used to calculate the cell biovolume (V).

$$V = \pi r^2 \times l \qquad \textit{Equation 3}$$

Where (r) is the radius and (l) is the length.

For the spherical shaped cells of *Staphylococcus aureus, Equation 2* was used.

2.4. Ingestion-only experiments

2.4.1 Ingestion at 2x10^7 particles/ml

Ingestion-only experiments at 2x10^7 particles/ml prey concentration employed all four prey types: Live (n=12), Picos (n=20), FLMs (n=3) and Dead prey (n=7) (full list in Appendix C, Table C.1). In triplicates, fluorescent prey were added to *T. pyriformis* at T = 0 min at a standard concentration of 2x10^7 particles/ml and incubated at room temperature (*ca.* 23^{o}C). Samples were fixed at 5 min with glutaraldehyde (0.5% v/v final conc). Prey/cell, vacuoles/cell and prey/vacuole at 5 min were determined (see Section 2.2.4).

2.4.2 Ingestion at different bacterial concentrations

Ingestion-only experiments at different prey concentrations employed *S. aureus* RFP (Live), *S. aureus* (Dead), *Synechococcus* sp.1479/11 (Pico [20]) and *Synechococcus* sp. S-KH3 (Pico [3]), details can be found in Tables 2.2, 2.3, 2.4. In triplicates, fluorescent prey were added to *T. pyriformis* at T = 0 min at varying concentrations (2x10^8, 1x10^8, 7x10^7, 4x10^7, 2x10^7, 1x10^7, 7x10^6, 4 x10^6, 2x10^6 and 1x10^6 cells/ml) and incubated at room temperature (*ca.* 23^{o}C). Samples were fixed at 5 min with glutaraldehyde (0.5% v/v final conc). Prey/cell, vacuoles/cell and prey/vacuole at 5 min were determined (see Section 2.2.4).

2.5. Pulse-chase experiments – ingestion and digestion

Pulse-chase experiments were performed with **Live** (n=12) and **Dead** prey (n=7) (Tests), and 0.5 µm diameter **FLMs** (Controls). The prey concentration was $2x10^7$ particles/ml and experimental temperature was c*a.* 23ºC. Picos were not tested because it is known they are indigestible to this ciliate (Thurman et al., 2010a).

2.5.1 Experimental protocol

Pulse (Ingestion): In triplicates, fluorescent bacterial prey (Live, Dead) were added to *T. pyriformis* at T = 0 to initiate feeding. Samples (100µl) were taken at T = 2.5 and 5 min and fixed with glutaraldehyde (0.5% v/v final conc.).

Chase (Digestion): At T= 6 min, the mixtures were diluted 1:50 with Chalkley's medium (Appendix A) to reduce the fluorescent prey concentration to $4x10^5$ particles/ml which is below the threshold concentration for feeding and stops further ingestion of fluorescent prey. Samples (1ml) were taken at 6.5 min, 8 min and then every 2 min until T = 20 min and fixed with glutaraldehyde (0.5% v/v final conc.).

Each pulse-chase experiment was accompanied by a control whereby the prey were indigestible FLMs. Additional samples at T = 25, 30, 40, 50 min allowed the vacuole passage time (VPT) to be determined (see Section 2.5.2.1.) and provided a 'no digestion' control to which possible digestion of bacterial prey could be compared (see Section 2.5.2.2)

2.5.2 Parameter determination

2.5.2.1 Vacuole passage time (VPT)

The indigestible FLMs were used to determine the vacuole passage time (VPT) of *T. pyriformis*. Digestive vacuole processing is divided into three phases: Phase 1: formation, Phase 2: processing, and Phase 3: egestion (Figure 2.1). The VPT is the time interval between the formation of the first fluorescent vacuole (start of Phase 1)

and its subsequent egestion (end of Phase 2/start of Phase 3) (Figure 2.1). The VPT of *T. pyriformis* was consistently 30 min in this study.

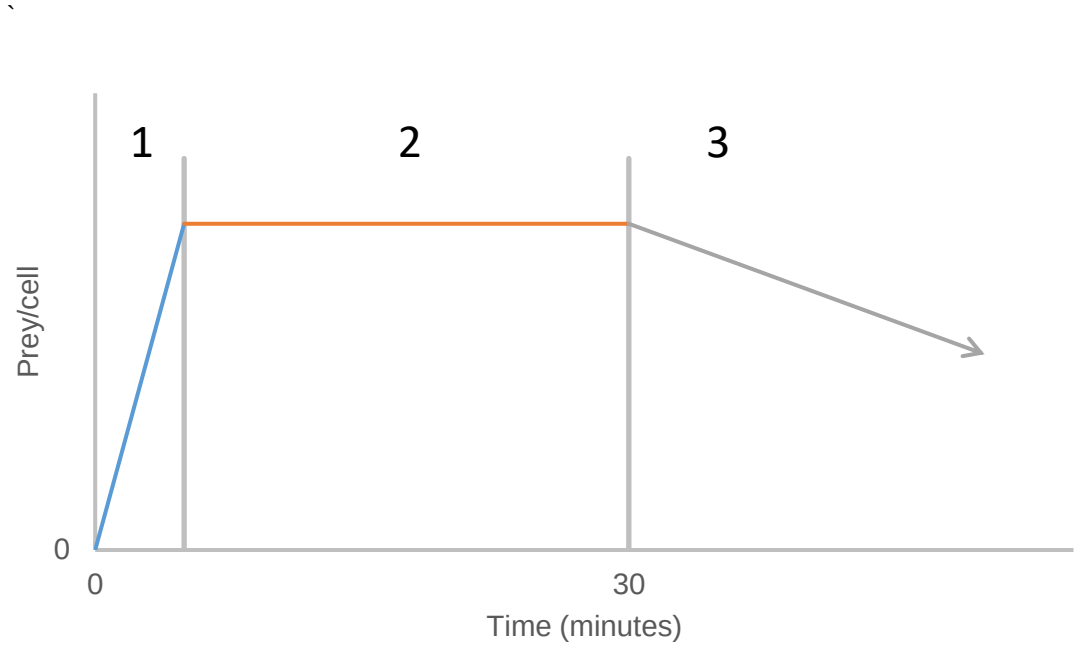


Figure 2.1 Example of a graph produced by a pulse-chase experiment using indigestible FLMs. The number of FLMs/cell (or vacuoles/cell) increases during the pulse due to ingestion (Phase 1). It then remains constant after the chase (6 min) due to the FLM-containing vacuoles passing through the cell (Phase 2). Finally, FLM-containing vacuoles begin to be egested by the cells and FLMs/cell decreases (Phase 3). Phase 1 and 2 denotes the vacuole passage time (VPT). The VPT of T. pyriformis was consistently 30 min in this study.

2.5.2.2 Ingestion and egestion rates

Ingestion/egestion rates were calculated by plotting each of the three replicas against time and subjecting them to linear regression analysis (gradient = the ingestion/egestion rate). An average of the three rates was then determined together with the standard error of the mean (SEM). To calculate FLM/vacuole egestion rates T_{zero} was taken as 30 min (beginning of Phase 3) and data for T=30, 40 and 50 min were plotted. For ingestion rates, data for T=0, 2.5 and 5 min were plotted ('Phase 1'). In addition, the prey/cell, vacuole/cell and prey/vacuole data at 5 min were combined with the ingestion-only data (Section 2.4) to investigate the effect of prey state on ingestion/vacuole formation.

2.5.2.3 Digestion rate and digestion period

A decrease in potentially digestible prey within *T. pyriformis* cells immediately after the chase (Phase 2) indicates digestion of that prey; as no egestion of digestive vacuole content occurs within the VPT (identified as no change in FLM/cell over the same time period). Beyond the VPT, any observed loss of prey might be due to a combination of digestion and egestion.

Digestion rates (as prey/vac/min) were calculated by plotting the number of prey/vacuole against time (from T = 6.5 min) for each triplicate and performing linear regression analysis on the linear decline. An average of the three rates was determined together with the standard error of the mean (SEM). In all experiments a linear decline was observed for a given time period (the digestion period) after which no further loss of prey was recorded.

Digestion rates were also expressed as either μm^3/vac/min, by multiplying the digestion rate by the biovolume of the prey; or % prey digested/vac/min by dividing the number of prey digested within the digestive period by the total number of prey/vac at T = 5 min.

Total prey digested (%) was calculated using *Equation 4*:

$$Total\ digestion\ (\%) = \frac{DR \times DP}{PV}$$ *Equation 4*

Where (DR) is the digestion rate (% prey/vac/min), (DP) is the digestion period (min) and (PV) is prey per vacuole at 5 minutes (prey/vac).

2.6. Growth experiments

A full list of prey used in growth experiments can be found in Table 3.8 (details for Live and Dead prey can be found in Table 2.2 and Table 2.3, respectively). Prey were fed to *T. pyriformis* at initial concentrations of $2x10^7$ and $2x10^8$ cells/ml to investigate the difference in ciliate growth rates.

In triplicates, prey were added to *T. pyriformis* (350 cells/ml for $2x10^7$ cells/ml prey concentration and 433 cells/ml for $2x10^8$ cells/ml prey concentration) at T = 0 h (early evening) and the cultures were incubated at *ca*. 23°C. Samples were taken at 1 or 1.5 h intervals between T = 14 h and T = 25 h (day 1). Further samples were taken at 2 hour intervals between T = 38 h and T = 46 h (day 2). All samples were fixed with glutaraldehyde (0.5% v/v final conc.). *T. pyriformis* concentration was determined with haemocytometer counts (Section 2.2.3) and graphs plotted of Ln cells/ml against time for each replica. Linear regression analysis of the increase in Ln cells/ml over time was applied and the specific growth rates (gradients) were combined to deduce an average and SEM.

2.7. Statistics

2.7.1 T-test

To determine significant differences between data, two-tailed, two-sample, equal variance t-tests were used with a confidence limit of 95% (P≤0.05).

2.7.2 Error

All errors were calculated as standard error of the mean (SEM) using *Equation 5*:

$$\mathrm{SE}_{\bar{x}} = \frac{s}{\sqrt{n}} \qquad \textit{Equation 5}$$

Where (s) is the sample standard deviation and (n) is the number of samples.

Chapter 3. Results

3.1. Red Fluorescent Protein (RFP) transformations

To expand the collection of RFP-expressing bacteria, transformations were attempted on eight bacterial strains (Section 2.1.3). VBMM agar + G60 was chosen as the medium to select for transformants because the *E. coli* strains (helper, mobiliser and donor) do not grow on it and the recipients were sensitive to Gentamycin (whereas the transformant would not be). Three bacteria, i.e. *Mesorhizobium* sp*., Morganella morganii* and *Shigella sonnei*, could not grow on VBMM so a different selective medium was required. *S. sonnei* grew on *Salmonella/Shigella* (SS) agar, but the *E. coli* strains could not, so SS agar + G60 was used to select for this strain's transformant. The other two strains proved more difficult and required a comparison of their antibiotic susceptibility profiles against those of the three *E. coli* strains (Table 3.1) in order to find an appropriate antibiotic to incorporate into LB medium, i.e., an antibiotic that the *E. coli* strains were sensitive to but the recipient was not.

M. morganii was found to be resistant to Colistin Sulphate (CO) at 25 µg/ml but the *E. coli* strains were not (Table 3.1), so the selective medium used was LB + CO25 + GM60. No suitable antibiotic combination was found for *Mesorhizobium* sp..

After three attempts to transform the 7 bacterial strains only *Enterobacter cloacae* was transformed successfully. The transformant and recipient both had identical morphology on agar plates and the transformant only had an additional resistance to Gentamycin (Table 3.2). An incubation temperature of 30^{o}C yielded the most % fluorescing cells (*ca.* 98%). Thereafter, this strain was added to the collection of RFP-expressing bacteria and used in this study.

Antibiotic	Bacterium						
	Mesorhizobium sp.	*M. morganii*	*S. sonnei*	*E. cloacae*	*E. coli* S17	*E. coli* RK2013	*E. coli* AKN132
Chlorampenicol	S	S	S	S	R	R	R
Erythromycin	R	R	R	R	R	R	R
Fusidic acid	R	R	R	R	R	R	R
Oxacillin	R	R	R	R	R	R	ND
Novobiocin	R	S	R	R	R	R	R
Ampicillin	R	R	S	R	R	R	R
Streptomycin	S	S	S	S	R	S	R
Tetracycline	S	S	S	S	S	S	S
Penicillin G	R	R	R	R	R	R	R
Clindamycin	R	R	R	R	R	R	R
Gentamicin	R	S	S	S	S	S	R
Trimethoprim	R	R	S	R	R	R	R
Sulphamethoxazole	R	R	R	R	R	R	R
Cephalothin	R	R	R	R	R	S	S
Colistin Sulphate	S	**R**	S	S	**S**	**S**	**S**
Cotrimoxazole	R	S	S	S	R	S	S
Sulphatriad	R	R	R	S	R	S	R

Table 3.1 Antibiotic susceptibility profiles of all bacterial strains involved in transformation experiments.

Antibiotic	Mast ring code	Enterobacter cloacae	
		Recipient	Transformant
Chlorampenicol	C	S	S
Erythromycin	E	R	R
Fusidic acid	FC	R	R
Oxacillin	OX	R	R
Novobiocin	NO	R	R
Ampicillin	AP	R	R
Streptomycin	S	S	S
Tetracycline	T	S	S
Penicillin G	PG	R	R
Clindamycin	CD	R	R
Gentamicin	GM	S	R
Trimethoprim	TM	R	R
Sulphamethoxazole	SMX	R	R
Cephalothin	KF	R	R
Colistin Sulphate	CO	S	S
Cotrimoxazole	TS	S	S
Sulphatriad	ST	S	S

Table 3.2 Antibiotic sensitivity profile of recipient Enterobacter cloacae and red fluorescent protein (RFP) expressing Enterobacter cloacae. These two strains have identical antibiotic sensitivity profile, apart from the resistance to gentamicin that the transformed strain gained from the RFP-containing plasmid.

3.2. Digestive vacuole processing using pulse-chase experiments

The time taken for a digestive vacuole to pass though the cell, the vacuole passage time (VPT), of *Tetrahymena pyriformis* was determined by feeding the ciliate indigestible 0.49µm diameter fluorescently-labelled microspheres (FLMs) in pulse-chase experiments (see Section 2.4.2). Figure 3.1 shows a typical graphical result whereby plotting the number of FLMs/cell or vacuoles/cell against time shows three phases: (1) ingestion, (2) passage and (3) egestion. Phases 1 and 2 combined denote the VPT of the first fluorescent vacuole formed and this was consistently 30 min in this study. Note that egestion rate (Phase 3) appears slower than formation rate (Phase 1).

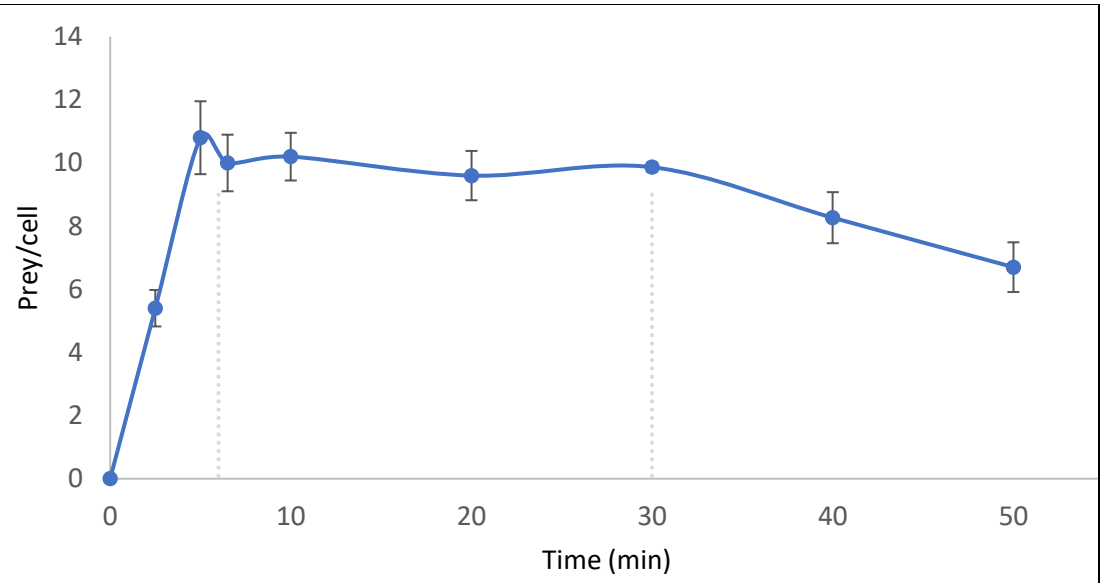


Figure 3.1 A typical pulse chase graph where T. pyriformis was fed fluorescently-labelled microspheres (FLMs) at a concentration of 2x10^7 particles/ml. Error bars = SEM. The number of FLMs/cell increases during the pulse due to ingestion (Phase 1). It then remains constant after the chase (dotted line at 6 min) due to the FLM-containing vacuoles passing through the cell (Phase 2). Finally, FLM-containing vacuoles begin to be egested by the cells and FLMs/cell decrease (Phase 3).

The following parameters were calculated from 13 pulse-chase experiments (Section 2.5) and 1 ingestion-only experiment (Section 2.4): FLM ingestion rate, vacuole formation rate, FLM egestion rate and vacuole egestion rate. Table 3.3 confirms that

vacuole egestion rate (average = 0.018 ± 0.003 vac/cell/min) is always slower than formation rate (average = 0.456 ± 0.024 vac/cell/min) which agrees with Thurman et al. (2010a) who suggested that this was due to vacuoles queuing at the single cytoproct prior to egestion. So, although the VPT for the first vacuole formed was consistently 30 min in this study, the VPT of subsequent vacuoles could be longer.

FLM Concentration (FLM/ml)	Ingestion rate (FLM/cell/min)	Vacuole formation rate (vac/cell/min)	Egestion rate (FLM/cell/min)	Vacuole egestion rate (vac/cell/min)
2.00E+07	1.93±0.19	0.33±0.04	0.11±0.01	0.007±0.002
2.00E+07	2.04±0.20	0.41±0.03	0.21±0.01	0.018±0.009
2.00E+07	2.16±0.23	0.36±0.02	0.10±0.00	0.015±0.000
2.00E+07	2.22±0.09	0.34±0.01	0.12±0.02	0.008±0.003
2.00E+07	2.69±0.19	0.44±0.04	0.09±0.03	0.015±0.000
2.00E+07	2.83±0.10	0.31±0.02	0.14±0.05	0.022±0.004
2.00E+07	2.83±0.18	0.55±0.01	0.21±0.06	0.020±0.003
2.00E+07	2.84±0.06	0.44±0.01	ND	ND
2.00E+07	2.91±0.13	0.51±0.04	0.21±0.01	0.037±0.011
2.00E+07	2.96±0.02	0.49±0.01	0.16±0.03	0.018±0.003
2.00E+07	3.14±0.03	0.41±0.01	0.26±0.04	0.035±0.003
2.00E+07	3.48±0.37	0.56±0.02	0.16±0.02	0.017±0.003
2.00E+07	3.52±0.47	0.59±0.05	0.18±0.01	0.022±0.002
2.00E+07	4.61±0.47	0.60±0.04	0.13±0.07	0.018±0.016

Table 3.3 FLM ingestion rates, vacuole formation rates, FLM egestion rates and vacuole egestion rates for Tetrahymena pyriformis when fed 0.49µm diameter indigestible FLMs at 2x10^7 particles/ml. Error = SEM. ND = not determined.

An average vacuole formation rate of 0.453 ± 0.026 vac/cell/min indicates that on average, *T. pyriformis* produces one vacuole in 2.21 min when fed 2x10^7 prey/ml (range: 1.67 min to 3.23 min). Hence a pulse at 6 min should allow for the formation of at least **two** complete vacuoles before the chase (relevant for digestion experiments, Section 3.4).

3.3. Phase 1: Ingestion

This study examined the processing of live/digestible RFP-expressing bacteria and compared it to other prey states. These included heat-killed/DTAF-stained bacteria (dead but digestible), FLMs (inert but indigestible) and live *Synechococcus* (live but indigestible). The mean biovolume (μm^3) of each prey state was significantly different to each other ($P \leq 0.05$) with the *Synechococcus* species (**Pico**) being the largest in biovolume, followed by RFP-expressing live bacteria (**Live**), then heat-killed/DTAF-stained bacteria (**Dead**), and finally FLMs (**FLM**) (Table 3.4). Therefore, an initial analysis of the effect of prey biovolume on instantaneous ingestion of prey and vacuole formation was required prior to analysing the effect of prey state.

Prey State	Code	n	Biovolume Mean (μm^3)	Biovolume Range (μm^3)
Inert/Indigestible	FLM	3	0.11 ± 0.028	0.065 – 0.410
Live/Indigestible	Pico	20	1.10 ± 0.100	0.588 – 2.342
Live/Digestible	Live	12	0.79 ± 0.075	0.272 – 1.701
Heat-killed/Digestible	Dead	7	0.27 ± 0.030	0.120 – 0.470

Table 3.4 Mean biovolumes of the four prey states (FLM, Pico, Live and Dead). Error = SEM. Biovolumes were significantly different from each other (P≤0.05).

3.3.1 Effect of prey biovolume on digestive vacuole formation and content

Prey ingestion and vacuole formation data for *T. pyriformis* at 5 min (initial prey concentration at $2x10^7$ particles/ml) were available from 73 experiments (pulse-chase and ingestion-only experiments) which encompassed the four prey states (Table 3.4). The three parameters deduced at 5 min were prey/cell (P/C), vacuoles/cell (V/C), and prey/vacuole (P/V). The latter parameter was calculated by dividing P/C by V/C for each replicate and it represents the number of prey within one complete vacuole.

Regression analysis of prey biovolume against each of the three parameters (for all 73 experiments; 4 prey states) showed no evidence that, within the first 5 min of feeding, prey biovolume affected any of these parameters (Figure 3.2).

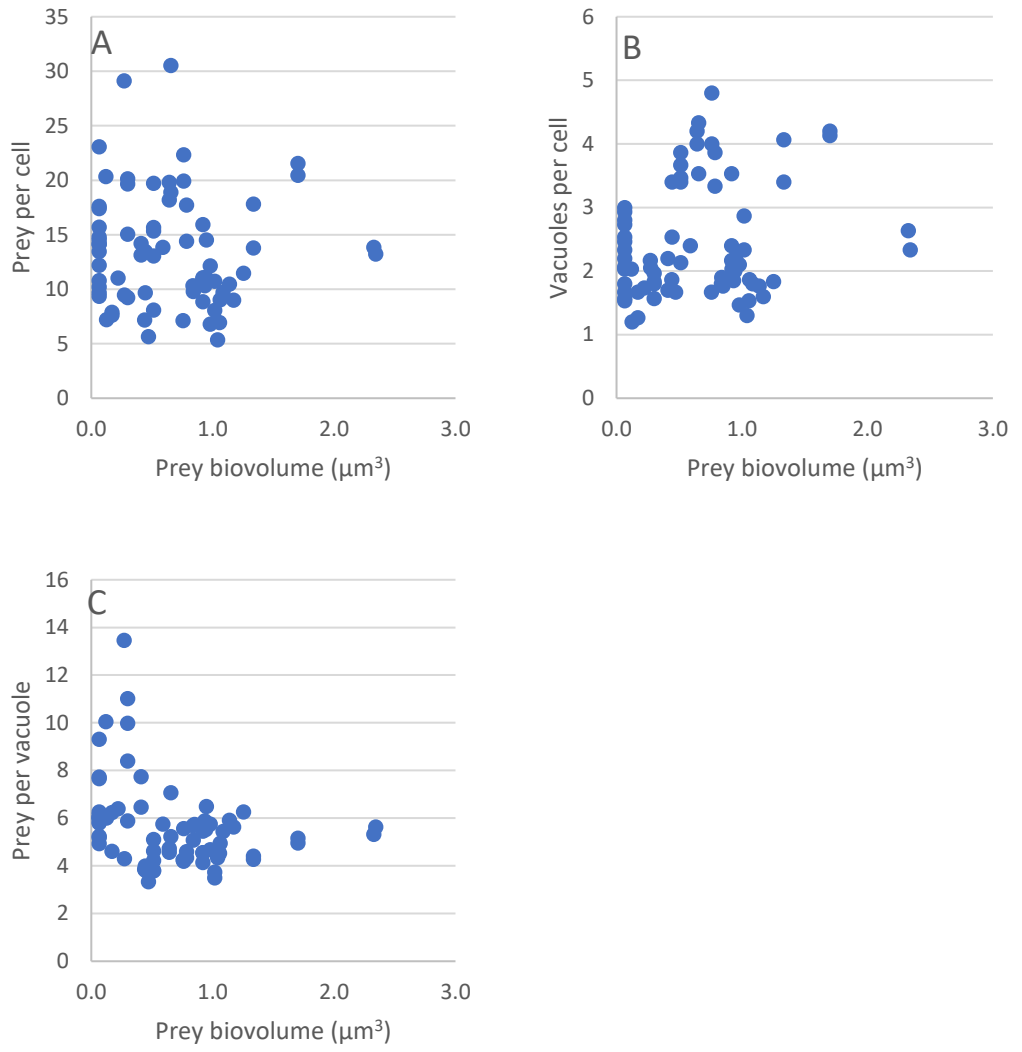


Figure 3.2 Analysis of prey biovolume against each of the three parameters (n=73); A) prey per cell, B) vacuoles per cell, C) prey per vacuole after 5 min of T. pyriformis feeding. There was no effect of prey biovolume on any of the three parameters.

It was noted that there was a large variation in prey/vacuole values when the prey biovolume was less than 0.5µm^3 but not above this biovolume (Figure 3.2C). When these data were separated out for each prey state (Figure 3.3) it became clear that the observed variation was mainly due to the Dead prey with prey/vacuole values ranging from *ca.* 3 to 14 (Figure 3.3B). Conversely, Picos (all >0.5 µm^3) only ranged from *ca.* 4 to 6 (Figure 3.3D), Live bacterial prey (which included cells ≤0.5 µm^3) ranged from *ca.* 4 to 7 (Figure 3.3C), and FLMs (all ≤0.5 µm^3) ranged from *ca.* 5 to 9 prey/vacuole (Figure 3.3A).

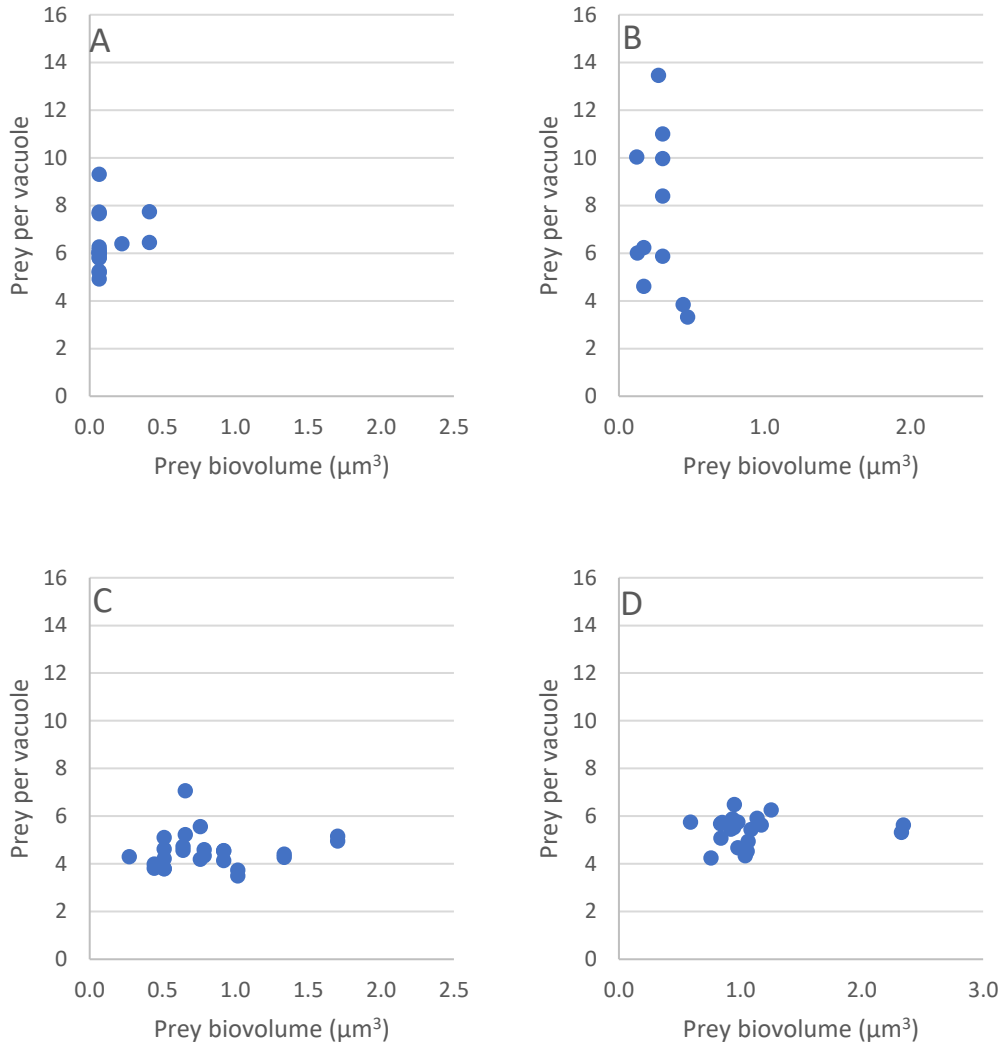


Figure 3.3 Analysis of prey biovolume against prey/vacuole for the four prey states A) FLMs, B) Dead, C) Live and D) Pico after 5 min of T. pyriformis feeding. Raw data were used, with each triplicate used as an individual data point.

