BACTERIVORY OF THREE FRESHWATER CILIATES ISOLATED FROM BLACKBAROUGH SPRUIT IN KWAZULU-NATAL, SOUTH AFRICA

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Submitted in fulfilment of the academic requirement for the degree of Doctor of Philosophy in Microbiology

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South Africa

November 2021

PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg campus, South Africa.

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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R.B. Bulannga

08 November 2021

As the candidate's supervisor I have approved this thesis for submission.

.....
Professor S. Schmidt

08 November 2021

DECLARATION 1 – PLAGIARISM

I, Rendani Bridghette Bulannga, declare that

i. The research presented in this thesis, except where otherwise indicated, is my own and has been generated by me as the result of my own original research.

ii. This thesis has not been submitted for any degree or examination at any other university.

iii. This thesis does not contain other persons' data, pictures, graphs, or other information unless specifically acknowledged.

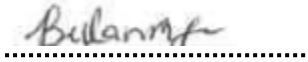
iv. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers.

v. Where other written sources have been quoted, then:

   a) their words have been re-written, but the general information attributed to them has been referenced.

   b) where their exact words have been used, their writing has been placed inside quotation marks and referenced.

vi. All the sources I have used or quoted have been indicated and acknowledged as complete references.



R.B. Bulannga

08 November 2021

DECLARATION 2 – PUBLICATION AND CONFERENCES

Bulannga RB, Schmidt S. 2022. Uptake and accumulation of microplastic particles by two freshwater ciliates isolated from a local river in South Africa. *Environmental Research* 204 (Pt B): 112123. doi: 10.1016/j.envres.2021.112123.

Rendani Bulannga and Stefan Schmidt. 2018. Characterization of three bacterivorous freshwater ciliates isolated from a stream in Pietermaritzburg (KwaZulu-Natal, South Africa). School of Life Sciences Research Day 22 May 2018, Pietermaritzburg Campus.

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R.B. Bulannga

08 November 2021

ABSTRACT

Bacterivorous ciliates are receiving increasing interest due to their essential interaction with bacteria in aquatic environments. They are common inhabitants of freshwater environments and artificial water systems and play a key role in regulating the bacterial population. They are now regarded as a reservoir for bacteria enabling bacterial survival and protection. Therefore, this study aimed to evaluate the predator-prey relationship between ciliates and various bacteria and their potential role in the survival of bacteria. Three holotrich ciliates were isolated from Blackborough Spruit stream water samples and identified via morphological characteristics as *Paramecium* sp., *Tetrahymena* sp., and *Coleps* sp. Analysis of the 18S rRNA gene sequence confirmed the identity of isolate RB1 as *Paramecium* sp. and isolate RB2 as *Tetrahymena* sp. The bacterivory experiments of the isolated ciliates in 5 mL Chalkley's medium at 25ºC in the dark revealed that the grazing activity of the ciliates resulted in up to 90% reduction in bacterial numbers for some bacterial species. Though ciliates did not show selective feeding on bacteria, growth varied with each species. In addition to bacteria, the isolated ciliates ingested viral particles, algae cells, fungal cells, spores, and microplastic particles. The particle size, which influenced grazing rates, ranged from viral-sized particles from 200 nm to 5 µm for *Tetrahymena* sp. RB2 and *Coleps* sp. RB3, while *Paramecium* sp. RB1 ingested particles up to 10 µm. Growth and grazing rates increased with an increase in bacterial concentrations from 10^7 to 10^{10} bacteria x mL^{-1}. The temperature influenced growth and grazing rates of ciliates; highest grazing and growth rates of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 were detected at 25ºC while highest grazing rates for *Coleps* sp. RB3 were detected at 35ºC. Ingested particles were collected and packaged in food vacuoles within the ciliate cells. However, not all bacterial cells ingested were digested in food vacuoles after 24 hours of feeding. Moreover, bacteria that were still viable within the ciliate were potentially protected from biocide treatments. The results obtained show that the presence of ciliates in freshwater systems is essential in the control of bacterial and even viral pollution. They nonetheless have the potential to act as a host for the survival and protection of bacteria and might contribute to the persistence and transmission of bacteria. Moreover, their inability to discriminate between biological

and non-biological particles may contribute to the bioaccumulation of microplastics particles along food webs in aquatic environments.

ACKNOWLEDGMENTS

First and foremost, I would like to give thanks and praise to God Almighty for the success of this thesis. I have experience your grace and love day by day and I will keep trusting you.

I would like to express my sincere gratitude to my supervisor, Professor Stefan Schmidt for his guidance that helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor for my Ph.D. study.

My sincere thanks to the staff of Microscopy and Microanalysis Unit (MMU) at Pietermaritzburg campus, Dr. Lorika Beukes and Ms Ntombozuko Cynthia Matyumza, for their assistance with microscopic analysis.

Many thanks to Dr. Maike Claussen for assistance with molecular analysis.

To my friends Lorika Beukes, Ntombozuko Cynthia Matyumza, Nonthando Ntuli, Isaac Sinusi and Ntaki Senoge, thank you for your continuous support and encouragement during my PhD studies.

I am extremely grateful to my family for their love, without their prayer and understanding it would not have been possible to complete my studies.

Finally, I gratefully acknowledge the funding received towards my PhD degree from DAAD-National Research Foundation.

TABLE OF CONTENTS

CHAPTER 2 – Isolation and characterization of ciliates from freshwater samples

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

Literature review

1.1. Introduction

Phagotrophic protists have long been recognized as an integral part of aquatic ecosystems. They are diverse and abundant in aquatic ecosystems and are considered as major links in matter and energy fluxes through the interaction of microorganisms from different trophic levels (Porter *et al*., 1985; Sherr and Sherr, 1994; Sherr and Sherr, 2002). This trophic interaction of