Although the prey biovolume did not directly influence the number of prey per vacuole (Figure 3.2C and Figure 3.3) it did influence the total volume (µm^3) of prey within those vacuoles (Figure 3.4). In other words, larger prey gave rise to larger vacuoles. For example, the vacuoles formed with the largest prey (Pico 20 at 2.34 µm^3) contained *ca.* 5 cells which was equivalent to the number of prey/vacuole obtained with smaller Picos (Figure 3.3D), but because of its size Pico 20 yielded the largest vacuoles (Figure 3.4).

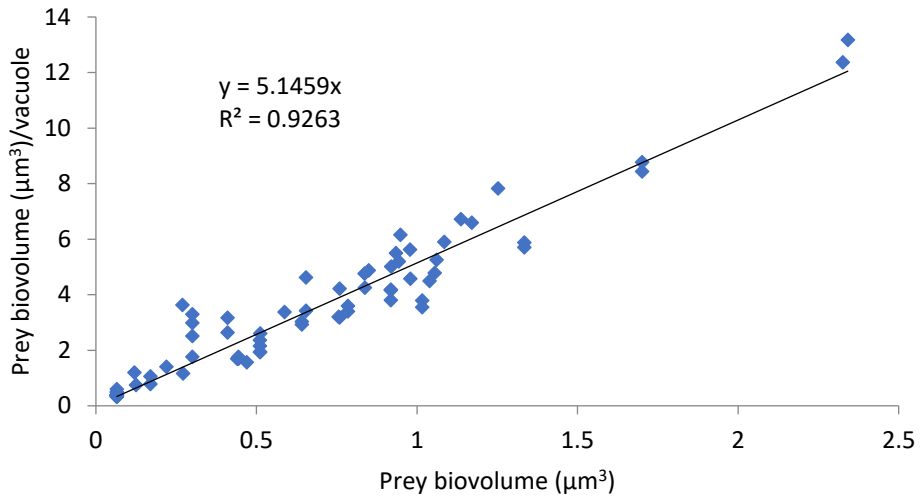


Figure 3.4 Analysis of prey biovolume against prey biovolume/vacuole after 5 min of T. pyriformis feeding showing that larger prey give rise to larger vacuoles.

3.3.2 Effect of prey state on digestive vacuole formation and content

3.3.2.1 General overview of between-prey state analysis

The effect of the four prey states (fed at $2x10^7$ cells/ml) on the three parameters (prey/cell, vacuoles/cell and prey/vacuole) after 5 min of feeding by *T. pyriformis* were evaluated and the average data presented in Table 3.5.

	Prey/cell	Vacuoles/cell	No. Prey/vacuole	Prey biovolume (μm^3)/vacuole
FLM	13.85±0.83	2.19±0.12	6.39±0.27	0.76±0.21*
Dead	13.66±2.31	1.73±0.09	7.53±0.98	1.93±0.31*
Live	15.82±1.04	3.40±0.15*	4.58±0.15*	3.62±0.37*
Pico	10.31±0.56*	1.89±0.07	5.41±0.14*	5.98±0.57*

*Table 3.5 The effect of four prey states (FLM, Dead, Live and Pico) on prey/cell, vacuoles/cell and prey/vacuole (as number of prey and biovolume of prey) after 5 min feeding by T. pyriformis. Error = SEM. *significantly different to all other prey states (P≤0.05).*

The processing of FLMs and Dead prey appeared equivalent with *T. pyriformis* ingesting *ca.* 14 prey in 5 min and depositing them into two vacuoles, yielding *ca.* 7 prey/vacuole, but vacuoles containing Dead prey were significantly larger (Table 3.5). The processing of both live prey states (Live bacteria and Pico) was different.

Specifically, prey/vacuole values were significantly lower than those with FLMs and Dead prey (Table 3.5), for different reasons.

Significantly fewer Pico cells (*ca.* 10 cells) were ingested by *T. pyriformis* in 5 min but they were still deposited into 2 vacuoles, yielding *ca.* 5 prey/vacuole and yielding the largest vacuoles of all prey states (Table 3.5). Conversely, Live prey were ingested to the same extent as FLM/Dead prey but they were deposited into more vacuoles, yielding the lowest prey/vacuole recorded but which were the second largest in size (Table 3.5). This suggests that live prey are processed differently to dead or inert prey at the ingestion stage, i.e. deposition of prey into vacuoles.

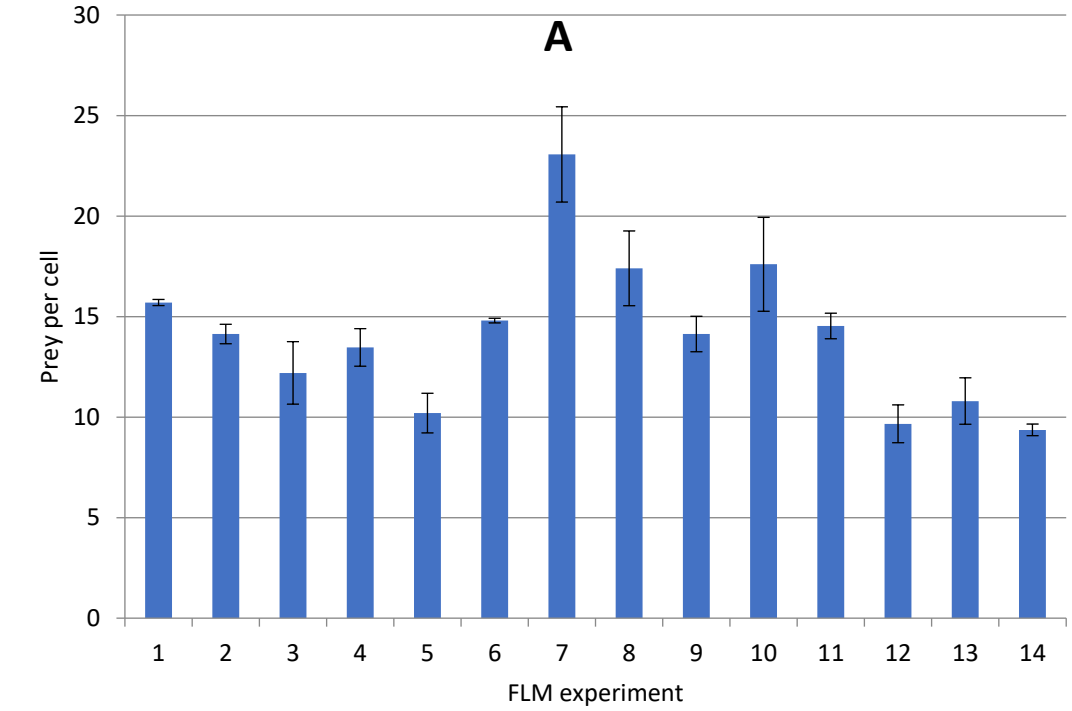
3.3.2.2 Effect of bacterial strain within each prey state

The level of inherent variation between experiments was explored with the FLM data and then the effect of bacterial strain was explored within each of the other three prey states.

3.3.2.2.1 FLMs

Fourteen experiments fed inert 0.49 µm diameter FLMs to *T. pyriformis*. Even though these FLMs were identical to each other, and all experiments employed a concentration of $2x10^7$ particles/ml, there was inherent variation in prey/cell, vacuoles/cell and prey/vacuole between experiments (Figure 3.5).

Prey/cell ranged from 9.37 (experiment 14) to 23.07 (experiment 7) (Figure 3.5A) yielding a net variation of 13.7 prey/cell. Vacuoles/cell ranged from 1.53 (experiment 2) to 3.00 (experiment 7) yielding a net variation of 1.47 vacuoles/cell (Figure 3.5B). The number of prey/vacuole ranged from 4.93 (experiment 5) to 9.31 (experiment 2) yielding a net variation of 4.38 prey/vacuole (Figure 3.5B). These variations were considered inherent to the use of different *T. pyriformis* cultures. Although the *T. pyriformis* population is clonal, slight variations in each culture's growth stage, level of satiation etc. were to be expected at the start of each experiment.



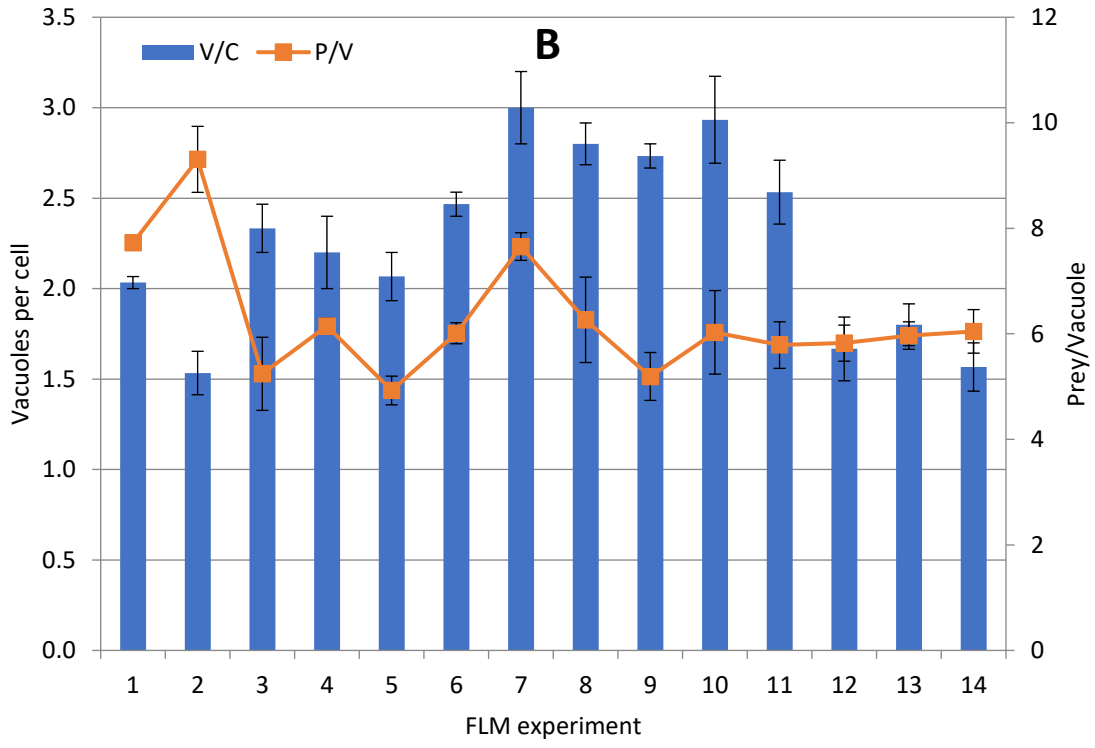


Figure 3.5 Prey per cell (A), vacuoles per cell and prey per vacuole (B) data for T. pyriformis feeding for 5 min on FLMs (0.49 µm diameter, 2x10^7 particles/ml) in 14 experiments. The average of 3 replicates for each experiment is presented. Error bars = SEM.

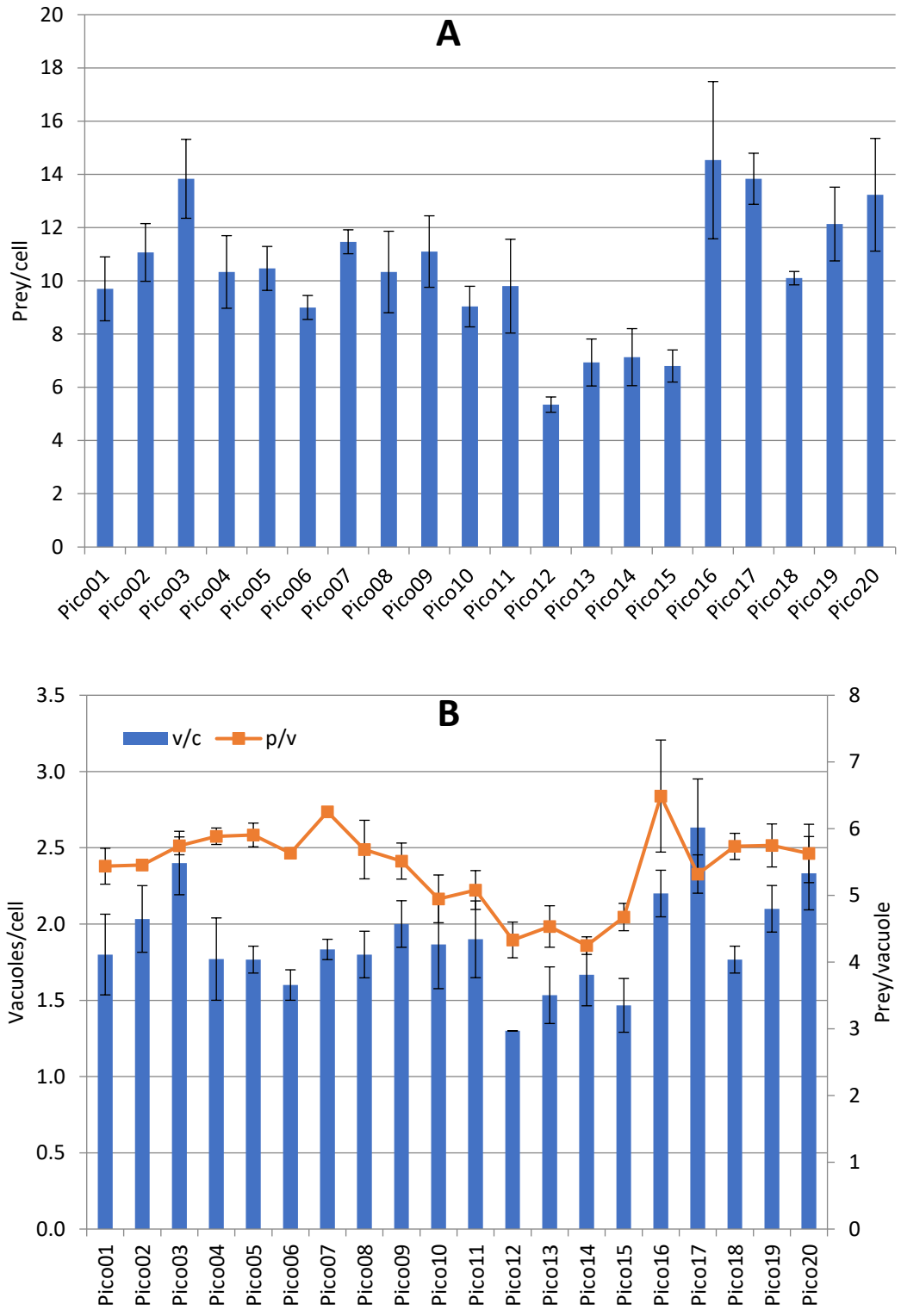


Figure 3.6 Prey per cell (A), vacuoles per cell and prey per vacuole (B) data for T. pyriformis feeding for 5 min on 20 strains of Synechococcus (Picos). The average of 6 replicates for each strain is presented. Error bars = SEM.

3.3.2.2.2 Picos

Examination of the processing of the twenty Pico strains (Figure 3.6) shows that the prey/cell ranged from 5.35 (strain 12) to 14.53 (strain 16) (Figure 3.6A), vacuoles/cell ranged from 1.3 (strain 12) to 2.6 (strain 17) (Figure 3.6B) and prey/vacuole ranged from 4.25 (strain 14) and 6.49 (strain 16) (Figure 3.6B). Comparing net variations to those inherent experimental variations (with FLMs) suggests that variation around *ca.* 2 vacuoles/cell is inherent whereas less variation is seen with regards to prey/cell and consequently prey/vacuole. This possibly indicates tighter control of these latter parameters by the ciliate, further suggesting that Picos might directly influence ingestion.

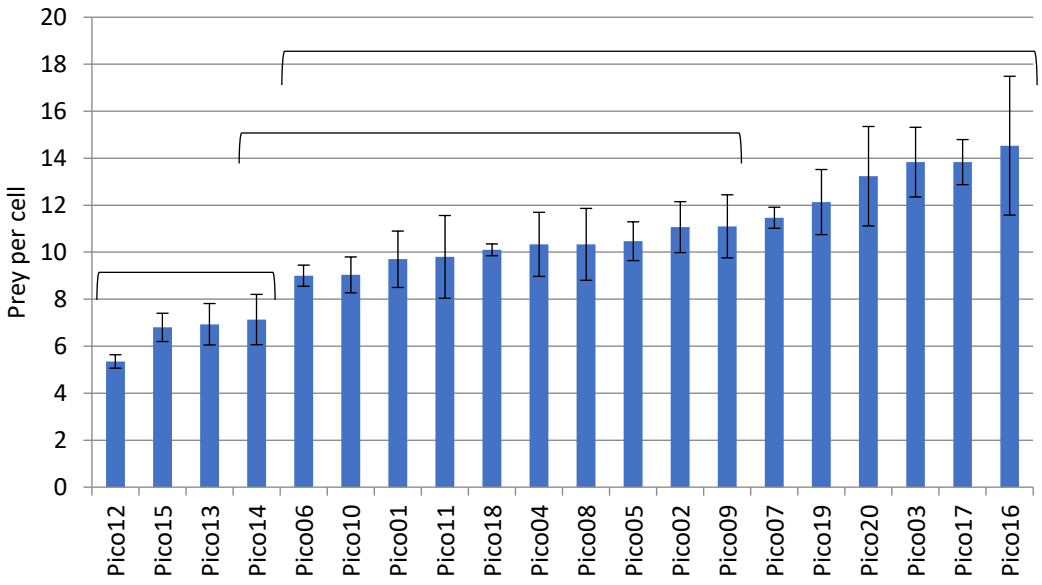


Figure 3.7 Prey per cell data for T. pyriformis feeding for 5 min on 20 strains of Synechococcus (Picos) with the least favoured strain on the left and the most favoured strain on the right. The average of 6 replicates for each strain is presented. Prey were divided into groups that were not significantly different to each other (groups are denoted by the symbol (⌈ ⌉). Error bars = SEM.

Figure 3.7 shows the same data as in Figure 3.6A but the prey are ranked from least favoured (Pico 12) to most favoured (Pico 16) however, significance tests (P≤0.05) only resulted in only one clear grouping (Picos 12, 13, 14 and 15) which separated out

from the rest (where there was much overlapping). Prey biovolume again played no part in these groupings and this can be highlighted most strongly in Figure 3.7 whereby Pico 3 is the smallest cell at 0.59 μm^3 and Pico 20 is the largest cell at 2.34 μm^3 yet their ingestion by the ciliate is equivalent (but the latter will produce larger vacuoles). The fact that only three strains are non-lysogenic (Strains 6, 8 and 17) and the rest contain prophage also does not appear to affect ingestion.

3.3.2.2.3 Live bacteria

Examination of the processing of the twelve live bacterial strains (Figure 3.8) shows that the prey/cell ranged from 9.4 (*A. hydrophila*) to 24.5 (*P. aeruginosa*) (Figure 3.8A), vacuoles/cell ranged from 2.6 (*A. hydrophila*) to 4.4 (*S. enterica* 74) (Figure 3.8B) and prey/vacuole ranged from between 3.62 (*A. hydrophila*) and 6.15 (*P. aeruginosa*) (Figure 3.8B). Comparing net variations to those inherent experimental variations (with FLMs) suggest that variation in prey/cell is inherent, i.e. the variation recorded between strains could be 'experimental error' which might explain why Live prey/cell values are not significantly different to those with FLMs and Dead cells (Table 3.5). The variation around *ca.* 3 vacuoles/cell is also inherent (and not significantly different) but the value is significantly higher than the *ca.* 2 vacuoles/cell recorded with other prey states (Table 3.5). Variation in prey/vacuole was much reduced compared to FLMs suggesting tighter control of this parameter by the ciliate, further suggesting that Live bacteria might directly influence ingestion; specifically influencing vacuole formation rate.

Figure 3.9 shows the same data for prey per vacuole as in Figure 3.8B but the bacterial strains are ranked from those having the least prey/vacuole (*A. hydrophila*) to those having the most (*P. aeruginosa*). However, differences were not significant and due to inherent experimental variation (as seen with the FLMs). The total biovolume of prey within each vacuole is also shown but there is no correlation between prey biovolume and prey/vacuole. Another factor, currently unknown, must be responsible

for controlling vacuole formation and filling. To explore whether the mechanism might involve bacterial cell surface receptors the Live data were compared to those for Dead cells as the process of heat-killing cells is known to damage/destroy surface receptors.

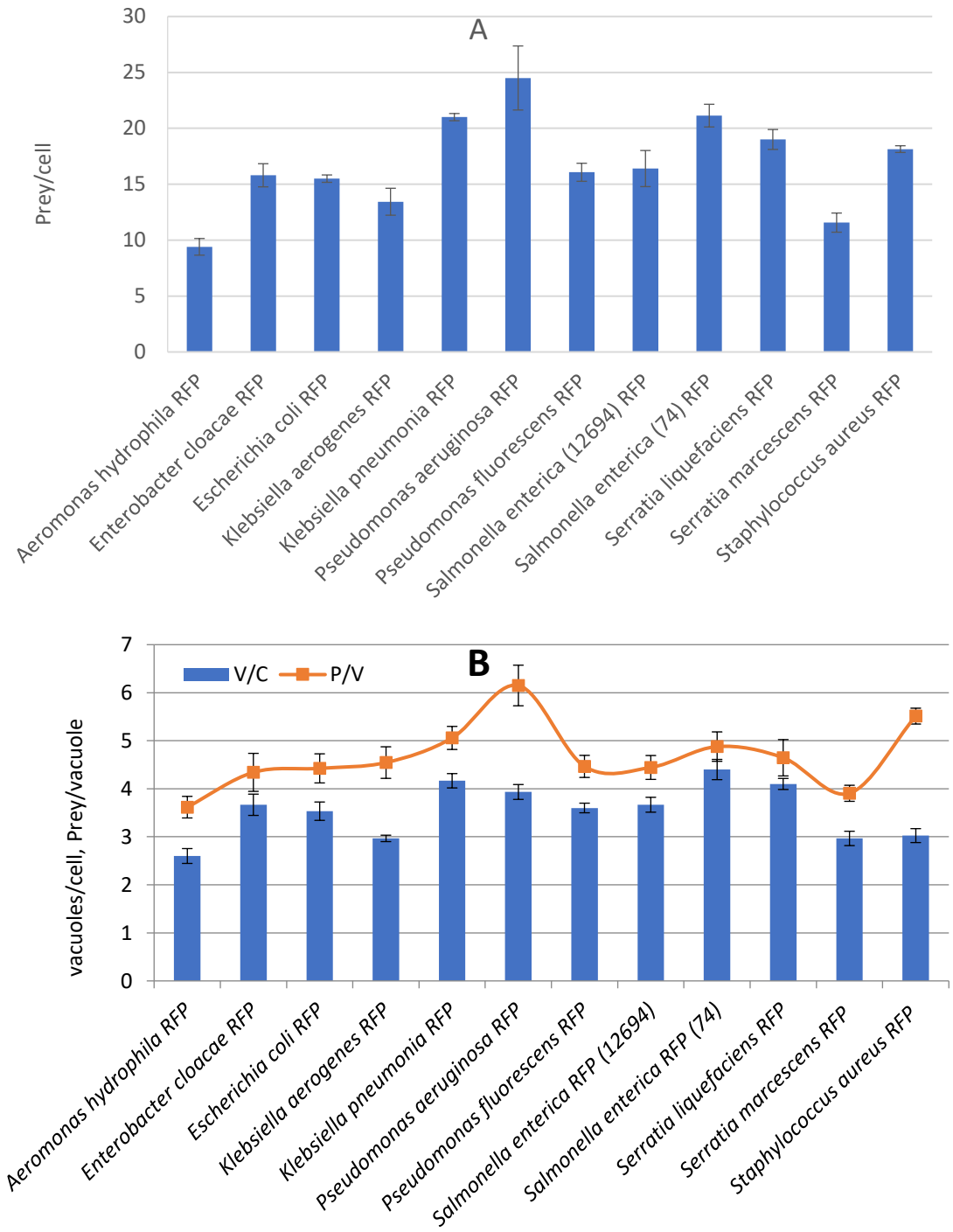


Figure 3.8 Prey per cell (A), vacuoles per cell and prey per vacuole (B) data for T. pyriformis feeding for 5 min on 12 live RFP-fluorescing strains of bacteria. The average of 6 replicates for each strain is presented. Error bars = SEM.

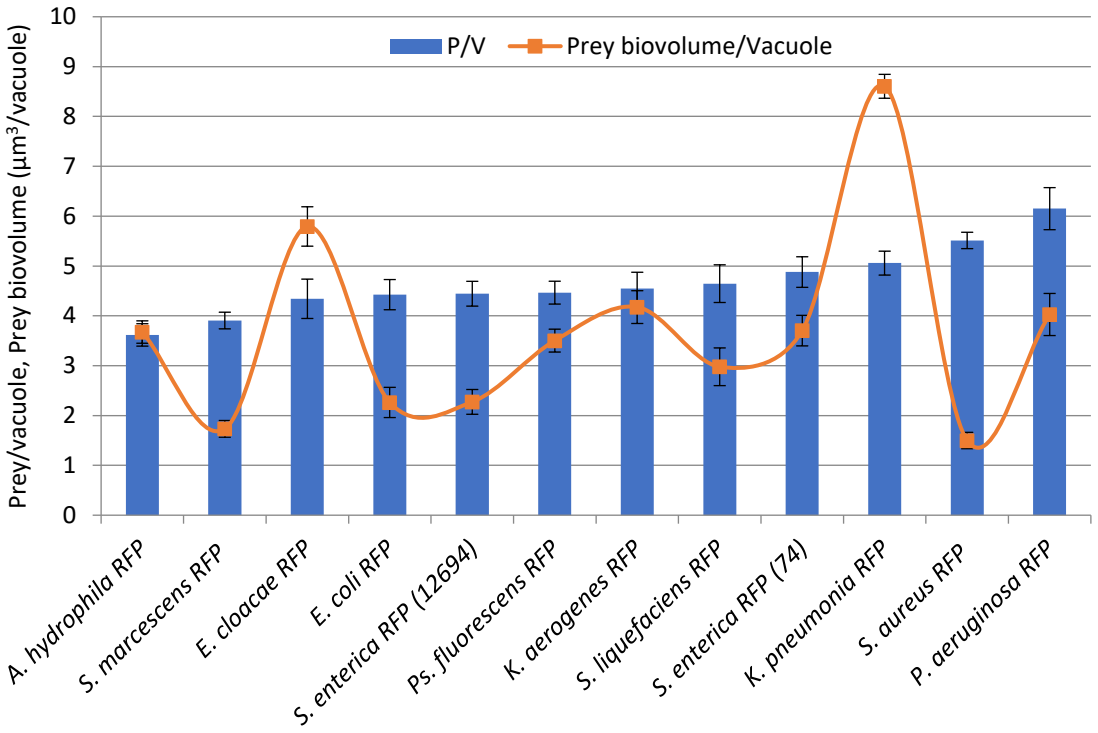


Figure 3.9 Prey per vacuole data for T. pyriformis feeding for 5 min on 12 strains of Live RFP-fluorescing bacteria with the strain yielding the least prey/vacuole on the left and the strain yielding the most prey/vacuole on the right. The biovolume (µm3) of prey within each vacuole is also shown. The average of 6 replicates for each strain is presented. Error bars = SEM.

3.3.2.2.4 Dead bacteria

The effect of heat-killing cells is best seen in Figure 3.10 which directly compares parameters obtained with four strains in Live and Dead states. The Live cells induced the formation of between 3.0 and 4.4 vacuoles/cell (Figure 3.10A) but all vacuoles contained 4.5-5.5 prey (Figure 3.10B). Conversely, Dead cells induced the formation of only 1.2-2.2 vacuoles/cell (Figure 3.10A) but the number of prey/vacuole varied considerably (6 to 13.5 prey/vacuole) (Figure 3.10B). *S. aureus* was the only prey to yield equivalent prey/vacuole with both states.

There was no obvious pattern seen with regards to the effect of prey state on prey/cell, which ranged from 8.3-21.3 irrespective of cell state (Figure 3.10C). This net variation

(12.8 prey/cell) was similar to that obtained with 0.49µm^3 diameter FLMs (13.7 prey/cell) indicating that the observed variation could be due to experimental error.

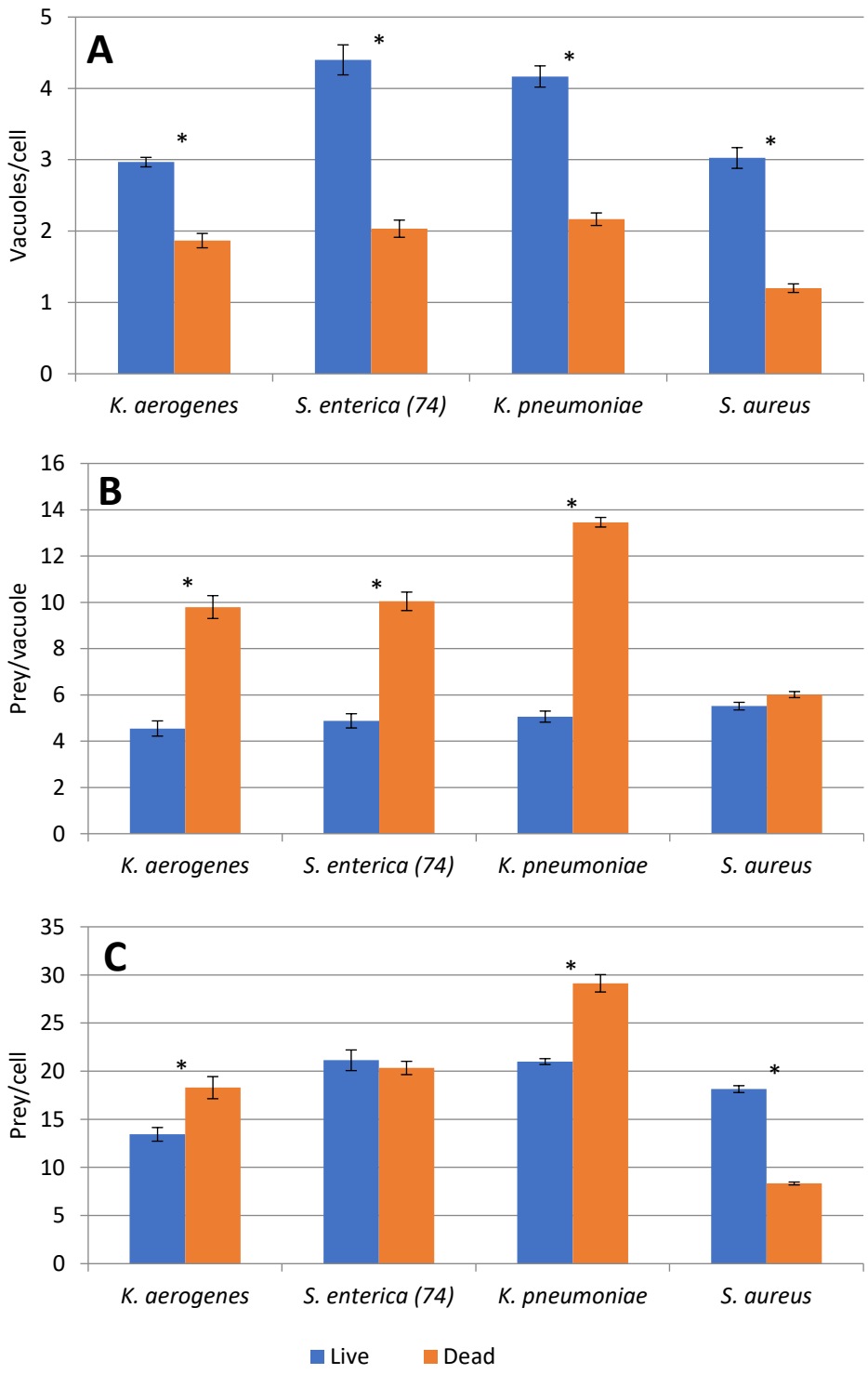


*Figure 3.10 Vacuoles per cell (A), prey per vacuole (B) and prey/cell (C) data for T. pyriformis feeding for 5 min on four strains of bacteria in both Live and Dead states. Error bars = SEM. *Significant difference between Live and Dead data (P≤0.05).*

However, there are clear trends with regards to the formation of vacuoles (Live cells always induced a higher number of vacuoles/cell, Figure 3.10A) and the number of prey deposited into those vacuole (Live cells always induce a lower number of prey/vacuole, Figure 3.10B), suggesting receptor-based mechanisms are involved in vacuole formation and filling.

Looking more closely at vacuoles/cell, Figure 3.11 shows data for all Dead and Live species tested. There is an obvious split between all Dead cells being on the left of the bar chart (lower number of vacuoles/cell) and all Live cells being on the right (higher number of vacuoles/cell) so the trend displayed in Figure 3.10 for four bacterial species appears to hold for other species. Variation in vacuoles/cell for Dead cells (*ca.* 1-2) was not significant and within the range of experimental error. In general, there appeared to be little effect of prey species or Gram status (Gram-positive: *S. aureus*, *L. monocytogenes* & *L. fermentum*) on vacuole formation whether the cells were Dead or Live.

Prey/vacuole results with Dead cells were highly variable (Figure 3.12) and outside the limits of experimental error. This was opposite to that found with Live bacteria whereby prey/vacuole did not differ significantly with prey strain (Figure 3.9). Data therefore suggest that strain differences may be being recognised by the ciliate only if the cells have been heat-killed, possibly suggesting that the heat-killing process leads to exposure of more deeper (species-specific) receptors in the cell membrane.

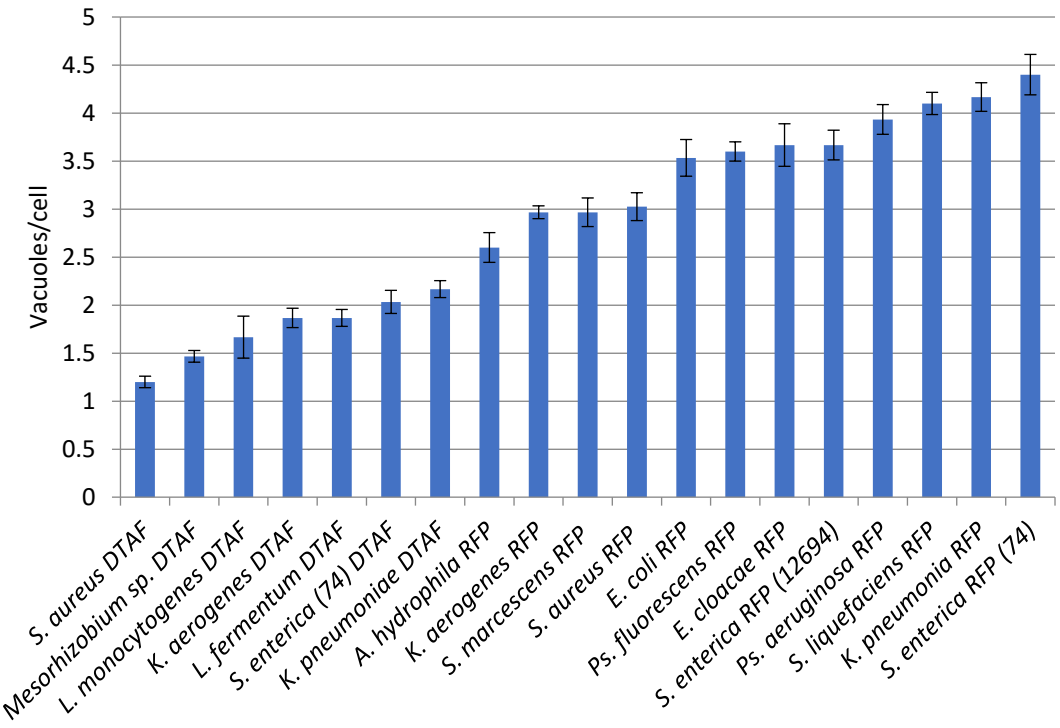


Figure 3.11 Vacuole per cell data for T. pyriformis feeding for 5 min on Live (RFP) and Dead (DTAF) bacteria with the strain yielding the least vacuoles/cell on the left and the strain yielding the most vacuoles/cell on the right. Error bars = SEM.

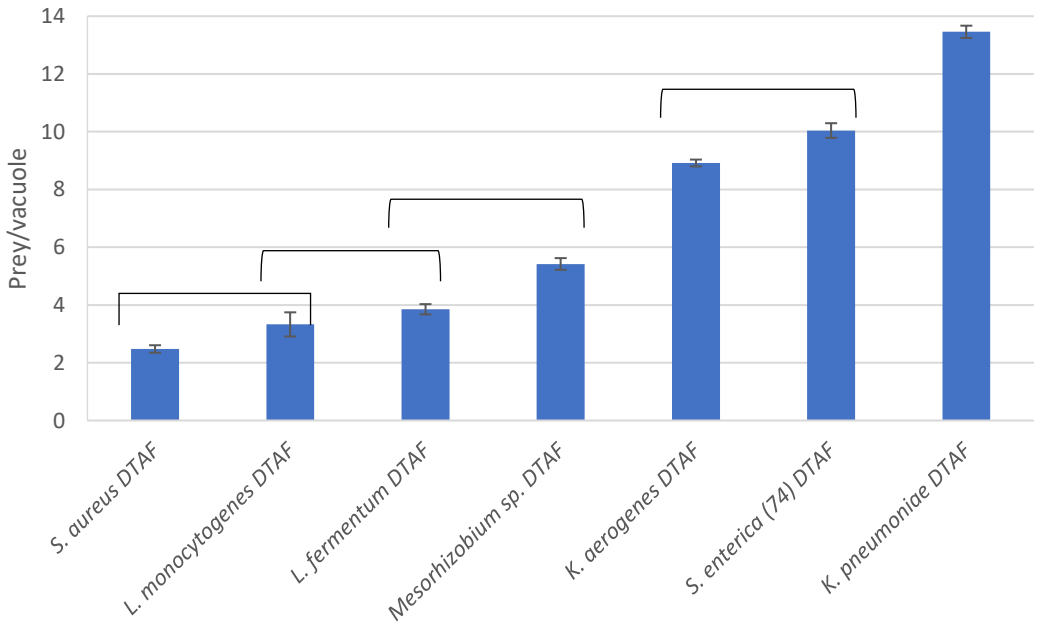


Figure 3.12 Prey per vacuole data for T. pyriformis feeding for 5 min on Dead (DTAF) bacteria with the strain yielding the least prey/vacuole on the left and the strain yielding the most prey/vacuole on the right. Error bars = SEM. Prey were divided into groups that were <u>not</u> significantly different to each other (groups are denoted by the symbol ⌐¬).

3.3.2.3 Effect of bacterial concentration within each prey state

The Live and Dead forms of *S. aureus* were fed to *T. pyriformis* at various concentrations to determine the maximum values of prey/cell, vacuoles/cell and prey/vacuole at 5 min and to evaluate whether *T. pyriformis* was at maximum feeding capacity with the $2x10^7$ prey/ml used in the ingestion experiments (Section 3.3.2.2). In those ingestion experiments the two states of *S. aureus* did not yield a significant difference in prey/vacuole values (Figure 3.10B). Their inclusion here would see whether this difference could be exaggerated at higher prey concentration. Picos 3 and 20 were also included in this experiment to test whether cell biovolume played a role in vacuole formation and content at different prey concentrations; with Pico 3 being the smallest cell at 0.59 μm^3 and Pico 20 being the largest cell at 2.34 μm^3. Their inclusion also allowed investigation of whether Picos affected vacuole formation rates (as do the other Live bacteria) at a different prey concentration to $2x10^7$ cells/ml. FLMs were not included in these experiments so data were taken from Table 3 of Thurman et al. (2010a) to provide maximum values for the parameters prey/cell, vacuoles/cell and prey/vacuole for this ciliate feeding on 0.49µm diameter FLMs at the same experimental temperature (Table 3.6, Appendix C Figure C.1)

Data showed that all four prey yielded hyperbolic relationships for each parameter against prey concentration (Figure 3.13) although the goodness of fit was lower with Dead *S. aureus*. Maximum values of prey/cell, vacuoles/cell and prey/vacuole are presented in Table 3.6 along with their corresponding values at $2x10^7$ prey/ml from the independent ingestion experiments (Section 3.3.2.2). Further data on Ks values can be found in Appendix C Table C.3.

Both Pico strains had induced their maximum number of vacuoles/cell (*ca.* 2.4 vacuoles/cell) in *T. pyriformis* at a prey concentration of $2x10^7$ prey/ml in the ingestion experiments (Table 3.6). Above this prey concentration the number of prey/cell kept

increasing to a maximum of 15-18 prey/cell but these cells were enclosed in this limited number of vacuoles so prey/vacuole increased to a maximum of *ca.* 7 prey/vacuole (compared to the *ca.* 5.5 recorded at $2x10^7$ prey/ml) (Table 3.6, Figure 3.13C). Both Pico strains also resulted in very similar maximum number of vacuoles/cell (2.50 and 2.57 vacuoles/cell respectively). Both also, on average, had a 10-fold lower Ks value for vacuole/cell than Live and Dead *S. aureus* (Appendix C Table C.3), indicating that maximum vacuole production for Picos was reached at much lower prey concentrations than with the other bacteria. This was very similar to that obtained with FLMs (Appendix C Table C.3) suggesting that vacuole formation rates over 5 min were not influenced by the presence of Picos. However, the maximum number of prey/vacuole was much higher for FLMs, suggesting that there is possibly less control with regards to the filling of vacuoles with this prey state.

Maximum value	Prey/cell	Vacuoles/cell	Prey/vacuole
Dead *S. aureus*	18.30±3.20	1.99±0.20	9.54±0.36
Live *S. aureus*	27.34±1.70	3.36±0.15	7.29±0.17
Pico 3	18.17±0.38	2.50±0.02	7.16±0.13
Pico 20	15.32±0.11	2.30±0.03	6.58±0.10
*FLM	41.80±1.41	2.57±0.28	19.53±0.43
at $2x10^7$ prey/ml			
Dead *S. aureus*	8.33±0.15	1.20±0.06	6.01±0.13
Live *S. aureus*	18.14±0.36	3.03±0.15	5.51±0.16
Pico 3	13.83±1.48	2.40±0.21	5.74±0.13
Pico 20	13.23±2.12	2.33±0.24	5.63±0.44
FLM	13.85±0.83	2.21±0.12	6.39±0.27

*Table 3.6 Mean values of prey/cell, vacuoles/cell and prey/vacuole at maximum and at $2x10^7$ prey/ml when T. pyriformis feeds for 5 min on S. aureus Live and Dead, Pico 3 and Pico 20. *using data for 0.49µm diameter FLMs in Table 3 of Thurman et al. (2010a). Error = SEM.*

Live *S. aureus* induced the formation of 3.03 vacuoles at $2x10^7$ prey/ml which was close to the maximum (3.36); but not as close as the Picos (Table 3.6). Above this prey concentration the prey/cell increased to a maximum of 27 prey/cell but once again these cells were enclosed in this limited number of vacuoles so prey/vacuole did

increase to a maximum of *ca.* 7 prey/vacuole. This was remarkably similar to the maximum prey/vacuole induced by the Picos (Table 3.6, Figure 3.13C).

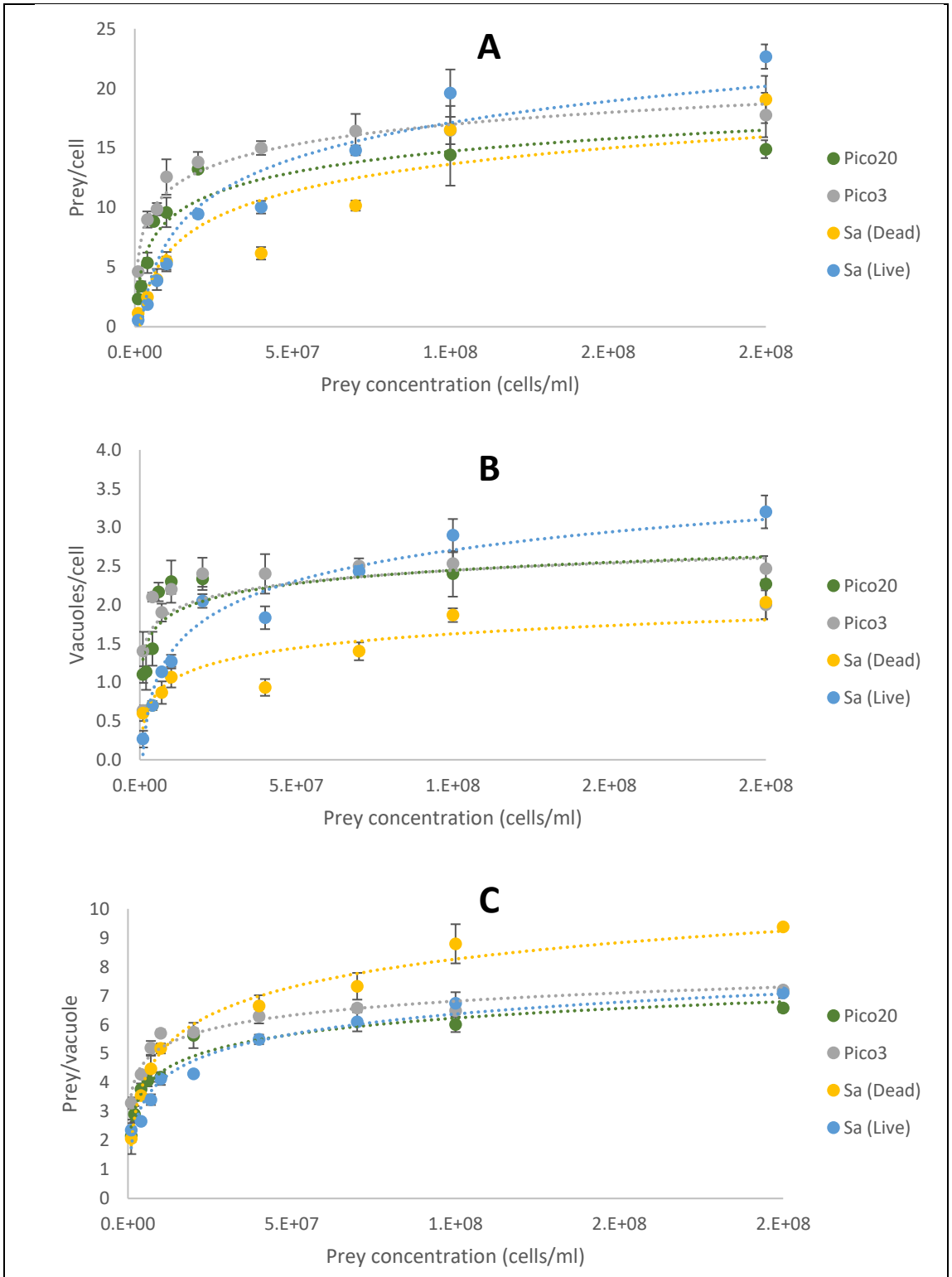


Figure 3.13 Prey per cell (A), vacuoles per cell (B) and prey/vacuole (C) data for T. pyriformis feeding for 5 min on various concentrations (cells/ml) of S. aureus Live and Dead, Pico 3 and Pico 20. The average of 3 replicates for each strain is presented. Error bars = SEM.

The response of Dead *S. aureus* was different to the other three prey strains. Firstly, it did not induce a near maximum number of vacuoles/cell in *T. pyriformis* at $2x10^7$ cells/ml in the ingestion experiments, but it did increase to a maximum of 2 vacuoles/cell at higher prey concentration (Table 3.6). Secondly, the maximum of *ca.* 9.5 prey/vacuole was significantly higher ($P \leq 0.01$) than the *ca.* 7 prey/vacuole recorded with its live counterpart and shows that the effect of prey state on this parameter can be exaggerated at high concentration (Table 3.6, Figure 3.13C).

Time constraints did not allow for further tests with other bacterial strains to evaluate the suggestion that all live prey (bacteria and Picos) might yield a maximum of 7 prey/vacuole in 5 min whereas Dead prey might yield different maximum prey/vacuole values. Instead, experiments evaluated whether the 'filling' of vacuoles was constant over time.

3.3.2.4 Effect of time on digestive vacuole formation and content

Staphylococcus aureus (Live and Dead) and Picos 3, 5 and 20 were fed to *T. pyriformis* at a concentration of $5x10^6$ prey/ml. Samples were taken every 5 min up to 20 min and prey/cell, vacuoles/cell and prey/vacuole were determined. Twenty-minutes was considered the maximum time for this experiment because Thurman et al. (2010a) recorded a minimum VPT of 25 min with this ciliate strain. The rationale for the chosen prey concentration was two-fold. Firstly, a high prey concentration ($2x10^7$ prey/ml) could possibly yield over 10 digestive vacuoles/cell by 20 min and could lead to counting errors for parameters such as prey/cell and prey/vacuole. Secondly, it was noted in Figure 3.13B that Picos 3 and 20 reached their maximum vacuole/cell at low prey concentrations ($4\text{-}6x10^6$ cells/ml) compared to Live or Dead *S. aureus* ($>5x10^7$ cells/ml). This required corroboration so Pico 5 (at 1.14 μm^3) was included in the experiment. The data gathered here at 5 min are not included in Figure 3.13 and represent an independent experiment

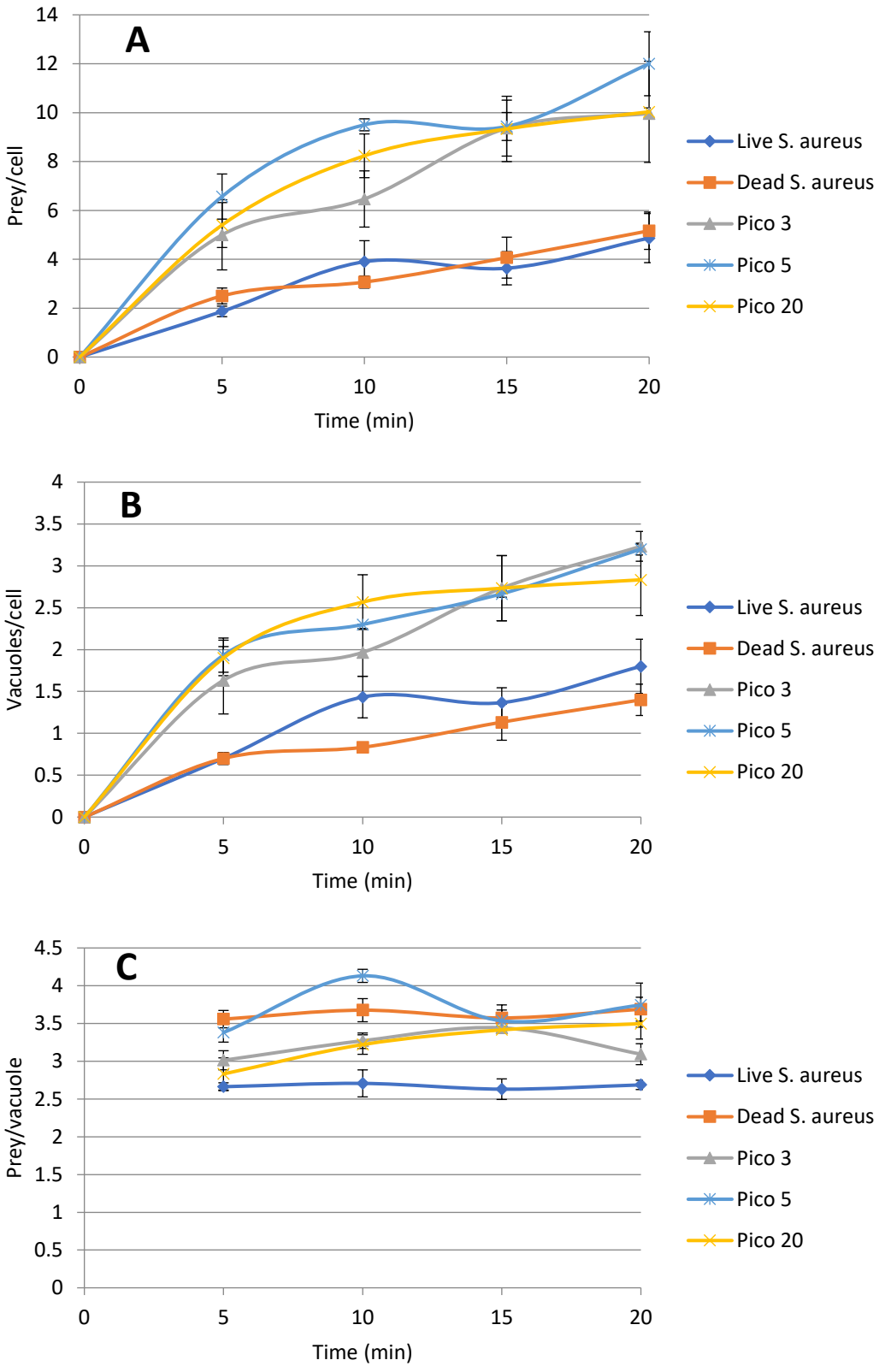


Figure 3.14 Changes in prey/cell (A), vacuoles/cell (B) and prey/vacuole (C) when T. pyrifomis feeds on S. aureus (Live and Dead) and Pico strains 3, 5 and 20 (at $5x10^6$ prey/ml) for 20 min. Error bars = SEM

The most striking feature of this experiment was that prey/vacuole values were constant over time (Figure 3.14C), i.e. newly formed vacuoles were consistently filled with a similar number of prey cells to older vacuoles, irrespective of the size of the prey, whether they were live (Live *S. aureus* and Picos) or dead, or even whether they were digestible (Live and Dead *S. aureus*) or indigestible (Picos). The inclusion of Pico 5 also confirmed that Picos are at near maximum vacuole formation at lower prey concentrations (Figure 3.14B).

The number of prey/vacuole did however differ between prey states with the Live *S. aureus* yielding a significantly lower number of prey/vacuole compared to the other four prey which were equivalent (Figure 3.14C). Because the Live and Dead *S. aureus* are digestible, the difference in prey/vacuole (which is a net value) may be due to differential digestion, i.e. Live *S. aureus* being digested quicker in the digestive vacuole – this was evaluated (see Section 3.4).

3.4. Phase 2: Prey digestion

3.4.1 Digestive period

Twelve live RFP-expressing bacterial strains (two experiments) and seven heat-killed strains (one experiment [except for *K. aerogenes* which was in four experiments]) were used to investigate the digestion process of *T. pyriformis* using pulse-chase experiments (see Section 2.5). Below shows the results of a typical pulse-chase experiment involving indigestible prey (FLMs) as a control (to determine VPT) and a digestible prey (live *S. enterica* [12694]). Both prey were ingested in Phase 1 (sampled at 0, 2.5 and 5 min). A chase at 6 min initiates Phase 2 whereby indigestible FLMs persist within the cell whereas digestible prey decline immediately. Considering the FLM-containing vacuoles indicate that no egestion is taking place until 30 min, the loss of *S. enterica* (12694) in Phase 2 is solely due to digestion. Note that digestion only appeared to occur up to 18 min, after which the number of prey/cell did not differ significantly. This is taken to indicate a digestive period (DP) of 18 min. Also, none of the combinations resulted in zero/prey/vacuole over the DP which is not surprising as this is an average population response (Thurman et al. 2010a).

The average digestion period for all 12 Live prey was 17.21±0.37 min (range 14-20 min) while that for the 7 Dead prey was significantly shorter at 13.85±0.53 min (range 11-16 min) ($P \leq 0.05$) (Table 3.7). There was no significant difference discerned between the strains within the Live prey state implying the range (14-20 min) might be due to standard experimental error. Significance testing could not be performed within the Dead prey state as all dead prey (apart from *K. aerogenes*) involved only one experiment and each of the three replicas had identical DPs so SEM = 0.

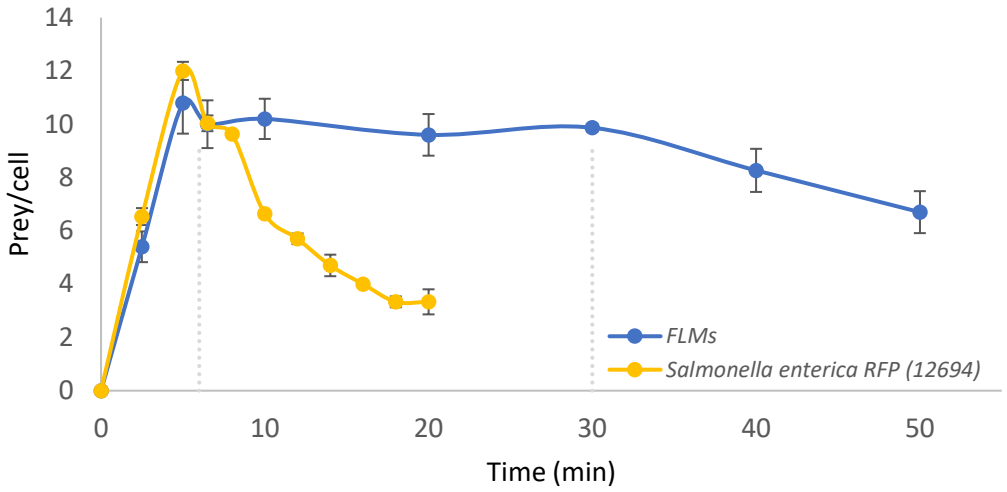


Figure 3.15 Processing of digestible S. enterica (12694) RFP (12694 RFP by T. pyriformis overlaid by the processing of indigestible FLMs. The number of S. enterica /cell increased during the pulse due to ingestion (Phase 1). It then decreased immediately after the chase due to digestion alone (Phase 2). Digestion continued up until ca. 18 min and then digestion appeared to stop. Error bars = SEM.

Prey	Prey state	Digestive period (min)
Aeromonas hydrophila	Live	16.0±0.00
Enterobacter cloacae	Live	15.0±0.45
Escherichia coli	Live	17.0±0.45
Klebsiella aerogenes	Dead	15.0±0.41
Klebsiella aerogenes	Live	18.0±1.12
Klebsiella pneumonia	Dead	13.5±0.00
Klebsiella pneumonia	Live	17.0±1.23
Lactobacillus fermentum	Dead	14.0±0.00
Listeria monocytogenes	Dead	15.0±0.00
Mesorhizobium sp.	Dead	11.0±0.00
Pseudomonas aeruginosa	Live	20.0±0.00
Pseudomonas fluorescens	Live	16.0±0.00
Salmonella enterica (12694)	Live	19.0±0.45
Salmonella enterica (74)	Dead	14.0±0.00
Salmonella enterica (74)	Live	18.0±0.89
Serratia liquefaciens	Live	20.0±0.00
Serratia marcescens	Live	18.0±0.00
Staphylococcus aureus	Dead	11.0±0.00
Staphylococcus aureus	Live	19.0±0.45

Table 3.7 Digestion periods of 12 Live and 7 Dead prey when fed to T. pyriformis at a concentration of 2x10^7 cells/ml. Digestion period were calculated from a number of experiments in triplicate for each prey: one experiment for Dead prey (but four for K. aerogenes); two for Live prey. Error = SEM.

3.4.2 Digestion Rates

Digestion data for *T. pyriformis* (initial prey concentration at $2x10^7$ particles/ml) were available from 34 pulse-chase experiments – 24 for Live prey (two for each bacterial strain) and 10 for Dead prey (four for *K. aerogenes*; one for each of the other bacterial strains). Digestion rates (prey per vacuole per minute) were determined from linear loss of prey per vacuole from 6.5 min to the end of the DP (Figure 3.17). The average of three replicas from one experiment were used, except for Dead *K. aerogenes* where the average of 12 replicas from 4 experiments were used.

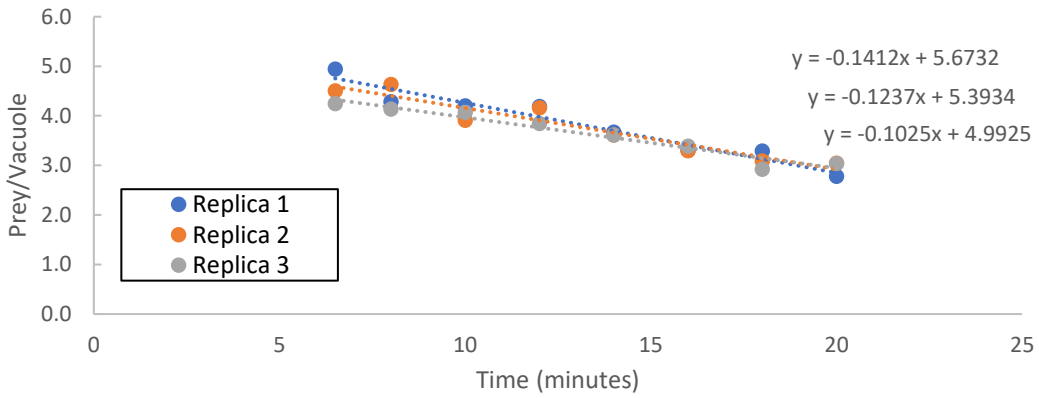


Figure 3.16 Example of how digestion rates (as prey/vacuole/min) were calculated for Salmonella enterica (12694) RFP using the linear decline in prey/vacuole over time between 6.5 min and the end of the digestive period. Triplicate data were used to calculate the average digestion rates and standard error of the mean.

'Digestion' can be presented one of three ways and Figure 3.17 shows these for the four bacterial strains that were present as Live and Dead cells. Firstly, digestion rate as prey/vac/min (Figure 3.17A) suggests that Dead cells are digested faster than their Live counterpart (except for *S. aureus*). If digestion rates are expressed as μm^3/vac/min (Figure 3.17B) results suggest the opposite, in that Live cells are digested faster than Dead cells (except for *S. aureus*). Both these methods do not account for variations between the number of prey/vacuole (higher for Dead cells) and prey biovolume/vacuole (higher for Live cells). To normalise the data, and investigate whether differential digestion was actually occurring, digestion rates were expressed

as % loss of prey/vacuole/min (Figure 3.17C); which yields equivalent data whether prey/vacuole or µm^3/vacuole is used. Table 3.8 shows the digestion rates for all prey tested and the total % of ingested prey/vacuole digested during the digestive period.

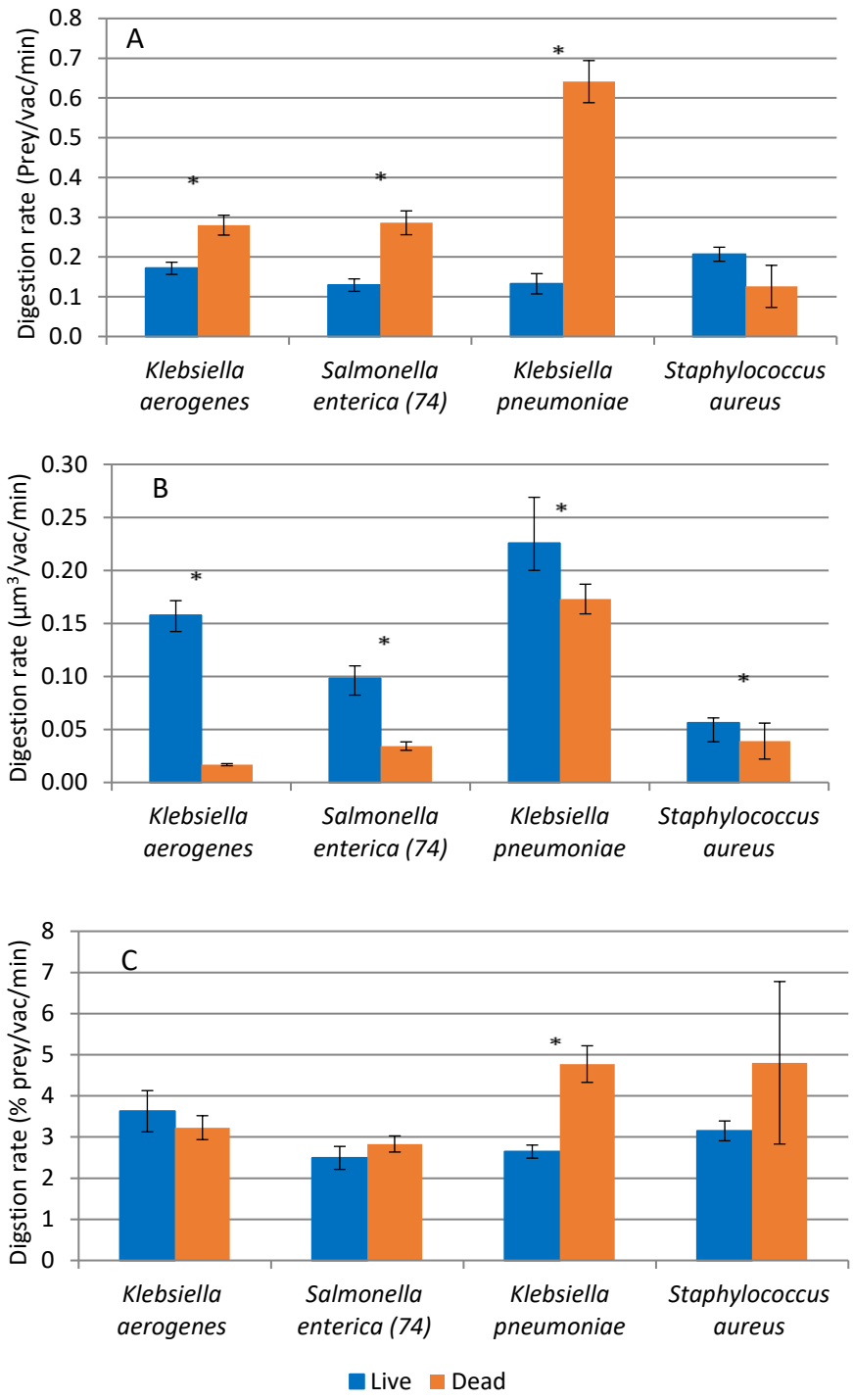


*Figure 3.17 Three ways 'digestion' can be presented: (A) as prey/vac/min; (B) as µm^3/vac/min; (C) as % loss of prey/vacuole/min. Error bars = SEM. *Significant difference between Live and Dead data (P≤0.05).*

The total digestion of Live and Dead cells was equivalent with *T. pyriformis* digesting on average *ca.* 52% of enclosed Live prey and *ca.* 50% of enclosed Dead prey within the digestive period (Table 3.8). However, the length of the digestive period with Dead prey was significantly shorter than that with Live cells (Section 3.4.1) and hence the digestion rates of Dead prey (%prey/vac/min), overall, appear significantly faster than with Live prey (P≤0.05); although only one out of four combinations show this in Figure 3.17. This means that when the number of cells/vacuole of Live *S. aureus* was lower than Dead *S. aureus* over a 20 min period in Figure 3.14C, it was not due to differential digestion between Live and Dead cells.

Prey	State	Total digestion (%)	Digestion rate (% Prey/vac/min)
Aeromonas hydrophila	Live	53±1	3.31±0.15
Enterobacter cloacae	Live	54±2	3.60±0.24
Escherichia coli	Live	37±3	2.19±0.31
Klebsiella aerogenes	Live	63±8	3.66±0.34
Klebsiella pneumonia	Live	48±2	2.92±0.48
Pseudomonas aeruginosa	Live	49±4	2.44±0.31
Pseudomonas fluorescens	Live	61±2	3.84±0.28
Salmonella enterica (12694)	Live	58±4	3.06±0.21
Salmonella enterica (74)	Live	45±5	2.53±0.21
Serratia liquefaciens	Live	53±4	2.64±0.13
Serratia marcescens	Live	40±2	2.22±0.16
Staphylococcus aureus	Live	64±4	3.36±0.24
Klebsiella aerogenes	Dead	48±4	3.23±0.29
Mesorhizobium sp.	Dead	46±3	4.19±0.27
Klebsiella pneumoniae	Dead	64±6	4.77±0.45
Salmonella enterica	Dead	40±3	2.83±0.20
Lactobacillus fermentum	Dead	60±10	4.32±0.69
Listeria monocytogenes	Dead	81±9	4.07±0.45
Staphylococcus aureus	Dead	53±22	4.80±1.97

Table 3.8 Digestion rates as % loss of prey/vacuole/min of twelve Live and seven Dead bacterial prey when fed to T. pyriformis at the concentration of $2x10^7$ cells/ml. Total digestion (% of enclosed prey/vacuole) also included. Error = SEM.

The digestion rate (% Prey/vac/min) within each of the Live and Dead groups were very similar. No significant differences between Dead strains were discerned and only two groupings (that were not significantly different from each other) were distinguished

within the Live prey (Figure 3.18). However, there was no obvious pattern, for example, the two *S. enterica* strains (74 and 12694) were in different groups, the two *Pseudomonas* species (*P. aerogenes* and *P. fluorescens*) were also in two groups (Figure 3.18). Both *Serratia* species (*S. marcescens* and *S. liquefaciens)* were in Group 2 but data for the *Klebsiella* species were inconclusive. In addition, *S. aureus*, the only Gram positive bacterium, clustered with the Gram-negative strains in Group 1 (Figure 3.18), thus showing no obvious effect Gram status.

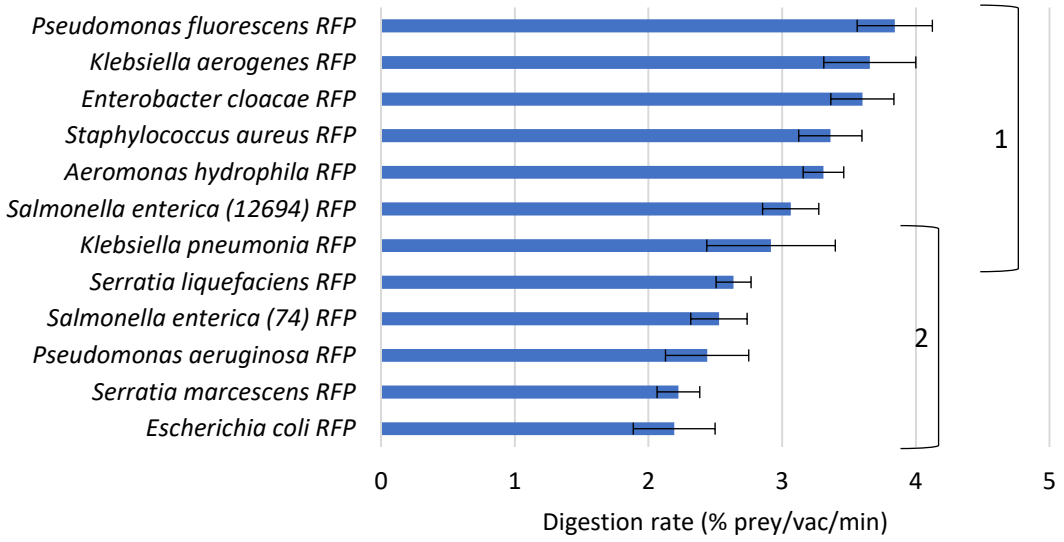


Figure 3.18. Digestion rates for 12 Live RFP bacteria. Error bars = SEM. Prey were divided into groups that were not significantly different to each other (groups are denoted by the symbol (⌈ ⌉).

3.4.3 Effect of vacuole content on digestion

Since there was very litte effect of species/strain on total digestion (%) and digestion rates (% Prey/vac/min) the effect of the number of prey, and the biovolume of prey, within the DVs were investigated. Figure 3.19 shows the relationships between prey/vacuole or prey biovolume/vacuole against % total digestion, for Live cells (Figure 3.19 A & C) and then Live and Dead combined (Figure 3.19 B & D). There is no relationship between prey/vacuole and % digestion (for either prey state, $R^2 \leq 0.1$), but there is a relationship between prey biovolume/vacuole and % total digestion for Live prey ($R^2=0.50$) which is reduced in the presence of Dead prey ($R^2=0.15$) whereby % total digestion declines with increasing prey biovolume enclosed in the vacuole.

Figure 3.20 shows the relationships between prey/vacuole or prey biovolume/vacuole against digestion rate, for Live cells only (Figure 3.20A & Figure 3.20C) and then Live and Dead combined (Figure 3.20B & Figure 3.20D). There is also no relationship between prey/vacuole and digestion rate (for either prey state, $R^2 \leq 0.1$), but there is a relationship between prey biovolume/vacuole and digestion rate for Live prey ($R^2=0.32$) which is maintained in the presence of Dead prey ($R^2=0.32$).

This may at first glance appear as a negative relationship but in reality more prey biovolume is being digested when the DVs contains larger prey. Figure 3.21 shows a strong positive relationship between total biovolume of prey within the DV and the volume of enclosed prey digested for both Live and Dead prey states ($R^2 = 0.89$). This implies that larger cells result in more biovolume being digested (and possibly assimilated) which in itself could lead to higher ciliate specific growth rates. This was tested in Section 3.5.

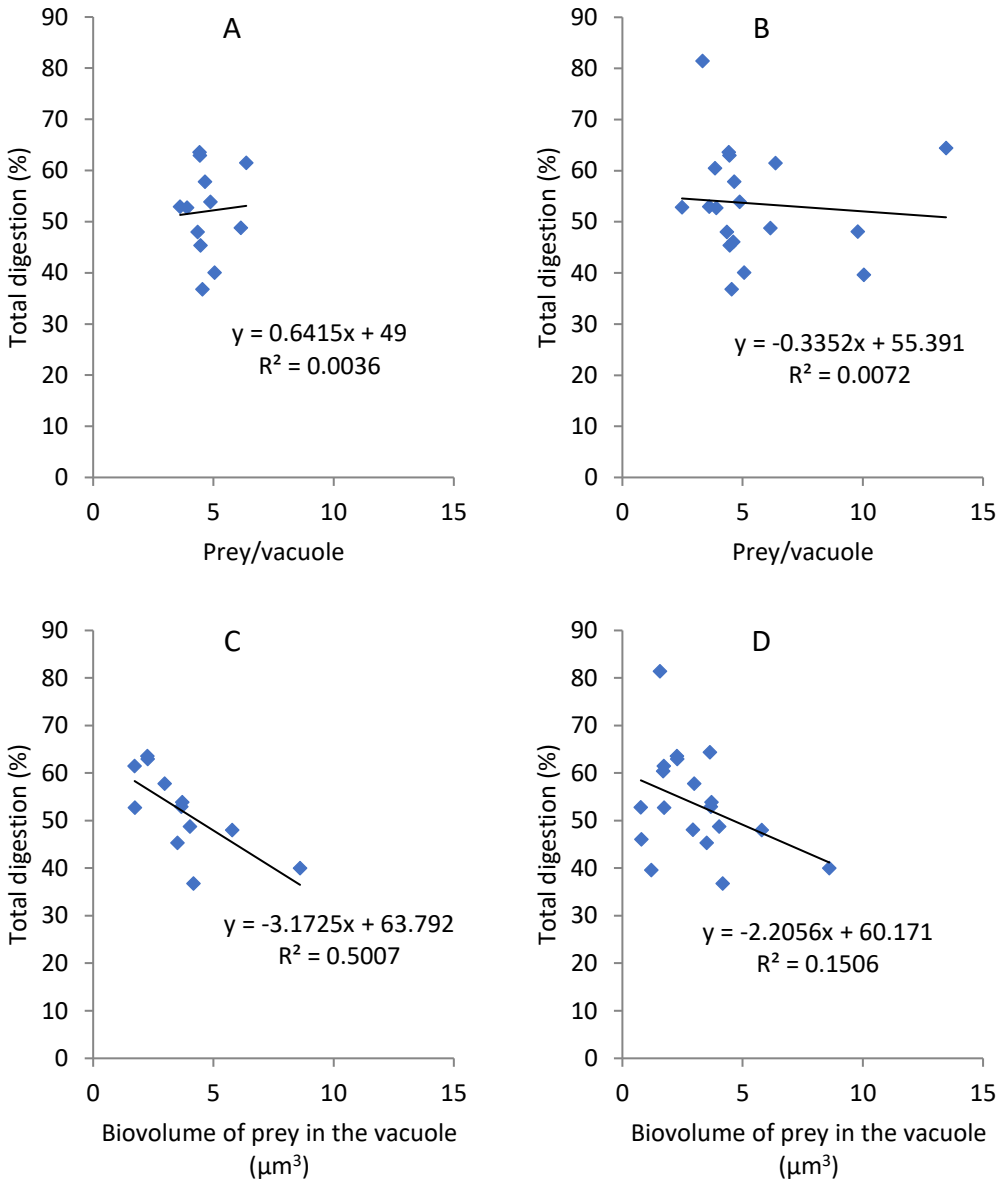


Figure 3.19 Total digestion (%) in the digestive period against prey/vacuole for T. pyriformis when fed (A) Live prey or (B) Live and Dead prey; total digestion (%) in the digestive period against biovolume of prey/vacuole for T. pyriformis when fed (C) Live prey; or (D) Live and Dead prey.

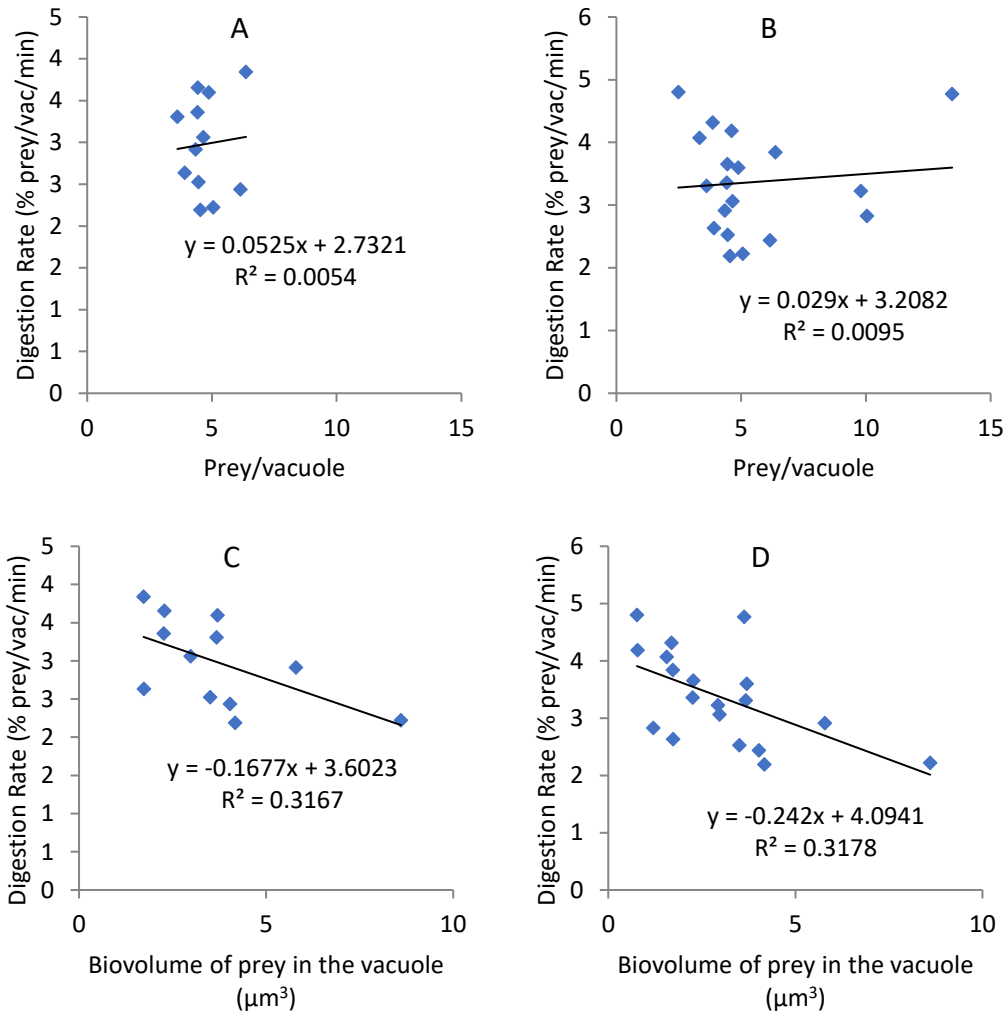


Figure 3.20. Digestion rate (%/cell/min) in the digestive period against prey/vacuole for T. pyriformis when fed (A) Live prey or (B) Live and Dead prey; digestion rate (%/cell/min) in the digestive period against biovolume of prey/vacuole for T. pyriformis when fed (C) Live prey; or (D) Live and Dead prey.

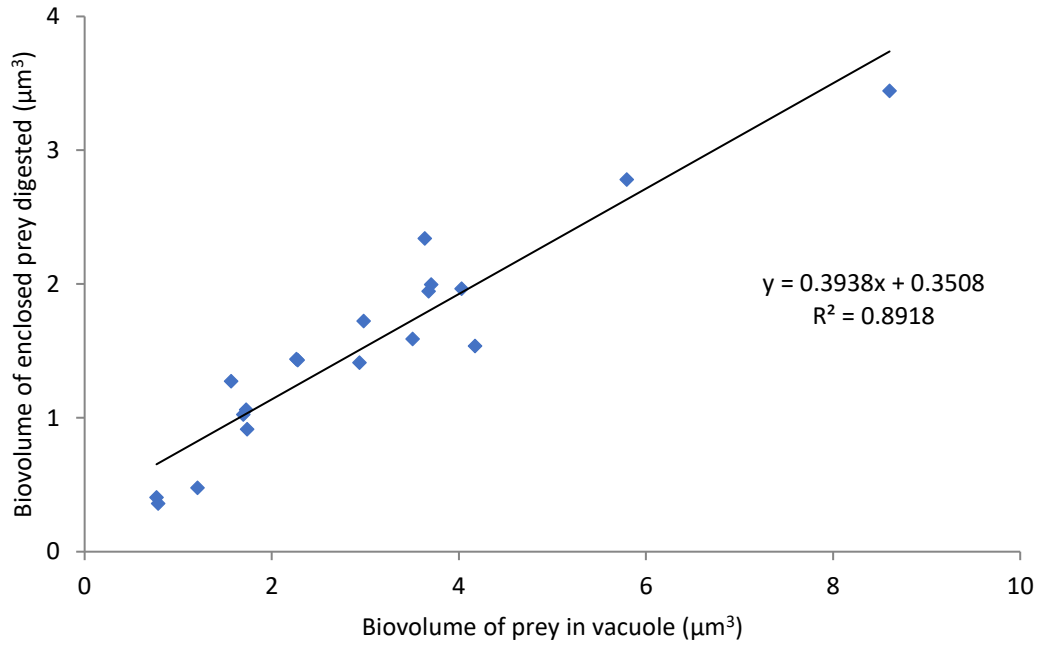


Figure 3.21. Biovolume of prey in digestive vacuoles vs biovolume of enclosed prey digested for both Live and Dead prey.

3.5. Growth

The twelve live bacterial strains and seven heat-killed strains were used to investigate the growth of *T. pyriformis*. Specific growth rates were calculated by linear regression from at least 6 data points of LN cells/ml against time, one for each of the three replicas (Figure 3.22). The average of three replicas was used to determine the average specific growth rate (h^{-1}) ±SEM.

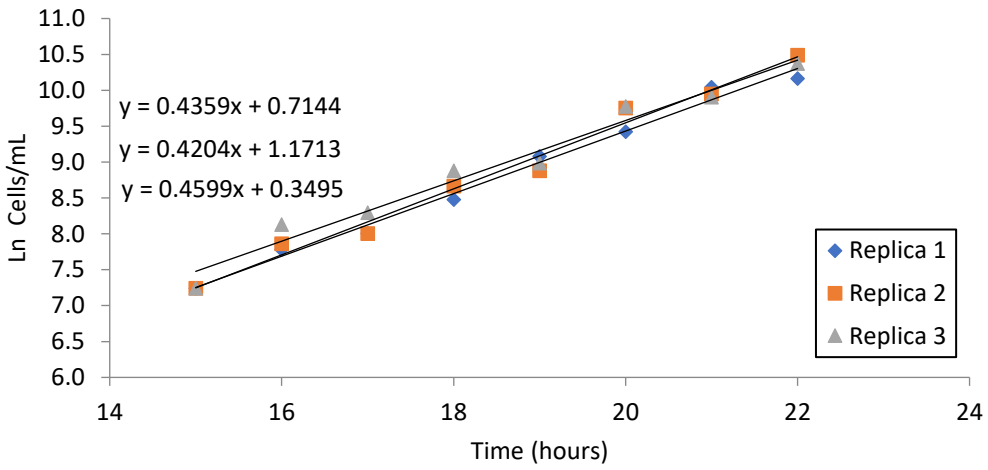


Figure 3.22. Example of how growth rates were calculated for Pseudomonas aeruginosa RFP using the linear increase in Ln cells/ml over time. Triplicate data were used to calculate the average growth rates and standard error of the mean.

3.5.1 Effect of bacterial concentration and species/strain

Table 3.8 shows the growth rates of the ciliate feeding on the different bacteria. The effect of prey concentration was tested with live prey only and as expected the growth rates with $2x10^8$ prey/ml were significantly higher ($P \leq 0.05$) than those for $2x10^7$ prey/ml. There were some significant differences in growth rate of *T. pyriformis* when fed different strains of bacteria. *S. aureus* (the only Gram-positive strain) yielded a significantly lower growth rate ($P \leq 0.05$) than all the other Gram-negative bacteria (Figure 3.23). The Gram-negative bacteria clustered into three groupings (that were not significantly different from each other), and just like the digestion rate data, no obvious pattern emerged. The two *S. enterica* strains (74 and 12694) were once again in different groups and so were the two *Serratia* species (*S. marcescens* and *S. liquefaciens).* The two *Pseudomonas* species (*P. aerogenes* and *P. fluorescens*) were now in the same group (Group 2) and data for the *Klebsiella* species were again inconclusive.

Dead prey were only fed an initial prey concentration of $2x10^7$ cells/ml. The only Gram-positive bacterium *S. aureus* resulted in a significantly lower specific growth rate than *K. aerogenes* ($P \leq 0.05$); significantly higher than *L. fermentum* and *L. monocytogenes* ($P \leq 0.05$); but not significantly different to *K pneumonia* or *S. enterica* (74).

Initial prey concentration:		$2x10^7$ cells/ml	$2x10^8$ cells/ml
Prey	State	Specific growth rate	Specific growth rate
Aeromonas hydrophila	Live	0.109±0.012	ND
Enterobacter cloacae	Live	0.226±0.008	0.277±0.009
Escherichia coli	Live	0.228±0.013	0.357±0.019
Klebsiella aerogenes	Live	0.147±0.009	0.412±0.040
Klebsiella pneumonia	Live	0.204±0.028	0.347±0.006
Pseudomonas aeruginosa	Live	0.164±0.015	0.284±0.006
Pseudomonas fluorescens	Live	0.164±0.008	0.439±0.011

Salmonella enterica (12694)	Live	0.139±0.007	0.398±0.028
Salmonella enterica (74)	Live	0.179±0.011	0.299±0.008
Serratia liquefaciens	Live	0.164±0.017	0.286±0.010
Serratia marcescens	Live	0.231±0.016	0.333±0.006
Staphylococcus aureus	Live	0.062±0.011	0.171±0.012
Klebsiella aerogenes	Dead	0.284±0.015	ND
Mesorhizobium sp.	Dead	0.189±0.031	ND
Staphylococcus aureus	Dead	0.184±0.018	ND
Klebsiella pneumonia	Dead	0.168±0.015	ND
Salmonella enterica (74)	Dead	0.168±0.019	ND
Lactobacillus fermentum	Dead	0.076±0.014	ND
Listeria monocytogenes	Dead	0.075±0.001	ND

Table 3.8 Specific growth rates of twelve Live and seven Dead bacterial prey when fed to T. pyriformis at the concentration of $2x10^7$ or $2x10^8$ cells/ml. Error = SEM. ND = not determined.

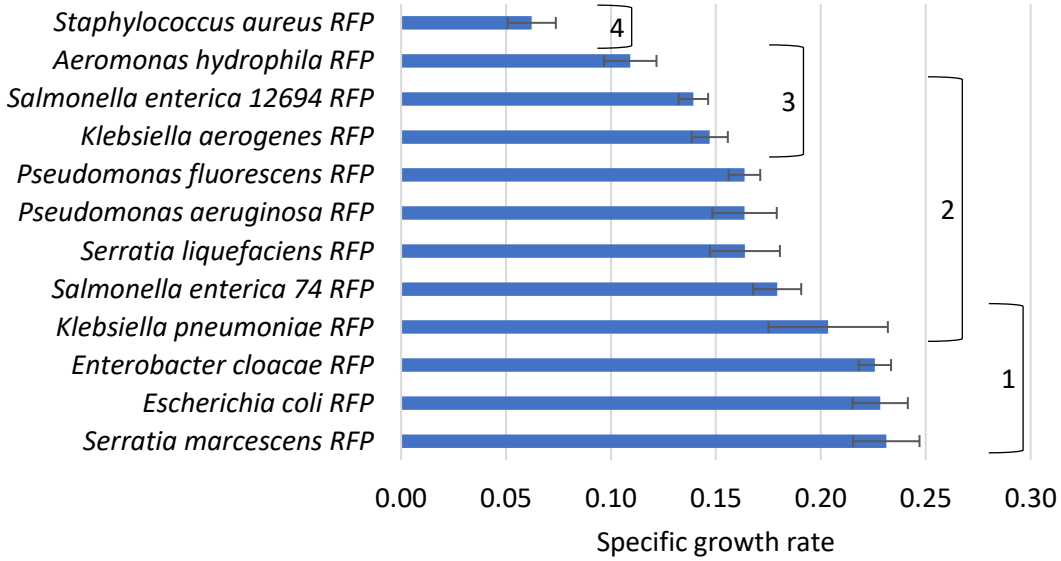


Figure 3.23. Specific growth rates for T. pyriformis feeding for 5 min on Live bacteria at an initial prey concentration of $2x10^7$ cells/ml. Error bars = SEM. Prey were divided into groups that were <u>not</u> significantly different to each other (groups are denoted by the symbol ⌈ ⌉).

3.5.2 Effect of bacterial prey state

In order to investigate the effect of bacterial prey state, the results from feeding *T. pyriformis* prey at $2x10^7$ cells/ml was focused on, because data were available for both states. When the two prey states were compared, the average specific growth rate of *T. pyriformis* on Live prey (0.1681±0.0089 h^{-1}) was not significantly different to the specific growth rate on Dead prey (0.1678±0.0168 h^{-1}) (P=0.988). The lack of effect of heat-killing cells can also be seen in Figure 3.25, which directly compares the specific growth rates obtained for the four bacterial strains in their Live and Dead states; none of which showed a significant difference in specific growth rates between the two states.

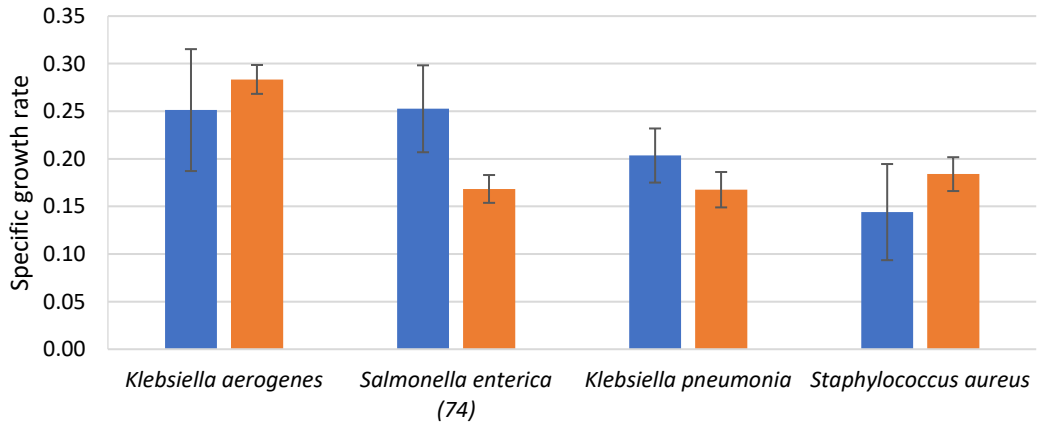


Figure 3.24 Specific growth rates for T. pyriformis feeding on four strains of bacteria in both Live and Dead states. All four bacterial prey show no significant difference in specific growth rate. Error bars = SEM.

3.5.3 Effect of digestion rate

There was large variation in specific growth rate values but no relationship between digestion rates and specific growth rates (R^2=0.20) was found (Figure 3.26A). It was suggested in Section 3.4.3 that if larger cells are enclosed in a DV then more biovolume will be digested (and possibly assimilated) and this might manifest itself in higher ciliate specific growth rates. If this hypothesis was true then there should be a positive relationship between µm^3 prey digested/vac and growth rates. However, no relationship was detected (Figure 3.26B). In fact, of all the parameters tested (including, biovolume of enclosed prey, biovolume of enclosed prey digested, prey biovolume, total digestion and digestion rate), none showed any relationship with either specific growth rate (Appendix C Figure C.3).

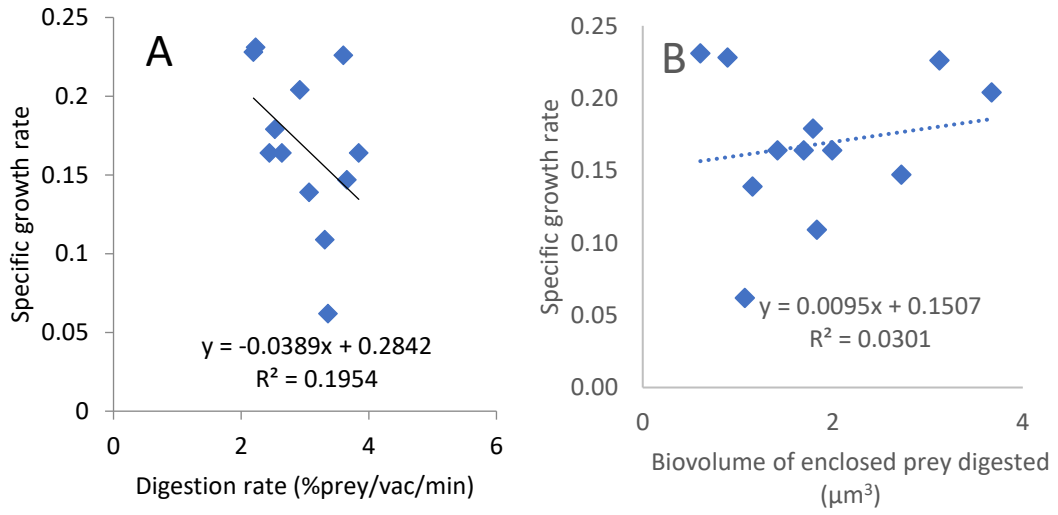


Figure 3.25 Digestion rates against (A) specific growth rates and biovolume of enclosed prey digested against specific growth rates (B) for T. pyriformis feeding on 12 Live prey at 2x10^7 cells/ml in both digestion and growth experiments.

Chapter 4. Discussion

Results presented in Chapter 3 showed that Live bacteria upregulate vacuole formation rate and affect vacuole filling, yielding a maximum of ca. 7 prey/vacuole during the first 5 min of feeding. This does not appear to be influenced by prey species/Gram status which suggests that if receptors are involved they are surface receptors common to all these bacteria.

Next, live Picos affect only vacuole filling, also yielding a maximum of ca. 7 prey/vacuole during the first 5 min of feeding. This does not appear to be influenced by prey strain which suggests that if receptors are involved they may be the same as those used by the Live bacteria. However, live Picos did not influence vacuole formation rate (as did Live bacteria) suggesting that either the receptor involved is missing from Picos or that it is masked in some way.

Dead cells appear not to affect vacuole formation rate (possibly due to the lack of surface receptors) but do affect vacuole filling. This appears to be species dependent (unlike Live bacteria and Picos) which suggests that if receptors are involved they might be deeper in the cell membrane and only exposed after heat-treatment.

Lastly, FLMs do not possess any receptors and vacuole formation in their presence may represent a basal mechanism based on the mere presence of particles. FLMs are deposited into digestive vacuoles and due to the lack of receptors suggests that hydrophobic interactions might be involved.

These data and their implications on growth are explored in the discussion below.

4.1. Ingestion of prey

4.1.1 Effect of prey biovolume

This study examined the effect of four prey-states (FLM, Pico, Live, Dead) on instantaneous ingestion (0-5 min) by the ciliate *Tetrahymena pyriformis*. The size of the prey did not govern the number of ingested prey per cell, number of digestive vacuoles (DVs) produced per cell or the number of prey within those DVs. Only vacuole size (μm^3) was influenced by prey size with larger prey giving rise to larger DVs.

The lack of effect of prey biovolume on prey uptake was surprising as many studies have stated that prey size can affect their ingestion by protists, with the ingestion of larger cells being generally preferred over smaller cells (Šimek and Chrzanowski, 1992, Jürgens and Šimek, 2000, Jürgens and Matz, 2002, Jürgens et al., 2008). However, not all studies agree that prey size is a strong selective criterion for protists (Monger and Landry, 1991, Flynn et al., 1996) and it does appear that the evidence for size-selective grazing is much stronger for flagellates than ciliates. For example, Gonzalez et al. (1990b), who studied both flagellates and ciliates, concluded that “both (flagellates and ciliates) appear to have proportionally higher clearance rates for larger-sized bacterial prey”, yet only a convincing positive relationship between bacterial biovolume (0.02-0.1 μm^3) and clearance rate (determined with fluorescently-labelled bacteria [FLBs] over 12 minutes) was presented for a natural assemblage of flagellates; data for such a relationship in ciliates were less convincing (Figure 4.1).

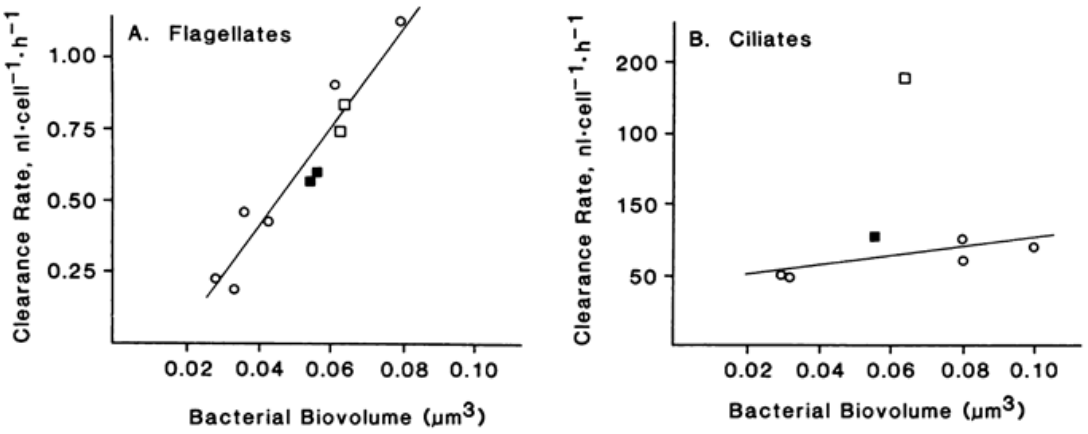


Figure 4.1 Data from Gonzalez et al. (1990b) showing the relationship between bacterial biovolume and clearance rates for (A) flagellates and (B) ciliates– showing a much weaker relationship for the latter.

It may be that prey larger than 0.1µm^3 are required to show significant size-selective grazing in ciliates. The current study used prey with biovolumes ranging from 0.65 to 2.34µm^3 but still no effect of biovolume on prey selection was discerned over the 5 minute feeding period. Jürgens and Šimek (2000), using even larger FLMs (ranging from 0.006 to 39.6µm^3) did report an increase in clearance rate of the ciliate *Halteria cf. grandinella* with increasing FLM biovolume up to a maximum/optimum rate with prey at 5.13µm^3. However, the authors acknowledged that these data were generated using only the top 10% of the population with the highest uptake rates as there was great heterogeneity in the number of FLMs ingested by the population (e.g. 1-30 particles/cell/min with 0.52µm^3 [1µm diameter] FLMs). Examination of maximum clearance values using the whole (100%) of the data set, over the 10 minute feeding time tested, showed less convincing evidence of size selective grazing, especially with FLMs having biovolumes similar to those of the prey used in the current experiment.

It may be that longer feeding times (>12 minutes) are required to show convincing size selective grazing in ciliates and indeed, evidence for its existence has been demonstrated in the ciliates *Halteria cf. grandinella* (Jürgens and Šimek, 2000) and

Stombidium sulcatum (but not *Uronema* sp.) (Christaki et al., 1998), both over 60 minutes. Interestingly, studies on size-selective feeding often show very similar uptake of different sized prey early on but then there is a later divergence. This not only happens with FLMs of different sizes but for different prey types as well. For example Figure 4.2 shows the uptake of a mixed assemblage of FLBs, *Synechococcus* and *Chlorella* by *Halteria cf. grandinella* (Jürgens and Šimek, 2000) and clearly shows that the number of prey ingested is very similar until 20 minutes, after which divergence is more obvious. However, due to the nature of the cells, it is difficult to discern whether the divergence is solely due to biovolume or not. Furthermore, over even longer periods of feeding time (2-42 hours), *Synechococcus* cell biovolume has been shown not to influence their ingestion by flagellates (*Goniomonas pacifica, Paraphysomonas imperforata, Pteridomonas danica*), a dinoflagellate (*Oxhyrhis marina*) and a ciliate (*Eutintinnis sp*.) (Zwirglmaier et al., 2009, Apple et al., 2011).

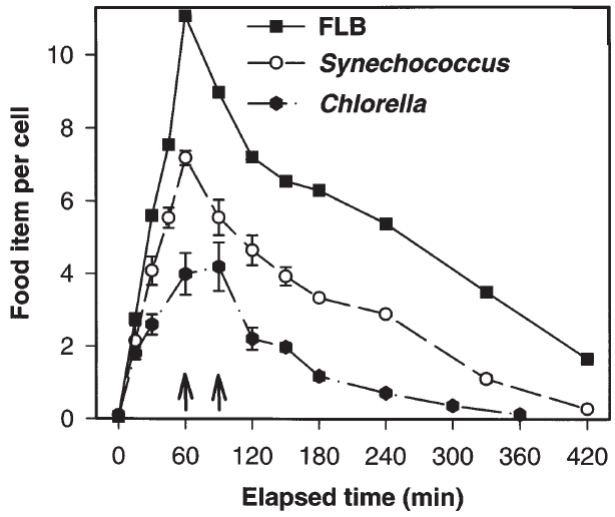


Figure 4.2 Results from pulse-chase experiments from Jürgens and Šimek (2000) showing the uptake of FLB, Synechococcus and Chlorella by Halteria cf. grandinella for 60 min before the chase. Ingestion of all three prey is very similar from 0 to 20 min, after which divergence is more obvious.

Indeed, an experiment in the current study which examined prey deposition into DVs over a 20 minute period (see Section 3.3.2.4.) found no change in prey/vacuole over time. These data suggest that if any prey-induced changes do occur due to prey

biovolume, it might not be an immediate phenomenon but one which develops over time (>20 min), i.e. via possible recognition of prey during digestion/assimilation stages (discussed further in 4.2.3.3). This may be a drawback of short-term feeding experiments, as used in the current study, where prey-induced changes in feeding behaviour may not be detected (Montagnes et al., 2008). But, on a positive note, 5 minute experiments did provide an opportunity to examine the effect of prey species and state (dead or live) on instantaneous ingestion by the ciliate without the complication of variable prey size.

4.1.2 Effect of live bacterial type – heterotrophic vs autotrophic

T. pyriformis ingestion rates on autotrophic *Synechococcus* cells were, on average, significantly lower than with live heterotrophic bacteria (and indeed FLMs and dead cells), with *ca.* 10 *Synechococcus* cells being ingested in 5 minutes compared to *ca.* 14 cells or particles with the other prey types.

Synechococcus cells are relatively large and in the current study the cell biovolumes of the cells were significantly larger than for live heterotrophic bacteria (Table 3.4). However, data showed that cell biovolume did not influence the parameters prey/cell, vacuoles/cell or prey/vacuole over 5 minutes so it cannot explain the difference in uptake between synechococci and heterotrophic bacteria (Section 3.3.1). Indeed, *Synechococcus* cell biovolume, motility, elemental ratio, protein content or hydrophobicity have been shown not to influence their ingestion by flagellates (*Goniomonas pacifica*, *Paraphysomonas imperforata*, *Pteridomonas danica*), a dinoflagellate (*Oxhyrhis marina*) and a ciliate (*Eutintinnis* sp.) (Zwirglmaier et al., 2009, Apple et al., 2011, Strom et al., 2012), suggesting that other properties play a role in the reduced grazing seen in the current study.

A feature that is shared by all *Synechococcus* strains, and not present in the live heterotrophic strains tested (except *A. hydrophila and S. marcescens*), is the presence of an S-layer which is an additional protein surface layer external to the outer

membrane (Sára and Sleytr, 2000). In *Synechococcus*, the two main proteins are SwmA, which is a 130 KDa glycoprotein which resides within the S-layer (Brahamsha, 1996, McCarren et al., 2005), and SwmB which is a giant protein (1.12 MDa) that protrudes out of the S-layer (McCarren and Brahamsha, 2007). Although both are required for non-flagellar swimming of these cells (Brahamsha, 1996, McCarren and Brahamsha, 2007) they have also been hypothesized to act as a shield against predation (Koval, 1993, Zwirglmaier et al., 2009, Strom et al., 2012). However, Strom et al. (2017) using knockout mutants (SwmA- and SwmB-) *Synechococus* WH8102 and feeding them to two ciliates (*Eutintinnus* sp. and *Salpingella* sp.) and three flagellates (*Ochromonas* sp., *Acanthoeca* sp. and *Pteridomonas* sp.) found that the lack of SwmA actually decreased ingestion in the two ciliates and two of the flagellates (not *Acanthoeca* sp.), i.e. an S-layer made the strain more rather than less vulnerable to predation. Removal of the SwmB protein had no effect on predation rates (Strom et al., 2017). Their results agreed with those of Koval (unpublished data cited in Beveridge et al. (1997)) who found that S-layer-bearing bacteria were ingested at equivalent or higher rates than their S-layer-negative mutants by the ciliate *Tetrahymena thermophila* and the flagellate *Paraphysomonas vestita*. In contrast, an earlier study by Strom et al. (2012) found that predation rates on the SwmB- mutant *Synechococus* WH8102 were significantly higher than for the wild-type in *Oxyrrhis marina*, suggesting that SwmB had a protective effect against predation by this dinoflagellate. Also, Koval and Hynes (1991) found the S-layer of several Gram-negative bacteria to be protective against predation by the bacterium *Bdellovibrio* and Tarao et al. (2009) found that modification or removal of the S layer by chemical means increased predation on actinobacteria by the flagellate *Poterioochromonas* sp..

Current information regarding the protective nature of an S-layer is contradictory and it may be that the type of predator involved may be influencing the differences seen. But in the current study synechochocci (with an S-layer) were ingested to a lesser extent

than live heterotrophic bacteria (without an S-layer). To the best of my knowledge, only two of the live heterotrophic bacterial species used in the current study possess an S-layer, *Aeromonas hydrophila* (Dooley et al., 1988) and *Serratia marcescens* (Kawai et al., 1998) with the major protein component being VapA and SlaA, respectively. No evidence for the existence of a protruding B protein exists for either. Interestingly, *A. hydrophila* and S. *marcescens* were the least favoured species by *T. pyriformis* (Figure 4.3) and even more interestingly, uptake rates of both strains were more like those of the synechococci (Picos), particularly *A. hydrophila*, compared to the other live bacteria (except *K. aerogenes*) suggesting that the presence of an S-layer might indeed deter grazing by this ciliate.

In the immune system, the response of phagocytes to bacteria possessing an S-layer seems mixed. First, it has been suggested to play an important role in the pathogenicity of bacteria in many ways, but most relevant is the protection it gives from complement-mediated phagocytosis (Thompson, 2002). Similarly, Blaser et al. (1988) suggested that the S-layer allows *Campylobacter fetus* to resist phagocytosis by leukocytes. Kotiranta et al. (1998) showed mixed results when testing the resistance of different strains of *Bacillus cereus* to human polymorphonuclear leukocytes. On the contrary, the S-layer of some bacterial strains seems to be able to induce an immune response. For example, S-layer proteins isolated from the pathogen *Clostridium difficile* induce the production of pro-inflammatory cytokines and chemokines and increase macrophage migration and phagocytotic activity *in vitro*. In addition, it can up-regulate surface markers on the macrophage, which are important in pathogen recognition and antigen presentation (Collins et al., 2014).

If, as suggested by most studies, it has a protective function against phagocytosis, it could be taken as being functionally similar to bacterial capsules which can also reduce ingestion by macrophages (Horwitz and Silverstein, 1980, Whitnack et al., 1981, Almeida and Oliver, 1993, Cunnion et al., 2003). Nevertheless, whether it be S-

layer or bacterial capsule, potential variation in these cell components between bacteria may lead to different degrees of ingestion, therefore the variation in ingestion of different bacterial species is discussed next.

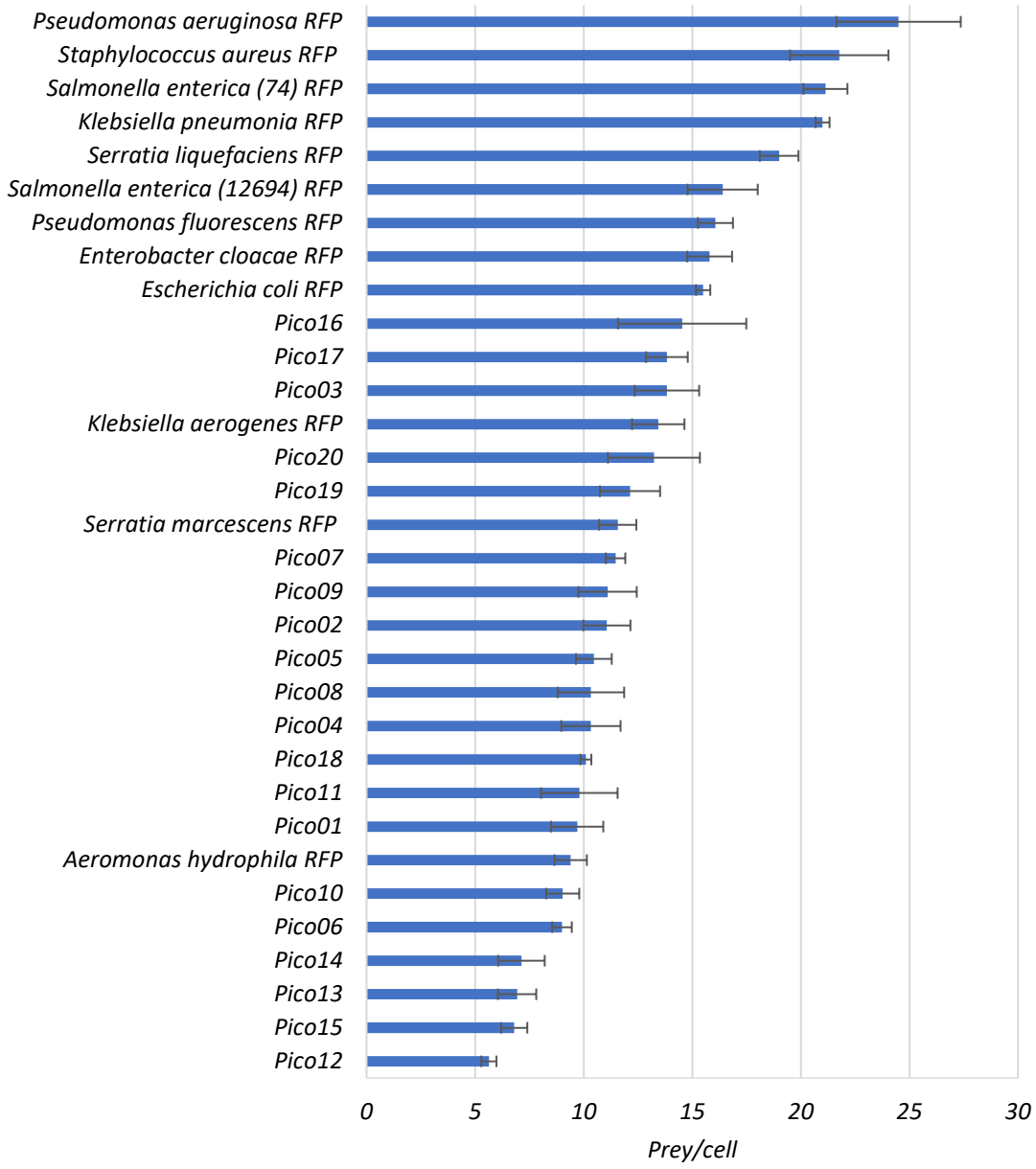


Figure 4.3 Prey per cell data for all Live and Pico prey when fed to T. pyriformis at a concentration of 2x10^7 cell/min for 5 minutes. A. hydrophila and S. marcescens (similar to Synechococcus, both with an S-layer) were the least favoured Live bacterial species by T. pyriformis.

4.1.3 Effect of prey species/strain

The current study found no statistical evidence that different species of live bacteria (or different species/strains of live *Synechococcus* sp.) were ingested differently over a 5 minute period by *T. pyriformis.* Indeed, Jezbera et al. (2005) could not find any evidence of preferential uptake by the ciliate *Cyclidium glaucoma* when fed a mixture of live *A. hydrophila* and *P. fluorescens* for 60 minutes. Thurman et al. (2010b) also showed that *T. pyriformis* could not discriminate between live RFP-expressing *K. aerogenes* and *E. coli* over a 10 minute feeding period. However, *T. pyriformis* did 'de-select' *Klebsiella ozaenae* (reclassified as *Serratia plymuthica*) after only 2-3 min of feeding. The mechanism behind selection remains elusive and it was unfortunate that *S. plymuthica* could not be incorporated into the current study (very poor cell fluorescence signal) but results from other work with amoebae (Pickup et al., 2007c) and the ciliates *Tetrahymena* sp. and *Chilodonella* sp. (Dopheide et al., 2011b) suggest that the mechanism might be related to the production of an external toxin/chemical cue by the bacterium.

Contrary to the above general lack of prey selection by ciliates, Zwirglmaier et al. (2009) found that two flagellates (*Paraphysomonas imperforata* and *Pteridomonas danica*) could selectively graze on various strains of *Synechoccocus* sp. over a 42 hour period and Jezbera et al. (2005) showed that two flagellates (*Bodo saltans* and *Goniomonas* sp.) preferentially ingested live *A. hydrophila* over *P. fluorescens* over a 60 minute period. Zwirglmaier et al. (2009) proposed that cell surface properties of the *Synechococcus* played a major role in selective ingestion by the flagellates because a spontaneous phage resistant mutant of strain WH7803, which showed modifications in its lipopolysaccharide (LPS) layer, was ingested while the wild-type strain was not. The change in LPS layer was specifically a loss of a significant proportion of the strain's O-polysaccharide side-chain units, or O-antigen, which are carbohydrates found on the outermost domain of the LPS (Wang et al., 2010).

LPS comprises Lipid A (embedded in the outer membrane) and the core polysaccharides (usually common to a specific genus) and the highly variable O-antigen (variable between species/strains), both of which protrude out of the membrane. Full-length O-antigens renders the LPS 'smooth', whereas the absence of O-antigens makes the LPS 'rough'. Bacteria with rough LPS are more readily engulfed by phagocytes in the immune system (Cunningham et al., 1975, Fields et al., 1986, de Bagüés et al., 2004) which agrees with the WT/Mutant observation made by Zwirglmaier et al. (2009). However, two studies have found the opposite, with smooth *Synechococcus* strains being grazed more than rough strains by an unidentified amoeba (Simkovsky et al., 2012, 2016).

There is great variation in the composition of the sugars in the O-antigen between species and strains of Gram-negative bacteria and it is therefore a major antigenic determinant (antibody-combining site) of the Gram-negative cell wall (Wang et al., 2010), i.e. it is a "Pathogen-Associated Molecular Pattern" (PAMP). Zwirglmaier et al. (2009) implied that O-antigen variation on the surface of *Synechococcus* might be the reason for selective ingestion by the flagellates and that this would support the findings of Wildschutte et al. (2004) who showed that epitope differences in the O-antigen of otherwise genetically distinct *Salmonella enterica* isolates led to differential ingestion by a range of amoebae, including *Acanthamoeba castellanii* and *Naegleria gruberi*. However, what was not acknowledged was that *T. pyriformis* showed no prey preference for the different serotypes at all (Wildschutte et al., 2004).

It may therefore be that prolonged cell-cell contact is required to differentiate between bacterial species based on O-antigen variation. This is a feature of direct interception feeding (carried out by flagellates and amoebae) but not a feature of filter feeding (carried out by many ciliates including *T. pyriformis*). This might be the reason why *T. pyriformis*, in the current study, failed to distinguish between species of live heterotrophic bacteria and strains of *Synechococcus* sp..

As stated above, O-antigen variation is an example of a PAMP. These are ligands that are recognised by “Pattern Recognition Receptors” (PRRs). In phagocytes of the immune system PRRs include Toll-like receptors (TLRs), Nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectins and scavenger receptors (SRs) (Stuart et al., 2005, Means et al., 2009). The latter two types are known to also exist in protists (Vogel et al., 1980, Bozzaro and Roseman, 1983, Janssen and Schleicher, 2001, Wilks and Sleigh, 2004, Sattler, 2012). However, O-antigen variation is only one of numerous PAMPs that can be detected by PRRs. Others include, flagellin, lipoteichoic acid, lipoproteins and peptidoglycan, mannose residues, N-formylmethionine, endogenous heat shock proteins, extracellular matrix molecules and nucleic acid variants, all of which are heterogeneously distributed within a bacterial cell (some on the cell surface, some not) (Wright, 1999, Mitchell et al., 2006). Not surprisingly, the term "PAMP" has been criticized because most bacteria, not only the pathogenic ones, express these molecules. A “virulence signal” capable of binding to the receptor, in combination with a “microbe-associated molecular pattern” (MAMP), has been proposed as one way to constitute a pathogen-specific response (Brennan and Anderson, 2004).

Considering that C-type lectins and scavenger receptors are known to exist in protists (including ciliates) and that there is a whole host of potential MAMPs within a bacterial cell to which they could bind, suggests that ingestion of bacterial prey by *T. pyriformis* could still be receptor-mediated; it just would not involve O-antigens.

4.1.4 Effect of prey state – live vs dead vs inert

4.1.4.1 Heat-killing bacterial cells

The preparation of dead prey by subjecting the bacterial cells to high heat (60ºC) for two hours, as used in the current study, is a harsh process. It not only kills the cells but also irreversibly changes the physical and biochemical characteristics of the bacteria. Perhaps most important to the current study is the alteration to the cell surface chemistry which could render them devoid of, or having reduced, lipopolysaccharide (LPS) and associated surface ligands (Katsui et al., 1981). For example, it has been shown that the ability of *S. aureus* and *E.coli* to bind collagen is significantly reduced after heat-killing (Holderbaum et al., 1986, Holderbaum et al., 1987, Visai et al., 1990). Conversely, if the cells are killed by fixation (e.g. glutaraldehyde) there is no interference with surface protein-protein interactions, suggesting no effect of surface ligands (Lönnbro et al., 2008). Indeed, lectin binding assays can be performed on live cells (Wootton et al., 2007), glutaraldehyde-fixed cells (Strom et al., 2017) and also ethanol-fixed cells (Peiser et al., 2006) further demonstrating that surface ligands remain intact upon fixation.

Therefore, for the purpose of the following discussion, heat-killed cells are considered to possess no surface ligands, only those that may have been buried deeper in the cell wall which have only become exposed upon heat-treatment and loss of the outer membrane. In contrast, live bacteria are considered to possess both surface and deeper ligands while FLMs possess no ligands at all.

4.1.4.2 Effect on digestive vacuole content

The number of prey per vacuole varied between the different prey types (Table 3.5). At very high prey concentrations live *S. aureus* and *Synechococcus* strains 3 and 20 yielded a maximum of 7 prey/vacuole whereas those with dead *S. aureus* contained 9 prey/vacuole and those with FLMs contained 19 prey/vacuole (Table 3.6). Data on prey/vacuole, with which to compare these data, are very rare as most studies present prey ingestion data as prey/cell. However Mitra et al. (2005) reported that the DVs of the amoeba *Entamoeba histolytica* very rarely contained more than 7 live *P. aeruginosa* cells/vacuole whilst the DVs of *E. dispar* never contained more than 7 *P. aeruginosa* cells. The 'magic number seven' has also been reported in macrophages feeding on apoptopic bodies (UV-irradiated thymocytes) whereby the maximum number ingested was 7 and this was not due to limitations in further engulfment as plenty of vacuolar membrane was still available (Marée et al., 2008). Indeed, no evidence of vacuolar membrane limitation was observed in the current study as 7 live prey cells were deposited into each DV whether they were small cells (*S. aureus* at 0.27µm^3) or large cells (*Synechococcus* 20 at 2.34µm^3).

No published data exist for DV content with dead cells but Klobutcher et al. (2006) reported that when *T. thermophila* fed on the spores of *Bacillus subtilis* the DVs were always smaller than when feeding on a mutant with a defective spore coat. Examination of the electron micrographs in that paper show that DVs contained *ca.*13 wild-type cells/vacuole and *ca.* 40 mutant cells/vacuole which is a similar trend to the current study whereby DVs contained more dead cells and live cells.

The process of DV filling appeared very controlled (tested with Live, Dead, *Synechococcus* only) with new DVs containing the same number of prey as older DVs over the 20 minutes tested (Figure 3.14). FLMs were not included in this 20 minute experiment (Section 3.3.2.4.), however Giorgione and Clarke (2008) did comment that there was little reproducibility in DV filling in *Dictyostelium* when feeding on polystyrene

latex beads (and FLMs are polystyrene latex beads) compared to live *E. coli* which was very reproducible (but no values were stated). If this is indeed the case, then 'controlled' DV filling might only occur with live and dead cells and the obvious difference between these and FLMs is that the latter are devoid of ligands. This suggests that DV filling is a receptor-mediated process but because live cells have surface ligands while dead cells do not, the receptors may be different.

4.1.4.2.1 Receptor-mediated uptake of live cells

The fact that no effect of genus, species or strain (nor Gram status) was recorded on the uptake of live heterotrophic or autotrophic bacteria suggests that if receptors are involved they are easily accessible and common to both Gram-positive and Gram-negative cells (whether or not they are covered by an S-layer). Indeed, Jiang et al. (2004) have suggested, using infrared spectroscopic, that the dominant functional groups of bacterial surfaces e.g. carboxyl, amide, phosphate, hydroxyl, and carbohydrate related moieties, are identical for both classes of bacteria.

Of these groups, the carbohydrate related moieties are those most often associated with recognition processes, specifically the transmembrane N-glycosylated proteins, or 'glycoproteins' (Durichen et al., 2016). Such glycoproteins span the outer membrane of bacterial cells (Alberts et al., 2002) and are also found in the S-layer e.g. SwmA is a glycoprotein (Messner and Sleytr, 1991, Brahamsha, 1996, McCarren et al., 2005). The sugars are attached to the protein at the amide nitrogen atom in the side chain of asparagine and all attached sugars have a common pentasaccharide core consisting of three mannose and two N-acetylglucosamine residues (Berg et al., 2002).

Vogel et al. (1980) was the first to discover carbohydrate binding proteins (lectins) on the surface of *Dictyostelium discoideum* that were related to prey uptake whereby addition of glucose inhibited the phagocytosis of live *E. coli*. Then, Bozzaro and Roseman (1983), using polyacrylamide gels containing different sugars covalently linked to the polymer, observed *Dictyostelium* cells binding to glucose, mannose and

N-acetylglucosamine and concluded that *Dictyostelium* possessed three different receptors, one highly specific for glucose and two less specific for N-acetylglucosamine and mannose (the only components of the pentasaccharide core of glycoproteins). Wilks and Sleigh (2004) were the first to test for the presence of lectins in the ciliate *Euplotes mutabilis*. Seven lectins showed unique binding patterns associated with the cytostome or digestive vacuoles however, only two lectins gave strong and consistent binding patterns, i.e., Wheat Germ Agglutanin (WGA) and Concanavilin A (ConA).

WGA binds to N-acetylglucosamine and sialic acid (Wright, 1984) which are both found in glycoproteins. Wilks and Sleigh (2004) found that WGA bound strongly to the lower basal half of 8-9 oral membranelles on the front section of the ciliary band. This region can detect the presence of stimuli and initiate a signal, translating this into a mechanical response (i.e. a reverse beat) to either accept or reject particles. Conversely, Durichen et al. (2016) did not report any binding of WGA to the cytostome of *T. pyriformis*. In their study, WGA bound only to distinct, smaller parts of the digestive vacuole membrane and more specifically to younger vacuoles.

ConA binds to internal and terminal, non-reducing manno-pyranosyl and gluco-pyranosyl residues, as well as glucose and mannose, which are found in N-glycosylated proteins (Goldstein et al., 1974, Wilks and Sleigh, 2004) showed a sequence of ConA binding events over time with the labelling first appearing at the cytostome (within 10s), then at the cytostome and on the digestive vacuole (by 40s), then only on the digestive vacuole. The label eventually disappeared.

Of the three sugar residues mentioned above (N-acetylglucosamine, glucose and mannose) most attention has been paid to mannose and its linear polymer mannan. Many studies have shown that the mannose receptor, which is a PRR, mediates phagocytosis in macrophages (Bar-Shavit et al., 1977, Bar-Shavit et al., 1980, Martinez-Pomares, 2012, Esparza et al., 2015, Garcia-Aguilar et al., 2016). For example, Bar-Shavit et al. (1977), (1980) showed the involvement of mannose in the

attachment of *E. coli* and *Salmonella typhi* to macrophages. Esparza et al. (2015) showed that *Mycobacterium tuberculosis* contains the mannosylated glycolipoprotein PstS-1 on its surface which binds readily to macrophages, and that phagocytosis of macrophages infected with *M. tuberculosis* and *M. tuberculosis* by 'healthy' macrophages is mediated by mannose (Garcia-Aguilar et al., 2016).

Mannose receptors and phagocytosis have also been investigated in protists (Bracha et al., 1982, Allen and Dawidowicz, 1990, Alsam et al., 2005, Declerck et al., 2007, Wootton et al., 2007, Medina et al., 2014). Bracha et al. (1982) showed that the attachment and ingestion of live *E. coli* by the amoeba *Entamoeba histolytica* required a membrane-associated mannose-binding receptor. Allen and Dawidowicz (1990) reported that addition of mannose and fructose inhibited the binding and ingestion of unidentified live yeast cells but (not latex beads) by *Acanthamoeba castellanii* in a stereospecific, concentration-dependent manner. Similarrly, Declerck et al. (2007) reported that addition of mannose inhibited the ingestion of live *Legionella pneumophila* by the amoebae *A. castellanii* and *Naegleria lovaniensis*. Similar studies have used oligosaccharide inhibition to show that the attachment of *Arcobacter butzleri* (Medina et al., 2014) and *Listeria monocytogenes* (Akya et al., 2009) to *A. castellanii* involves the participation of mannose and mannose-binding proteins.

Wootton et al. (2007) demonstrated mannose binding receptor involvement in the ingestion of the phytoflagellate *Isochrysis galbana* by the dinoflagellate *Oxyrrhis marina* by showing that (i) blocking the mannose receptor using mannose-BSA (bovine serum albumin) reduced prey ingestion by 60%; (ii) *O. marina* ingested twice as many latex beads coated with mannose-BSA compared to those coated with N-acetylgalactosamine-BSA and; (iii) when pre-incubated with mannose-BSA, *O. marina* was no longer able to discriminate between latex beads coated with different lectins.

Furthermore, Martel (2009) showed that nitrogen-deficient *I. galbana* cells were richer in cell-surface mannose than nitrogen-sufficient cells and *O. marina* has been

observed to select against *I. galbana* in mixed prey feeding experiments when the prey is nitrogen-deficient (Flynn et al., 1996, Hansen et al., 1996, Martel, 2006). However, if a mannose-specific feeding receptor is involved, one would expect that mannose-rich microalgae to be captured and ingested more readily, which they are not. Nevertheless, the authors concluded that the cells were too 'sticky' and excess cell-surface mannose residues may have sheared off the cells and inhibited the binding efficiency of feeding receptors; something which was also demonstrated to occur by Wootton et al. (2007).

Evidence suggests that the mannose receptor might be a good candidate for involvement in the controlled uptake of live bacteria into the DVs of *T. pyriformis*, as mannose is ubiquitous in the surface membranes of both Gram-negative and Gram-positive cells (Mirelman et al., 1980, Allavena et al., 2004) and in the S-layer (Brahamsha, 1996, McCarren et al., 2005), so it is a ligand that is 'available' to the ciliate whether the prey cell possesses an S-layer or not. It also does not appear to be species-specific as blocking the mannose receptor results in disrupted ingestion of a wide range of prey, e.g*. A. butzleri*, *I. galbana*, *E. coli,* yeast, *L. monocytogenes, L. pneumophila* and even infected macrophages (Bracha et al., 1982, Allen and Dawidowicz, 1990, Declerck et al., 2007, Wootton et al., 2007, Akya et al., 2009, Medina et al., 2014). It might be considered as a receptor that just recognises 'live food', whether or not that food is digestible.

4.1.4.2.2 Receptor-mediated uptake of heat-killed cells

The deposition of heat-killed bacterial prey into the DVs of *T. pyriformis* was different to that observed with live bacteria in that (i) there were more dead cells/vacuole than live cells/vacuole and (ii) the number of prey/vacuole differed between species with values ranging from 3 to 14 prey/vacuole (at $2x10^7$ prey/ml). Even so, it still appeared to be a controlled process (tested with dead *S. aureus* over 20 minutes), suggesting a receptor-mediated process.

As mentioned in Section 4.1.4.1. the nature of the bacterial killing process will affect the integrity of the cell wall and associated ligands. Heat-killing destroys the outer cell surface layer (Katsui et al., 1981), while chemical fixation with for example, glutaraldehyde or ethanol (Peiser et al., 2006, Strom et al., 2017) maintains the integrity of the surface ligands. However, data within Chung and Kocks (2011) does question the use of ethanol fixation as ethanol (just like heat) was found to disrupt the outer cell surface ligands of the Gram-negative bacteria, *E. coli*, *Serratia marcescens*, and *Pseudomonas aeruginosa*. In their study, a receptor known as Eater (found on *Drosophila melanogaster* phagocytes) was shown to bind to heat-killed or ethanol-treated Gram-negative bacterial cells but not to live or formaldehyde-fixed cells suggesting it was binding to a deeper ligand exposed after heat/ethanol treatment (which is normally masked in live cells). Interestingly, Eater could bind to live Gram-positive cells (*S. aureus* and *Enterococcus faecalis*). Because of this, the proposed ligand for Eater is peptidoglycan (or peptidoglycan-associated molecules) because it is more abundant and accessible in live Gram-positive cell walls, whereas in live Gram-negative cells walls it is much less abundant and deeply buried under the outer LPS membrane (Silhavy et al., 2010).

Eater is an example of a Class A scavenger receptor (SR) and SRs are PRRs (just like the mannose receptor). SRs are trimeric, integral membrane glycoproteins and have been grouped into 10 Classes (A-J) based on the current understanding of structure

and biological function (Platt and Gordon, 1998, Kang and Lee, 2011, Zani et al., 2015). The most extensively studied have been the class A SRs, specifically SR-A, which binds to both Gram-positive and Gram negative bacteria (discussed here); and MARCO, which binds to inert particles (discussed in Section 4.1.4.2.3).

Class A SRs have been shown to be involved in macrophage phagocytosis (Palecanda et al., 1999, Beamer and Holian, 2005, Areschoug and Gordon, 2009, DeLoid et al., 2009, Silverstein and Febbraio, 2009, Zani et al., 2015, Peruń et al., 2016). Most studies have employed either inert latex microspheres or heat-killed bacterial cells so it has been unclear whether SRs are also involved in the uptake of live prey. There are only two published studies which have investigated this. DeLoid et al. (2009), using a general blocker for all Class A SRs, was the first to show that SRs were involved in the ingestion of heat-killed *S. aureus* by peritoneal macrophages but not live *S. aureus*. Then, Peruń et al. (2016) showed that SR-A was the major, if not the only, receptor involved in uptake of heat-killed *E. coli* and *S. aureus* and was redundant in the uptake of their live counterparts. The ligand for SR-A, just like Eater, is considered to be peptidoglycan (or peptidoglycan-associated molecules) because it binds both Gram-positive and Gram-negative cells (Adachi and Tsujimoto, 2002, Shimaoka et al., 2003, Philips et al., 2005, Jiang et al., 2006, Baur et al., 2014).

In the current study, the number of prey/vacuole with heat-killed cells varied from 3-14, with the lower values being associated with the Gram-positive cells. Based on the observations above one would expect Gram-positive cells to yield a higher prey/vacuole if a Class A scavenger receptor is involved in the filling of the vacuoles, but the opposite was seen (Figure 3.12). It may be that (i) the heat-killing process destroyed much of the peptidoglycan in both Gram-positive and Gram-negative cells and rendered them more equivalent; (ii) more peptidoglycan was degraded in Gram-positive because of the lack of protection from an LPS outer membrane; or (iii) the

species variation in either peptidoglycan or the molecules associated within the peptidoglycan causes the variation in prey/vacuole.

Both (i) and (ii) are unquantifiable differences and would be very difficult to discern. However, it is possible to investigate (iii) in terms of the variation in peptidoglycan or the molecules associated within the peptidoglycan between different bacteria. Chung and Kocks (2011) showed that the Eater N-terminal fragment displayed differential binding to different types of polymeric peptidoglycan whereby it bound to peptidoglycan isolated from *E. coli*, *Bacillus subtilis* and *S. aureus*, but not from *Micrococcus luteus.* Thus, differences were discerned between genera within the Gram-positive group.

Both Gram-positive and Gram-negative bacteria are able to modify their peptidoglycan by N-deacetylation, N-glycolylation, and O-acetylation (Vollmer, 2008, Davis and Weiser, 2011, Martin et al., 1973, Rosenthal et al., 1982) and as a result, peptidoglycan can be highly variable between different bacterial species. For example, the glycan strands of Gram-negative species have a 1,6-anhydro ring at the terminal MurNAc residue, whereas Gram-positive species attach other cell-wall polymers (e.g. teichoic acids, capsular polysaccharides) via phosphodiester bonds to their GlcNAc or MurNAc residues (Vollmer et al., 2008). Furthermore, *Neisseria gonorrhoeae*, as well as in several other Gram-negative bacteria (*Moraxella, Neisseria, Pseudomonas* and *Proteus*) have been observed to modify their peptidoglycan by O-acetylation (Martin et al., 1973, Rosenthal et al., 1982).

Peptidoglycan or molecules within peptidoglycan might therefore play an important role in the recognition of different bacterial species. In heat-killed cells these deep receptors would be immediately ‘available’ to the ciliate; possibly explaining why differences in vacuole filling was discerned between dead species in the current study. However in live cells, these receptors would be masked until digestive processes in the DV unmasked them (discussed further in Section.4.2.3.2); possibly explaining why there was no effect of prey species on vacuole filling with live prey.

4.1.4.2.3 Non-specific-receptor-mediated uptake of inert particles

FLMs

In the current study, *T. pyriformis* readily ingested FLMs at a relatively constant rate (*ca.* 2.7 FLMs/min during 5 minutes of feeding). There are also a wealth of other studies showing that ciliates can ingest artificial prey devoid of any surface ligands (Jonsson, 1986, Pace and Bailiff, 1987, Christaki et al., 1998, Jürgens and Šimek, 2000, Thurman et al., 2010a). Macrophages are also able to ingest non-opsonised and non-biological particles like silica (Gilberti et al., 2008, Gilberti and Knecht, 2015), quartz (Haberzettl et al., 2007) and latex particles (Parod and Brain, 1983). This has led to the proposal that live and inert particles are ingested differently by phagocytes.

Vogel et al. (1980) were the first to propose that, in *Dictyostelium,* there were two functionally distinct routes prey could be recognized and internalised. One route was a 'non-specific'-receptor-based route which was primarily governed by hydrophobic interactions between the predator and the prey, while the other was a lectin-receptor-based route (previously discussed in Section 4.1.4.2.1.).

The possibility of two 'entry routes' into ciliates is an interesting concept, particularly since Jürgens and Šimek (2000) showed that when the ciliate *Halteria cf. grandinella* was fed a 1:1 mixture of similar sized artificial beads and live *Synechococcus*, no interference in prey ingestion of either prey was observed (i.e. both particles were ingested at equivalent rates to when they were present as the single prey). This study might have inadvertently shown that live prey and artificial prey enter the cell via different pathways.

There are a small number of receptors that may be responsible for 'non-specific'-receptor-based ingestion of particles. However, in professional phagocytes, for which most data on phagocytic receptors is available, the scavenger receptor (SR) family has been shown to be major players. In particular, Arredouani et al. (2005) identified the Class A SR MARCO as the dominant receptor of un-opsonized latex beads in human

alveolar macrophages. MARCO (like other class A SRs) possesses collagen-like and cysteine-rich domains in its extracellular regions. At the collagen-like domain, a cluster of lysine-residues form a positive-charged groove to interact with negatively-charged particles (Krieger, 1994, Taylor et al., 2005b). The exact mechanism by which MARCO is able to bind latex beads (devoid of surface ligands) is unclear but it has been suggested that hydrophobicity plays a major role (Kobzik, 1995).

Many studies have shown that macrophages ingest more hydrophobic/negatively charged microspheres than hydrophilic microspheres (Watanabe et al., 1984, Sano et al., 1986, Yamada et al., 1993, Roser et al., 1998, He et al., 2010). However, data regarding the effect of inert prey hydrophobicity on protistan feeding is scarce and no robust evidence exists.

Hammer et al. (1999) fed the dinoflagellate *O. marina* various artificial particles (i.e. silicate particles and microspheres) with different hydrophobicities and showed that the dinoflagellate had a higher ingestion rate on particles with decreasing hydrophobicity, however, particle volume was not standardised in the study. Eisenmann et al. (2001) fed the ciliate *Epistysis* sp. an equal mixture of equal-sized green hydrophobic- and red hydrophilic-FLMs and found that the DVs contained equal numbers of both particle type suggesting no effect of hydrophobicity. Matz et al. (2002) who fed 0.75µm polystyrene beads of differing hydrophobicity (0 to -55 mV zeta potential) to the nanoflagellate *Spumella* sp. and found that only extreme charges beyond -45 mV reduced flagellate ingestion rates. Such extreme charges have not been associated with natural bacterial prey.

It may be that ‘hydrophobicity’ is too broad a feature to define an effect on ingestion and that it is the ‘charge’ of the particle that might be the key factor. Indeed, there is a trend towards equal-sized neutral- and negatively-charged particles to be ingested equally, but positively-charge particles to be ingested at lower rates by *T. pyriformis* (Rasmussen and Modeweg-Hansen, 1973, Durichen et al., 2016). Fok et al. (1988)

have also reported that DV formation rates are 26% lower if the ciliate *Paramecium multimicronuceatum* is fed positively-charged beads (compared to same-sized neutral or negatively-charged particles).

Although the actually mechanism is unknown, it is possible that the receptor used to ingest inert particles in ciliates is similar to MARCO and could similarly possess a positive-charged groove to interact with negative-charged particles. Positively charged particles would 'repulse' against the positively charged groove and this would lead to reduced attachment and ingestion.

Live and Dead Bacteria

Surface charge has been shown to differ between different bacterial species (Van Loosdrecht et al., 1987, Daffonchio et al., 1995, Zita and Hermansson, 1997a, Zita and Hermansson, 1997b, Soni et al., 2008, Choi et al., 2015). Daffonchio et al. (1995) collated contact angle (a measurement of hydrophobicity) data from several published studies and showed differences in contact angles for various live bacterial species. In particular, some species that were also used in this study were: most hydrophobic *Listeria monocytogenes* followed by *S. aureus* and then *E. coli*.

Soni et al. (2008) showed that surface charge may differ depending on the physiological state of the bacterium (i.e. live or dead [killed using sodium azide solution]): the zeta potentials (i.e. surface charge) of dead *E. coli* and *Salmonella* sp. (-3.37±0.05 and -1.30±0.01 respectively) were significantly higher than their live counterparts (-2.70±0.46 and -0.31±0.03 respectively), i.e. dead cells are more negatively charged that live cells.

Hammer et al. (1999) offered live bacteria/algae with differing surface charge to *Oxyrrhis marina* and no effect of surface charge on ingestion rates was reported. Similarly, Matz and Jürgens (2001) assessed the effect of 14 unidentified live bacterial surface charge on heterotrophic nanoflagellate ingestion (*Bodo saltans*, *Spumella*

pudica and *Ochromonas* sp.). It was reported that there was no significant dependence on prey surface charge. Lastly, Matz et al. (2002) reported that feeding heat-killed bacteria of different surface charge to *Spumella* sp. again showed no effect. These results may initially be surprising as it does not seem to conform to what is shown in leukocytes and macrophages, where hydrophobic particles are more favourable (Van Oss, 1978, Watanabe et al., 1984, Sano et al., 1986, Monger and Landry, 1990, Yamada et al., 1993, Roser et al., 1998, He et al., 2010).

Data on the effect of surface charge on ingestion of bacterial prey is varied. As mentioned above, even though it may be possible for live and dead bacteria to be ingested via this surface charge mediated pathway, it may well be that the receptor mediated pathways suggested in Sections 4.1.4.2.1 and 4.1.4.2.2 are the dominant pathway for the ingestion of live and dead bacteria. Thus, the attempts to investigate surface charge in the aforementioned studies may be unavoidably affected.

Nevertheless, once the particles are ‘recognised’ by the ciliate, they are ingested and placed into digestive vacuoles. The action of ‘recognising’ and ‘ingesting’ may be separate and regulated as such. Hence vacuole formation and the mechanism whereby it may be controlled is discussed next.

4.1.4.3 Effect on digestive vacuole formation rate

Vacuole formation rates (and egestion rates) in the presence of $2x10^7$ FLMs/ml were consistent between experiments (Table 3.3) and in line with those previously reported for *T. pyriformis* (Thurman et al., 2010a). They were at near maximum at this particle concentration (V_{max}, 0.575 ± 0.053 vac/cell/min, K_s, $3x10^6$ FLMs/mL, Table C.3. in Appendix C) where one vacuole is formed in *ca.* 2.21 minutes (Table 3.3). This maximum rate falls within published maximum rates for different strains of *T. pyriformis* feeding on inert particles with one vacuole being formed in 1.67-3.23 minutes (Chapman-Andresen and Nilsson, 1968, Ricketts, 1971, Nilsson, 1972, Ricketts and Rappitt, 1976, Hoffmann et al., 1974, Rasmussen, 1976). Gonda et al. (2000) also reported that *Tetrahymena thermophila* formed vacuoles at a maximum rate of one every 3.3 minutes and (Fok et al., 1987) reported that *Paramecium caudatum* produced one vacuole in 2.5 minutes. Contrary to this, an incredibly fast DV formation rate of one vacuole being formed every 0.43 min has been reported by Kodama and Fujishima (2005) when *Paramecium bursaria* was fed India ink.

Subtle differences in DV formation rates for the *Tetrahymena* species may be due to environmental factors such as changes in temperature (Lee, 1942b, Preer Jr, 1975) and pH (Lee, 1942a, Brutkowska, 1963) which were not always stated, but the overall reproducibility in vacuole formation rate across *Tetrahymena* strains suggests a 'basal vacuole formation' mechanism might occur in this ciliate which is initiated by the mere presence of particles. Although particles are known to induce vacuole formation in both *Paramecium* and *Tetrahymena* (Mueller et al., 1965, Rasmussen, 1976, Nilsson, 1979, Fok et al., 1988), cells can form vacuoles even in their absence, albeit at a much slower rate and the resultant vacuoles are very small (Ishida et al., 2001).

In the current study, under equivalent environmental conditions, the shortest time taken to form one vacuole ranged from 2.0 to 2.51 minutes with dead *S. aureus*, Pico 3 and Pico 20 (Table 2.6) which falls within the published range for *T. pyriformis* feeding on

inert particles, suggesting that the basal vacuole formation mechanism occurs with these prey. However, vacuole formation with live *S. aureus* was much quicker (one vacuole in 1.49 min) and outside published values with inert particles suggesting an upregulation in vacuole formation rate. Indeed, experiments with $2x10^7$ prey/ml showed that all the live heterotrophic bacterial species produced more vacuoles in 5 minutes (average of 3.4 vacuoles/cell) than their heat-killed counterparts, FLMs and live autotrophic prey (average of 1.94 vacuoles/cell) (Table 3.5). Similar upregulation of DV formation has been reported by Kodama and Fujishima (2005), whereby the *P. bursaria* formed one vacuole every 0.43 min when the cells were fed India ink, but formed one vacuole every 0.25 min when the cells were fed live *Chlorella* sp..

The mechanism behind such upregulation is unknown but considering the speed at which it occurred in the current study, it suggests that if the process is receptor-mediated the receptor is 'available' on the outer surface of the live heterotrophic bacterial cell wall no matter the Gram status or species/strain of the bacterium. It also suggests that, because this receptor is not 'available' on live *Synechococcus* cells, the receptor can be covered by an S-layer suggesting that it exists in the outer membrane of Gram-negative bacteria and on the surface of the peptidoglycan in Gram-positive bacteria.

Even though *Synechococcus* cells did not up-regulate DV formation in 5 minutes, this might have been too short a period to detect a change. Interestingly, Thurman et al. (2010a) presented a Figure (Figure 4.4) which showed the formation of FLM-containing DVs and *Synechococcus* (Pico 5)-containing DVs over a 20 minute period at $2x10^7$ particles/ml and just as prey selection graphs can diverge after a short period of time (Figure 4.1), DV formation graphs can also. Figure 4.4 shows that DV formation is similar at 5 minutes with both prey types (and equivalent to those in the current study) but after this, divergence is evident with Pico 5 inducing the formation of 7.5 vacuoles by 20 minutes compared to only 4 vacuoles with FLMs. This does not seem to be

related to the biovolume of the prey, as Pico 5 is larger (1.14µm^3) than FLMs (0.065µm^3) so the reduced number of FLM-containing vacuoles is not due to a limitation of vacuolar membrane.

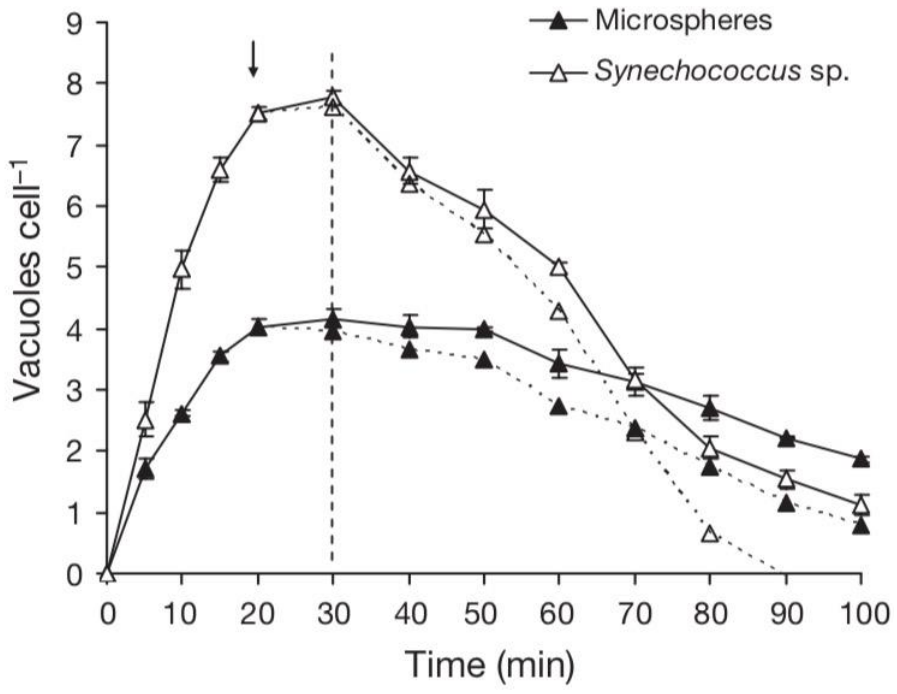


Figure 4.4 Results from pulse-chase experiments from Thurman et al. (2010a) on vacuole formation when T. pyriformis was fed FLMs and Synechococcus (Pico) No. 5 at 2x10^7 particles/ml for 20 min before the chase. Vacuole formation is similar for both prey at 5 minutes but then divergence is evident with Pico 5 inducing more vacuoles by 20 minutes compared to FLMs.

If vacuole formation rates with FLMs are the 'norm' then this suggests that Pico 5 (in Figure 4.4) is upregulating vacuole formation after 5 minutes which also suggests that it might do this via a recognition processes within the DV and not external to it. This could be due to the un-masking of the outer membrane receptor upon 'digestion' in the vacuole and even though this strain is indigestible to *T. pyriformis* (Thurman et al., 2010a), there is no evidence to suggest that its S-layer remains intact upon egestion. Such up-regulation of the vacuole machinery might also explain why egestion rates

with Pico 5 were more rapid than with FLMs (Figure 4.4); a result that Thurman et al. (2010a) could not explain at the time. Even so, the current study did not observe up-regulation of DV formation in *T. pyriformis* over 20 minutes when fed with Pico 5 at $5x10^6$ cells/ml (Figure 3.14) which might suggest that perception of any ligand by the phagosome might depend on the volume of prey within it, i.e. more prey results in more stimulation. It may have been that a longer period than 20 minutes was necessary to demonstrate any upregulation in DV formation at this lower prey concentration.

4.1.4.4 Cross talk between proposed ingestion pathways

It is suggested above (Section 4.1.4.2) that there are two distinct routes for prey to enter a protist, the 'non-specific'-receptor-based route which is primarily governed by electrostatic charge interactions with scavenger receptors (SRs) like MARCO, and a lectin-receptor-based route. However, these two routes may not be mutually exclusive and 'cross-talk' may occur. Vogel et al. (1980) not only showed that lectins were involved in live prey ingestion by *Dictyostelium* but they also showed that when the lectin-route was inhibited the WT was able to ingest live bacteria via the 'non-specific'-receptor-based route. This suggests that both entry routes are co-existing, and perhaps the lectin-based route is that 'chosen' for bacterial prey while inert prey only enter via the non-specific-receptor route (due to their interaction with a MARCO-like receptor). However, mannose receptors have been reported to be involvement in phagocytosis of un-opsonized heat-killed yeast (*Saccharomyces cerevisiae*) by macrophages (Giaimis et al., 1993), thus suggesting that these cells can also enter via the lectin-route.

4.2 Digestion of prey

4.2.1 Phagosome maturation

The phagocytic process is initiated by the recognition of prey ligands (PAMP/MAMP) by cell surface receptors (PRR) (discussed in Section 4.1.4.2 and 4.1.4.3) and prey are subsequently deposited into digestive vacuoles (DV) (or 'phagosomes'). Because the nascent DV membrane is essentially the same as plasma membrane (Muller et al., 1980a, 1980b), DVs inherently lack the microbicidal and degradative capacity required for prey digestion. These capabilities are acquired subsequently by a process called 'phagosome maturation'; a sequence of membrane fusion and fission events with components of the endocytic compartment (e.g. acidosomes, lysosomes) (Pauwels et al. 2017). Phagosome membranes are selectively fusogenic and acquire surface proteins which allow the phagosome to selectively interact with the next endosome in the maturation sequence, i.e. the surface proteins change over time (Desjardins et al., 1997, Maniak, 2003). The dynamic way in which phagosome surface proteins change can be influenced by various factors, including the nature of the enclosed prey (or 'cargo') (Pauwels et al. 2017).

Most work has examined pathogenic 'cargo', particularly the intra-vacuolar pathogens which actively re-model the phagosomal membrane to mimic those of resident organelles; this prevents phagosome maturation and allows the pathogen to replicate within the phagosome (Blander and Medzhitov, 2006). Examples of such pathogens include *Leigionella pneumophila* and *Mycobacterium tuberculosis* whereby both affect phagosome maturation by impeding acidification and phago-lysosomal fusion. The Dot/Icm type IV secretion system is the single most important pathogenic determinant of *L. pneumophila* (Ensminger and Isberg, 2009) as it injects multiple effector proteins into the host cell where they subvert host vesicle trafficking and signal transduction pathways (Nagai and Kubori, 2011). Approximately 300 different Icm/Dot substrates have been experimentally validated (Lifshitz et al., 2013). One is SidK which

specifically inhibits V-ATPase activity and stops the acidification process (Xu et al., 2010). *M. tuberculosis* also produces several secreted proteins with PtpA being the one that inhibits V-ATPase (Forrellad et al., 2013). Components of the complex mycobacterial cell wall have also been identified as virulence factors that can arrest phagosome maturation. These include mannose-capped lipoarabinomannan (ManLAM) (Fratti et al., 2003) and trehalose-6,6-dimycolate (TDM) (Axelrod et al., 2008).

Even so, the vast majority of bacteria that protists would encounter *in situ* would not possess such virulence factors and studies on the effect of such innocuous bacteria on phagosome maturation are rare. To date, twenty five studies have examined the effect of innocuous 'cargo' on phagosome maturation in macrophages and dendritic cells with most of these using polystyrene beads coated with specific ligands (Pauwels et al. 2017). Of these, only a handful have used ligands relevant to the uptake of innocuous bacterial prey by protists, i.e. those coated with LPS or mannan (bacterial PAMPs). There is a general consensus that mannan does not affect phagosome maturation (Dykstra et al., 2011, Dill et al., 2015) which is not surprising as the mannose receptor has for a long time been considered to be only a phagocytic receptor (Section 4.1.4.2.1); although ManLAM (in *M. tuberculosis*) has been shown to affect phagosome maturation (Fratti et al., 2003). LPS has been shown to induce a quicker acidification and stronger oxidative burst, but has no effect on overall ligand digestion (Dill et al., 2015). However, Blander and Medzhitov (2004) showed that coating apoptotic cells with *E. coli*-derived LPS did not result in a change in phagosome maturation rate.

The current study evaluated the effect of different 'innocuous' bacterial strains in two states (live and dead) on prey digestion (and indirectly, phagosome maturation) in *T. pyriformis* and once again, for the purpose of the following discussion live bacteria are considered to possess both surface and deeper ligands while heat-killed cells are considered not to possess surface ligands but only those that may have been buried

deeper in the cell well (which have only become exposed upon heat-treatment and loss of the outer membrane).

4.2.2 Effect of prey species/strain

The current study found no evidence that different species of dead bacteria affected their % total digested and digestion rate in *T. pyriformis* and no effect of Gram-reaction was seen. This conflicts with the results of Thurman et al. (2010a) who showed that DTAF-stained *S. aureus* was digested at a significantly slower rate than Gram-negative bacteria by *T. pyriformis*, but agrees with their finding that there was no significant difference in the digestion rates of the three DTAF-stained Gram-negative strains tested (*E. coli* K12, *Mesorhizobium* sp. and *P. aeruginosa*).

Comparison of the digestion rates of live bacteria resulted in two groupings each containing strains that were not significantly different from each other but there was no obvious pattern as to the composition of those groups (Figure 3.18). Jezbera et al. (2005) found no evidence of differential digestion of live *A. hydrophila* and *P. fluorescens* by the ciliate *Cyclidium glaucoma* and flagellate *Goniomonas* sp.. In the current study both these bacterial species were also not digested differentially by *T. pyriformis*; both were within Group 1 (Figure 3.18). Jezbera et al. (2005) however, did find that another flagellate (*Bodo saltans*) digested *P. fluorescens* at a faster rate than *A. hydrophila* but inspection of their data shows that rates were, in reality, equivalent for the first 20 min (4 data points) and then dropped rapidly (1 data point), leading to an overall faster rate. In the current study, live *S. aureus* (within Group 1) was digested at slower rates than only those Gram-negative strains in Group 2; no difference was seen with those in Group 1. So once again, no obvious pattern emerged.

Comparison of the current study's calculated digestion rates to published data is impossible because studies report digestion rate in a number of ways. Most have measured prey digestion as prey/ciliate/min (Sherr et al., 1988, Gonzalez et al., 1990a, González and Suttle, 1993, First et al., 2012) which does not take into accout the

variations in, (i) the number of vacuoles per cell, (ii) the number of prey/vacuole and (iii) prey biovolume/vacuole at the start of the chase. Those that have expressed digestion rate as prey/vacuole/min (Thurman et al., 2010a, Dixon, 2010) have accounted for (i) and (ii) but not (iii), while those that have expressed digestion rates as % loss of food/cell/min (Dolan and Coats, 1991, Dolan and Simek, 1998, Xinyao et al., 2006), have accounted for (ii) and (iii) but not (i). Therefore any apparent differential digestion between prey species previously reported might be questionable. In the current study there was very little evidence for differential digestion between either live or dead bacterial species using digestion rates expressed as % prey/vacuole/min.

4.2.3 Effect of prey state – Live vs Dead prey

Differential digestion has been examined previously (Boenigk et al., 2001b, Thurman et al., 2010a, Boenigk et al., 2001a, Sherr et al., 1992), however, the effect of prey state has not been directly investigated. Prey state is of particular importance as numerous protistan digestion (and ingestion) studies utilise heat-killed-DTAF-stained bacteria as surrogate prey, in substitution of live prey (Sherr et al., 1987, Sherr et al., 1988, Gonzalez et al., 1990a, Gonzalez et al., 1990b, González and Suttle, 1993, Strom, 2001, Dixon, 2010, Thurman et al., 2010a).

4.2.3.1 Effect on total prey digestion and digestion rate

T. pyriformis digested on average *ca.* 52% of enclosed live prey and *ca*. 52% of enclosed dead prey within the digestive period (DP). However, the length of the DP with dead prey was significantly shorter than that with live cells (discussed further in Section 4.2.3.2) and hence the digestion rates of dead prey (as %prey/vac/min), overall, appeared significantly faster than with live prey ($P \leq 0.05$). This suggests that the heat-killing of cells makes them more digestible which seems logical, as there is no outer membrane to digest. However, examination of the direct comparison between the four strains (in both live and dead states) only showed a significantly faster rate for dead *K. pneumoniae* (Figure 3.17). Digestion rates were equivalent for the other three

species (*K. aerogenes, S. enterica* [74] and *S. aureus*). This does question the robustness of the statistical difference between the aggregated group results.

When the biovolume of prey in digestive vacuoles (whether live or dead) was plotted against the biovolume of enclosed prey digested (Figure 3.21), there was a very strong linear relationship, no matter what the prey state was. This implies that larger biovolume of prey result in more biovolume of prey being digested (and possibly assimilated), which in itself could lead to higher ciliate specific growth rates and cell yields (see Section 4.3). This behaviour may be due to the recognition of ligands in the membrane of the prey cells or peptidoglycan, where this recognition results in altered digestion efforts (discussed in detail in Section 4.2.3.3.). It may be that a larger biovolume of cells within the DV provides more membrane material, and thus more ligands or peptidoglycan for recognition.

4.2.3.2 Effect on digestion period

The only strong significant difference observed with regards to DV maturation in the current study was that the digestion periods (DP) with dead bacteria were significantly shorter (by *ca.* 5 min) than the DPs with live bacteria. The DP includes Phases I to III of the DV processing model suggested by Fok et al. (1982) and the fact that it was significantly shorter with dead bacteria suggests that this prey type was altering the timing of one or all of the three phases. It is unlikely that one of the phases was completely omitted because (i) dead cells showed evidence of digestion which suggests that lysosomal fusion occurred (Phase III) and this cannot happen unless the DV is acidified (in Phases I-II) (Fok et al., 1982, Fok and Shockley, 1985). Indeed, Giorgione and Clarke (2008) have shown that macrophage phagosomes containing inert particles, devoid of any ligands, still go through acidification and lysosomal fusion. However, none of the phases proposed by Fok et al. (1982) are a ‘one-off’ fusion event. They are an orchestrated sequence of fusions between the DV and different vesicles over time, particularly during Phase III. For example, it is known that optimum

pH conditions for each of the numerous digestive enzymes involved with digestion differ and in macrophages different enzymes are delivered into the phagosome in a sequential fashion by fusion with lysosomes containing the different enzymes (Tjelle et al., 2000, Vieira et al., 2002).

It is therefore hypothesised that the lack of surface ligands on dead cells prevents the DV from going through the complete sequence of fusion events with lysosomes and only allows fusion with 'late' lysosomes; by-passing 'early' lysosomes. Contrary to this, live prey with their full complement of receptors go through the full sequence of fusions with surface receptors allowing fusion with 'early' lysosomes, and deep receptors (the only ones available in dead cells) allowing fusion with 'late' lysosomes. This would result in longer DPs with live cells, as reported here. Although only a hypothesis, it is interesting to note that there is evidence that *Dictyostelium discoidium* (Souza et al. 1997, Rupper et al. 2001) and macrophages (Oh and Swanson, 1996) can detect vacuolar content and make adjustments to vacuole trafficking. Also, Wilkes and Sleigh (2004) recorded that the binding of ConA to DVs of the ciliate *Euplotes mutabilis* (feeding on live *Vibrio natriegens*) peaked at 10 minutes and then declined. One of the ligands for ConA is mannose which, in the current study, is the proposed surface receptor for the uptake of live bacteria (Section 4.1.4.2.1). Its disappearance after 10 minutes could be an indication that the ligand is being digested and judging by the model of Fok et al. (1982) (and Figure 1.4) this time period equates to the early stages of Phase-III. Its disappearance suggests that it might be more involved with DV fusion with 'early' lysosomes, rather than 'late' lysosomes. If this is the case, then it is likely that the LPS *per se* is digested in a similar fashion and any associated ligands are not involved with DV fusion with 'late' lysosomes. These late fusion events might be reliant on those deep ligands which have been unmasked during the digestion process. One such deep ligand was proposed to be peptidoglycan (or associated ligands) (Section 4.1.4.2.2).

4.2.3.3 Prey recognition within the digestive vacuole

The recognition of deep ligands such as peptidoglycan by an SR-A-like receptor has been proposed to be a potential mechanism by which *T. pyriformis* might identify different species of bacteria (Section 4.1.4.2.2). Peptidoglycan (also known as murein), is made of glycan strands cross-linked by short peptides and forms a protective structure surrounding the cytoplasmic membrane. The sugar component consists of alternating glycosidic β1,4-linked residues of *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) (Vollmer et al., 2008). Lysozyme is an enzyme that specifically degrades bacterial cell walls by breaking the β(1-4) linkage between residues of N-acetylmuramic acid and N-acetylglucosamine peptidoglycan (Bera et al., 2005, Stryer, 1981). Chung and Kocks (2011) showed that treatment of *E. coli* with lysozyme led to the binding of the SR Eater in a concentration-dependent manner, with lysozyme concentrations of up to 1.5μM 'unmasking' its ligand(s). This suggests that digestion of live bacteria in the DV (in Phase III), which involves lysozyme, can unmask deep ligands such as peptidoglycan (and associated ligands) in the later stages of Phase III.

In the current study it was hypothesised that bacterial species/strains might be distinguishable based on peptidoglycan (and associated ligands) because many bacteria are able to modify their peptidoglycan and as a result peptidoglycan can be highly variable between different bacteria species (Vollmer, 2008, Davis and Weiser, 2011). In heat-killed cells these ligands were immediately available to the ciliate and this resulted in species variations in prey/vacuole (Section 4.1.4.2.2). Considering the mannose receptor was proposed to be a receptor that just recognises 'live food' (no matter whether it is digestible or not) (Section 4.1.4.2.1), and it could not differentiate between different species, it may be that species/strain recognition of live bacteria normally occurs within the DV, during digestion, when the deeper ligands are unmasked. This would cause a 'delay' in 'normal' prey species recognition and might

possibly explain why prey uptake and vacuole formation graphs diverge after a given period of time (Figures 4.2 and 4.4). It is also interesting to note that a common virulence factor of pathogenic bacteria is their indigestibility. For example, *Bacillus anthracis*, *Streptococcus pneumoniae* and *Listeria monocytogenes* are Gram-positive pathogens that have been shown to N-deacetylate the N-acetylglucosamine residues of their peptidoglycan and as a consequence they are resistant to lysozyme (Zipperle Jr et al., 1984, Vollmer and Tomasz, 2000, Boneca et al., 2007). Being resistant to digestion would prevent the unmasking of deeper ligands and, if as hypothesised here these deep ligands are species/strain specific, this would mean that these pathogens would never reveal their true identity to their host cell.

Thurman et al. (2010a) alluded to the fact that *T. pyriformis* might have the ability to recognise enclosed prey in DVs and adjust its digestion processes accordingly. In that study, the digestion rates of heat-killed-DTAF-stained *Mesorhizobium* were deduced after the ciliate had been pre-fed indigestible FLMs, indigestible *Synechococcus* or digestible live *Mesorhizobium* sp. for 5, 15 or 30 minutes. The digestion rates of the *Mesorhizobium* sp. were equivalent in all ciliate cultures except for those pre-fed with indigestible FLMs or *Synechococcus* for 30 minutes. These were significantly higher due to a ‘burst’ of rapid digestion over the first 5 min, suggesting that an elevated level of digestive machinery was being presented to new vacuoles after 30 minutes of starvation. The authors proposed two hypotheses that might explain how this was achieved. (i) That *T. pyriformis* may have up-regulated digestive enzyme activity in response to the prolonged feeding on indigestible prey, since Fok and Paeste (1982) had shown that levels of lysozyme in *Paramecium* spp. increased under nutrient stress and, (ii) since there was evidence that *Dictyostelium discoidium* and macrophages (Oh and Swanson, 1996, Souza et al., 1997, Rupper et al., 2001) can detect vacuolar content and make adjustments to vacuole trafficking, it was hypothesised that digestive machinery might have been immediately re-directed from the vacuoles containing

indigestible prey to those newer ones containing digestible prey. Considering that vacuolar content (specifically heat-killed cells) in the current study might have short circuited the phagosome-lysosome fusion events (see Section 4.2.3.2), hypothesis (ii) seems quite feasible but whatever the mechanism, results imply that feeding history might significantly influence DV processing and digestive efficiency.

If prey species recognition normally occurs within the DV as opposed to outside it, this might go some way to explain the contradictory evidence for/against prey species selection or prey-size selection by protists, in that short-term feeding experiments (e.g. 20 minutes in the current study), are just too short to detect any prey-induced changes in feeding behaviour, as highlighted by Montagnes et al. (2008).

4.3 Growth

4.3.1 Effect of prey species/strain

The current study examined the growth of *T. pyriformis* on the 12 live and 7 dead heterotrophic bacteria for which digestion rates had been calculated (at $2x10^7$ prey/ml). Since there had been very litte effect of species/strain on total digestion (%) and digestion rates (% Prey/vac/min) (Section 3.5.1), there was no expectation that any effects on ciliate growth rate would be recorded. However, the live Gram-positive *S. aureus* resulted in a significantly lower growth rate, compared to all the Gram-negative strains. It's growth rate was also equivalent, whether it (*S. aureus)* was live or dead. Other studies have acknowledged that Gram-positive bacteria give rise to lower growth rates in flagellates and ciliates (Gonzalez et al., 1990a, Iriberri et al., 1994) and Pickup et al. (2007c) reported that *S. aureus* was completely indigestible to the amoebae *A. castellanii* and *Vermamoeba (Hartmannella) vermiformis*, and was egested apparently unharmed. More pathogenic strains of *S. aureus* can also replicate within these amoebae (Anacarso et al., 2012). Growth rates on dead bacteria also showed an effect of Gram status with all the Gram-positive bacteria giving rise to lower growth rates compared to the Gram-negative strains.

4.3.2 Effect of prey state – Live vs Dead

There was very little effect of prey state (Live vs Dead) on digestion (Section 3.5.2) with *T. pyriformis* digesting *ca.* 51% of the enclosed prey within the digestive period, so it was expected that there would be little effect of growth rate. Indeed, the growth rates of *T. pyriformis* on both prey states were equivalent. This agrees with the limited published studies investigating the differences in growth of protists when fed live or heat-killed bacteria. For example, Ferrier-Pages and Rassoulzadegan (1994) showed that in a ciliate *Strombidium sulcatum* and a *flagellate Pseudobodo sp.* that heat-killed bacterial prey supported growth but gave lower yields than live bacteria. Similarly, Allali et al. (1994) reported that in *S. sulcatum*, growth rates were indistinguishable between

cultures fed live bacterial prey and heat-killed bacterial prey. This possibly indicates the more complete nature of the prey cell for digestion and assimilation.

4.3.3 Effect of prey biovolume

No matter whether the prey were live or dead, a very strong positive correlation had been recorded between total biovolume of prey within the DV and the volume of enclosed prey that was digested (Figure 3.21). This suggested that larger cells (in larger vacuoles) would result in more biovolume being digested and possibly assimilated which in itself might lead to higher ciliate specific growth rates. However, after comparing these parameters there was no relationship. There was also no relationship between growth rate and total digestion values and digestion rates.

4.3.4 From digestion to division

The lack of a direct relationship between total digestion/digestion rate and growth rate is not entirely surprising because as far as cellular processing is concerned, they are far removed. The current study did not evaluate the rate of assimilation and indeed the fate of digested material, i.e. what proportion was released by the cells and this is the 'missing link' in the study. Such investigations can be performed with radiolabelled prey and using such a technique Zubkov and Sleigh (1999) showed that although two flagellates (*Caecitellus parvulus* and *Pteridomonas danica*) consumed between 55-75% of the available prey (live *Vibrio* sp.) their Gross Growth Efficiencies (GGE), i.e. the fraction of ingested prey devoted to growth, were only 22-29%, with these flagellates regenerating and releasing *ca*. 70-85% of the nutrients present in their food out of their cells. Such remineralisation is a hallmark of protists participation in the 'microbial loop' (Johannes, 1964, Nagata and Kirchman, 1992, Dolan, 1997, Twining and Fisher, 2004) (Section 1.2.1).

However, it is interesting that in the most comprehensive survey of published GGE values to date (Straile, 1997), the average flagellate (and dinoflagellate) GGE was

similar to that of ciliates, standing at *ca*. 30%. Indeed, this was very similar to GGEs reported for very different taxa such as cladocerans, copepods and rotifers (Straile, 1997). Mathematical modellers even use 30% as an estimate of GGE when simulating carbon transfer processes within aquatic foodwebs (Buitenhuis et al., 2010). Based on data in the current study that only *ca.* 50% of ingested prey is digested, and using an estimated 30% for GGE, this suggests that the majority of nutrients (50% out of 70%) is released from the protist as carbon-rich fecal pellets, with only 20% of ingested prey material being completely mineralised to orthophosphate and ammonium due to a loss of CO_2 during respiration (Sherr et al., 1983, Fagerbakke et al., 1996, Dolan, 1997). Considering that in addition to egesting carbon-rich vacuoles, protists 'actively' recycle nutrients as dissolved organic matter to the bacterial communities (Johannes, 1964, Nagata and Kirchman, 1992, Dolan, 1997, Twining and Fisher, 2004) it is intriguing to wonder whether nutrient recycling is at all active or whether it is just a mechanical response. Even so, all released materials are substrates that bacteria can utilise and this helps sustain an active prey population for the predator.

4.4 Conclusions

This study set out to investigate the effect of different prey types on their ingestion and digestion by the ciliate *Tetrahymena pyriformis* and how this affected ciliate specific growth rate. The findings suggest that there is a receptor system for recognition of prey particles leading to ingestion on the one hand, and a separate recognition system inside digestive vacuoles (deciding what the ingested material is), is on the other hand. This is known to occur with macrophages (Pauwels et al., 2017) but it has also been proposed to occur in free-living protists (Zwirglmaier et al., 2009, Strom et al., 2017).

4.4.1 Ingestion conclusions

The study showed that there was a lack of effect of prey biovolume and Gram status on prey ingestion by *T. pyriformis*. However, there was evidence that different prey types (Live, Dead, Pico and FLM) were ingested differently. Live heterotrophic bacteria seemed able to quickly upregulate DV formation rate (in 5 minutes) which was something not demonstrated by any of the other prey types. It was hypothesised that this is receptor-mediated and that the ligand might be in the outer membrane (in Gram-negative cells) or on the surface of the peptidoglycan (in Gram-positive cells). Either could be masked by an outer S-layer which results in no upregulation of DV formation rate like seen in Pico prey.

All prey types, except FLMs, showed controlled vacuole filling over a 20 minute period which suggests this is receptor mediated. Live heterotrophic and autotrophic prey yielded a significantly lower maximum prey/vacuole than heat-killed cells, and whereas significant differences in prey/vacuole between species were discerned for heat-killed cells, no such differences were discerned for live species. Putative receptors were hypothesised to be (i) the mannose receptor (ligand, mannose) for the controlled uptake of live bacteria and (ii) a scavenger-like receptor (ligand, peptidoglycan) for the uptake of heat-killed bacteria.

The predominant ingestion pathways for the different prey types are hypothesised to be (i) the lectin-receptor-based route for live prey (mannose-receptor binding with mannose/mannan) and, (ii) the non-specific-receptor-mediated route for the uptake of inert prey (MARCO-like SR, surface-charge interaction). However, these pathways may not be exclusive to one prey type and cross-talk between the two seems likely to occur. The route in for heat-killed cells is more difficult to discern and there may even be a third route in. Evidence points to an SR-A-like receptor being involved in the uptake of heat-killed cell. Being an SR, and not a lectin, suggests entry via the non-specific-receptor-mediated route however, uptake did not appear 'non-specific' as significant differences between prey species with regards to vacuole filling was observed.

4.4.2 Digestion conclusions

The current study found no evidence that different species of live or dead bacteria affected their % total digested and digestion rate in *T. pyriformis* and no effect of Gram-reaction was seen. Overall, *T. pyriformis* digested *ca.* 52% of enclosed live prey and *ca*. 50% of enclosed dead prey within the digestive period. There was however a very strong relationship (for both live and dead states) between biovolume of prey in the vacuole and the total biovolume of prey digested in the vacuole which implied that larger cells result in more biovolume being digested (and possibly assimilated) which in itself could lead to higher ciliate specific growth rates. This however was not found to be the case. The only parameter which differed between the prey states was the length of the DP with the DPs of dead prey being significantly shorter than those with live cells. It was hypothesised that that the lack of surface ligands on dead cells prevented the DV from going through the complete sequence of fusion events with lysosomes and this shortened the DP. It was also hypothesised that the unmasking of deep ligands by lysozyme action within the DV might uncover the identity of the cargo and

this might be a reason why numerous pathogenic bacteria alter their peptidoglycan layer in order to resist the action of lysozyme.

4.4.3 Growth conclusions

Considering the lack of differential digestion of prey in the digestion experiments it was no surprise that the prey had little effect on ciliate growth rates. There was also no obvious relationship between growth rate and any parameter tested (prey biovolume in the vacuole, % prey biovolume digested in the vacuole, % total digestion of prey, digestion rate) suggesting that digestion and growth are too far removed with regards to cellular processes. Even though assimilation was not determined, it was estimated that the majority of prey material released by protists is within spent faecal pellets, as opposed to being directly remineralised and released as inorganic nutrients.

Appendix A. Media recipes

BG11 Broth

Stock solutions:	(1) $NaNO_3$	75.0g	Each in 500ml distilled water
	(2) K_2HPO_4	2.00g	
	(3) $MgSO_4 \cdot 7H_2O$	3.75g	
	(4) $CaCl_2 \cdot 2H_2O$	1.80g	
	(5) Citric acid	0.30g	
	(6) Ammonium ferric citrate green	0.30g	
	(7) $EDTANa_2$	0.05g	

(9) Trace metal solution:

Na_2CO_3	1.00g	In 1000ml distilled water
H_3BO_3	2.86 g	
$MnCl_2 \cdot 4H_2O$	1.81 g	
$ZnSO_4 \cdot 7H_2O$	0.22 g	
$Na_2MoO_4 \cdot 2H_2O$	0.39 g	
$CuSO_4 \cdot 5H_2O$	0.08 g	
$Co(NO_3)_2 \cdot 6H_2O$	0.05 g	

Use 10mL of stock solutions (1) to (8) and 1 mL of stock solution (9) per 1L BG11 broth.

Make up to 1 litre with deionized water. Adjust pH to 7.1 with 1M NaOH or HCl.

Autoclave at 121°C for 15 minutes

CHALKLEY'S MEDIUM

Stock solution:	NaCl	2.00g	In 100ml
	KCl	0.08g	distilled
	$CaCl_2$	0.12g	water

Add 5mL of stock solution to 1 litre distilled water. Autoclave at 121°C for 15 minutes.

DIAGNOSTIC SENSITIVITY TEST (DST) AGAR

Diagnostic susceptibility test agar	37.5g

Add to 1 litre distilled water. Autoclave at 121°C for 15 minutes. Cool to 47°C before pouring aseptically.

LYSOGENY BROTH (LB)

NaCl	10g
Typtone	10g
Yeast extract	5g

Add to 1 litre distilled water. Autoclave at 121°C for 15 minutes.

LYSOGENY BROTH (LB) AGAR

NaCl	10g
Typtone	10g
Yeast extract	5g
Agar No. 2	15g

Add to 1 litre distilled water. Autoclave at 121°C for 15 minutes. Cool to 47°C before pouring aseptically.

LYSOGENY BROTH (LB) AGAR WITH ANTIBIOTICS

Ampicillin stock	100 µg/mL
Chloramphenicol stock	6 µg/mL
Gentamycin stock	60 µg/mL
Kanamycin stock	50 µg/mL

To make each antibiotic agar, autoclave 1 litre LB agar at 121°C for 15 minutes. Cool to 55°C before adding 1 mL of filter sterilized antibiotic stock. Pour aseptically.

VOGEL-BONNER MINIMAL MEDIUM (VBMM) AGAR

Stock solution (10x):	$MgSO_4 \cdot 7H_2O$	2g
	Citric acid	20g
	Trisodium citrate	30g
	KH_2PO_4	100g
	$NaHNH_4PO_4 \cdot 4H_2O$	35g

To make a 10 times VBMM stock solution, add salts to 800mL of warm distilled water. Stir and warm till dissolved and then adjust to pH 7 with sodium hydroxide. Adjust volume to 1 litre and autoclave at 121°C for 15 minutes. Store at room temperature.

For 1 litre of solution for VBMM plates, add 15g of agar too 900mL of distilled water, and then add 100mL of 10 times VBMM stock solution. Autoclave at 121°C for 15 minutes. Cool to 47°C before pouring aseptically.

VOGEL-BONNER MINIMAL MEDIUM - GENTAMYCIN (VBMM G60) AGAR

Gentamycin stock	60 µg/mL

Add 15g of agar too 900mL of distilled water, and then add 100mL of 10 times VBMM stock solution (above). Autoclave at 121°C for 15 minutes. Cool to 55°C before adding 1 mL of filter sterilized gentamycin stock. Pour aseptically.

Appendix B. 5-([4,6-Dichlorotriazin-2-yl]amino) fluorescein (DTAF) staining

Preparation of heat-killed DTAF stained bacteria

Solution 1

Na_2HPO_4	0.71g	in 100ml
NaCl	3.00g	distilled
		water

Autoclave at 121°C for 15 minutes.

Solution 2

tetrasodium pyrophosphate	0.89g	in 100ml
NaCl	0.85g	distilled
		water

Autoclave at 121°C for 15 minutes.

Appendix C. Data and analysis

Prey	State	Source	Strain
Aeromonas hydrophila (RFP)	Live	NCIMB	9240
Enterobacter cloacae (RFP)	Live	MAST labs	
Escherichia coli (RFP)	Live	P Hill	AKN132
Klebsiella aerogenes (RFP)	Live	NCTC	9528
Klebsiella pneumoniae (RFP)	Live	NCTC	5055
Pseudomonas aeruginosa (RFP)	Live	NCIMB	10412
Pseudomonas fluorescens (RFP)	Live	R Pickup	FH1
Salmonella enterica (RFP)	Live	NCTC	74
Salmonella enterica (RFP)	Live	NCTC	12694
Serratia liquefaciens (RFP)	Live	MAST labs	
Serratia marcescens (RFP)	Live	NCIMB	1377
Staphylococcus aureus (RFP)	Live	P Hill	
Klebsiella aerogenes	Dead	NCTC	9528
Klebsiella pneumoniae	Dead	NCTC	5055
Lactobacillus fermentum	Dead	NCIMB	
Listeria monocytogenes	Dead	NCIMB	
Mesorhizobium sp.	Dead	J Parry	B1
Salmonella enterica	Dead	NCTC	74
Staphylococcus aureus	Dead	NCTC	6571
Yellow/green microspheres (0.50 µm)	FLM	Fluoresbrite™ Polyscience Inc.	-
Yellow/green microspheres (0.75 µm)	FLM	Fluoresbrite™ Polyscience Inc.	-
Yellow/green microspheres (0.92 µm)	FLM	Fluoresbrite™ Polyscience Inc.	-
Synechococcus sp.	Pico	K Harper	S-KH1
Synechococcus sp.	Pico	K Harper	S-KH2
Synechococcus sp.	Pico	K Harper	S-KH3
Synechococcus sp.	Pico	K Harper	S-KH4
Synechococcus sp.	Pico	K Harper	S-KH5
Synechococcus sp.*	Pico	K Harper	S-KH6
Synechococcus sp.	Pico	K Harper	S-KH7
Synechococcus sp.*	Pico	K Harper	S-KH8
Synechococcus sp.	Pico	K Harper	S-KH9
Synechococcus sp.	Pico	K Harper	S-KH10
Synechococcus sp.	Pico	K Harper	S-KH11
Synechococcus sp.	Pico	K Harper	S-KH12
Synechococcus sp.	Pico	K Harper	S-KH13
Synechococcus sp.	Pico	K Harper	S-KH14
Synechococcus sp.	Pico	K Harper	S-KH15
Synechococcus sp.	Pico	CCAP	1479/12
*Synechococcus leopoliensis**	Pico	CCAP	1405/1
Synechococcus elongatus	Pico	CCAP	1479/1A
Synechococcus sp.	Pico	CCAP	1479/10
Synechococcus sp.	Pico	CCAP	1479/11

Table C.1. All prey used in ingestion experiments together with their state, source and strain.

A

Prey per cell (P/C)				Vacuoles per cell (V/C)				Prey per vacuole (P/V)				Bio-volume (BV)			
FLM	**DTAF**	**PICO**	**RFP**	**FLM**	**DTAF**	**PICO**	**RFP**	**FLM**	**DTAF**	**PICO**	**RFP**	**FLM**	**DTAF**	**PICO**	**RFP**
15.70	13.77	9.70	8.87	2.03	2.20	1.80	2.17	7.72	6.29	5.44	4.14	0.065	0.30	1.085	0.918
14.13	15.07	11.07	8.10	1.53	1.80	2.03	2.13	9.31	8.40	5.46	3.80	0.065	0.3	0.919	0.511
13.10	20.13	13.83	7.43	1.93	1.83	2.40	2.30	6.78	11.01	5.74	3.24	0.065	0.3	0.588	1.016
11.63	19.67	10.33	4.17	1.83	1.97	1.77	1.17	6.39	9.97	5.89	3.57	0.065	0.3	0.934	1.016
11.03	7.90	10.47	12.03	1.60	1.27	1.77	2.47	7.00	6.23	5.91	4.87	0.065	0.17	1.137	1.334
12.93	7.63	9.00	8.77	1.93	1.67	1.60	2.33	6.69	4.61	5.63	3.75	0.065	0.17	1.171	1.334
17.47	29.13	11.47	11.27	2.47	2.17	1.83	3.00	7.07	13.46	6.25	3.76	0.065	0.27	1.253	0.511
15.20	20.33	10.33	10.27	2.27	2.03	1.80	2.73	6.70	10.04	5.69	3.77	0.065	0.12	0.837	0.511
12.17	7.17	11.10	9.69	2.00	1.87	2.00	2.10	6.10	3.85	5.51	4.65	0.065	0.44	0.943	0.918
13.03	5.67	9.03	11.60	1.93	1.67	1.87	2.43	6.73	3.33	4.95	4.77	0.065	0.47	1.062	0.918
13.47	4.20	9.80	18.27	2.23	1.70	1.90	3.83	6.00	2.48	5.08	4.78	0.065	0.125	0.838	1.701
6.87	14.73	5.63	18.00	1.27	2.87	1.30	4.37	5.38	5.14	4.33	4.12	0.065	1.33	1.039	1.701
8.77	13.10	6.93	19.90	1.43	2.77	1.53	3.63	6.12	4.74	4.53	5.48	0.065	1.33	1.055	0.655
9.37	9.23	7.13	14.03	1.57	1.57	1.67	2.80	6.05	5.88	4.25	5.00	0.065	0.3	0.756	0.655
11.03		6.80	11.90	1.73		1.47	2.83	6.39		4.68	4.21	0.22		0.979	0.785
14.20		14.53	8.13	2.20		2.20	2.03	6.46		6.49	4.00	0.41		0.948	0.785
13.13		13.83	10.03	1.70		2.63	3.07	7.73		5.32	3.28	0.41		2.326	0.512
		10.10	15.77			1.77	3.47			5.73	4.56			0.850	0.512
		12.13	21.67			2.10	3.63			5.75	5.93			0.978	0.759
		13.23	15.53			2.33	3.70			5.63	4.22			2.342	0.759
			12.70				2.96				4.30				0.641
			13.87				3.44				4.03				0.641
			10.07				3.03				3.32				0.444
			8.47				2.43				3.49				0.444
			14.77				2.93				5.04				0.272
			13.63				1.83				7.44				0.272

B

	Prey per cell (P/C)				Vacuoles per cell (V/C)				Prey per vacuole (P/V)				Bio-volume (BV)			
	FLM	**DTAF**	**PICO**	**RFP**	**FLM**	**DTAF**	**PICO**	**RFP**	**FLM**	**DTAF**	**PICO**	**RFP**	**FLM**	**DTAF**	**PICO**	**RFP**
Mean	13.85	13.66	10.31	15.82	2.19	1.73	1.89	3.40	6.39	7.53	5.41	4.58	0.11	0.27	1.10	0.79
SD	3.43	7.66	2.51	5.29	0.49	0.30	0.33	0.77	1.12	3.27	0.61	0.78	0.12	0.12	0.45	0.38
SEM	0.83	2.31	0.56	1.04	0.12	0.09	0.07	0.15	0.27	0.98	0.14	0.15	0.03	0.04	0.10	0.07

C

	Prey per cell (P/C)				Vacuoles per cell (V/C)		
	DTAF	**PICO**	**RFP**		**DTAF**	**PICO**	**RFP**
FLM	0.930	0.001	0.183	**FLM**	0.009	0.032	0.000
DTAF		0.081	0.330	**DTAF**		0.184	0.000
PICO			0.000	**PICO**			0.000
	Prey per vacuole (P/V)				**Bio-volume (BV)**		
	DTAF	**PICO**	**RFP**		**DTAF**	**PICO**	**RFP**
FLM	0.196	0.002	0.000	**FLM**	0.002	0.000	0.000
DTAF		0.008	0.000	**DTAF**		0.000	0.000
PICO			0.000	**PICO**			0.014

*Table C.2. Data and analysis of four prey states when fed to T. pyriformis for 5 min: live and indigestible (Pico), live and digestible (Live), dead and digestible (Dead) and inert and (FLM) for four parameters: prey per cell (P/C), vacuoles per cell (V/C), prey per vacuole (P/V) and bio-volume. **A**) Raw data for 77 prey**. B**) Mean, standard deviation (SD) and standard error of the mean (SEM) for each of the prey states and each parameter. **C**) T-test results (P values) for the four parameters between each of the four prey states. P values in red demote significant differences (P≤0.05).*

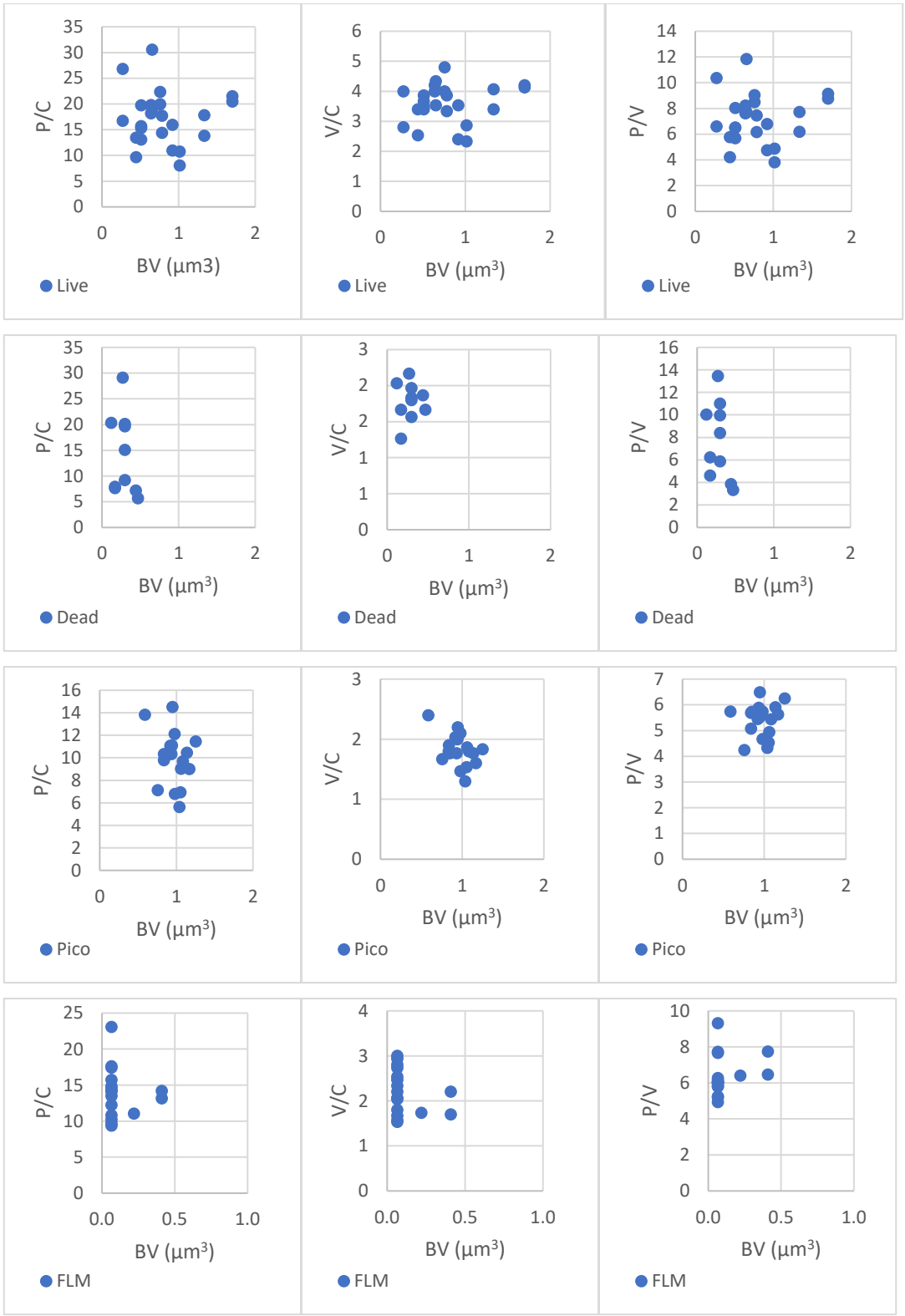


Figure C.1. Regression analysis of prey biovolume against each of the three parameters for all four prey types. There were no effect of prey bio-volume on any of the three parameters. P/C = Prey per cell. V/C = Vacuoles per cell. P/V = Prey per vacuole. BV = Prey bio-volume.

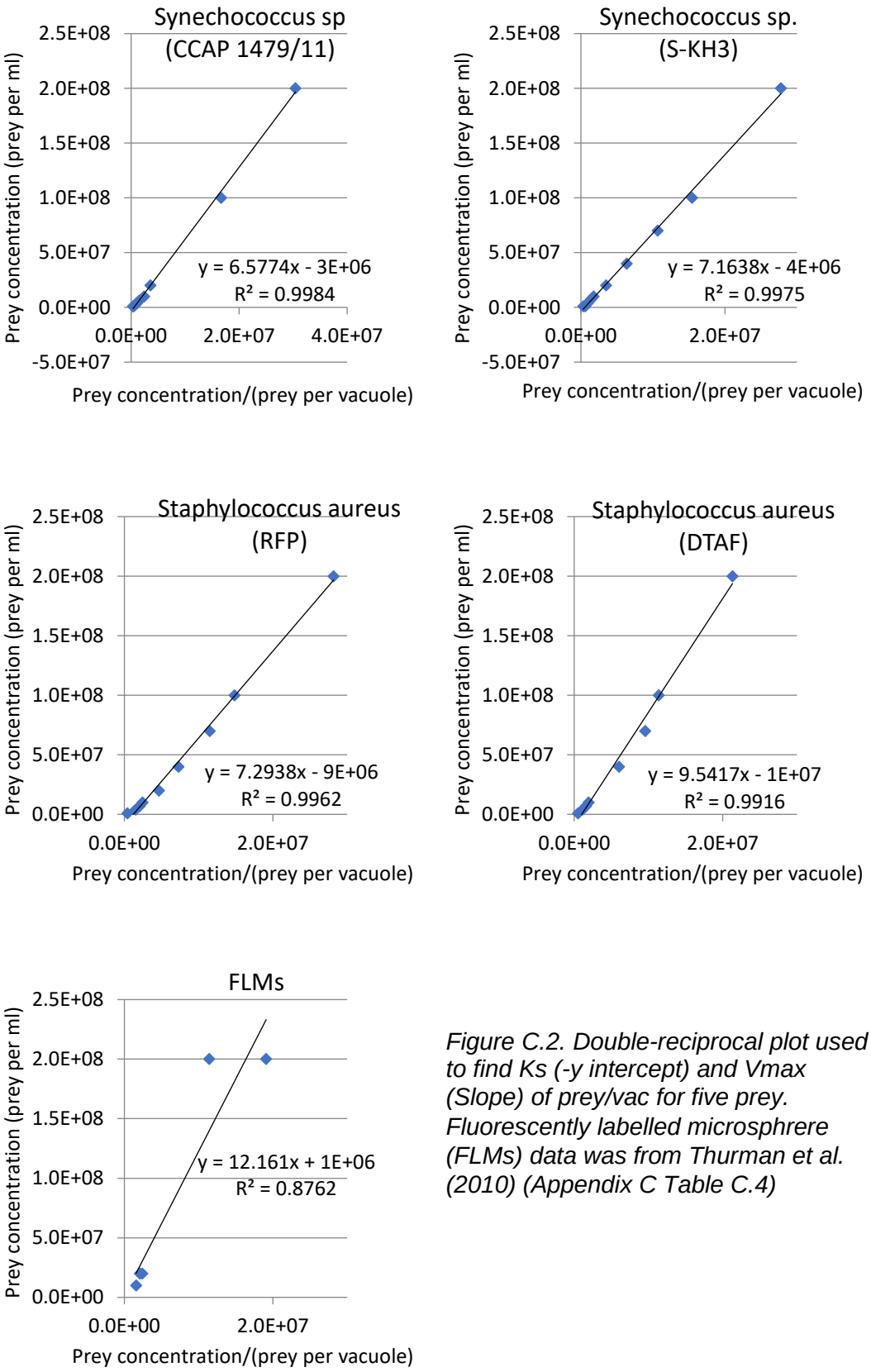


Figure C.2. Double-reciprocal plot used to find Ks (-y intercept) and Vmax (Slope) of prey/vac for five prey. Fluorescently labelled microsphrere (FLMs) data was from Thurman et al. (2010) (Appendix C Table C.4)

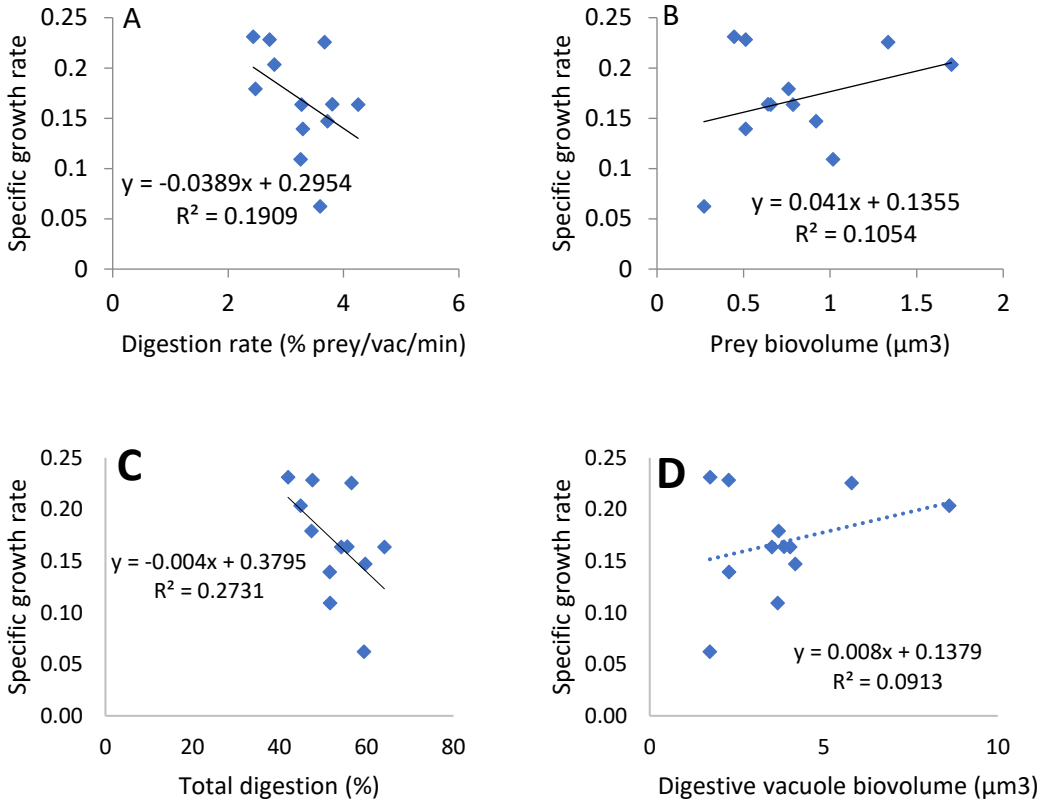


Figure C.3. Regression analysis of specific growth rates against (A) digestion rates, (B) prey biovolume, (C) total digestion, (D) digestive vacuole biovolume when T. pyriformis was fed live bacterial prey at 2x10^7 cells/ml. There were no relationships found between the parameters.

Prey	Vacuoles/cell		Prey/cell		Max ingestion rate
	Ks (x10^5)	Vmax	Ks (x10^6)	Vmax	(Prey/cell/min)
Pico 20	7.37±8.94	2.30±0.03	5.75±0.56	15.3±0.11	3.07±0.02
Pico 3	11.9±5.43	2.50±0.02	6.98±1.66	18.2±0.38	3.63±0.08
Live *S. aureus*	172±40.6	3.36±0.15	48.1±7.18	27.3±1.70	5.46±0.34
Dead *S. aureus*	132±90.1	1.99±0.20	28.3±17.7	18.3±3.20	3.66±0.64
FLMs	60.4±19.0	2.45±0.38	-13.2±8.02	38.4±2.26	7.68±0.45

Table C.3. Maximum and Ks (cells/ml) values for vacuoles/cell, prey/cell and ingestion rate (prey/cell/min) when T. pyriformis feeds for 5 min on Live and Dead S. aureus, Pico 3 and Pico 20. FLMs data was taken from Thurman et al. (2010). Error = SEM. Double-reciprocal plot used to find Ks and Vmax can be found in Appendix C Figure C.2

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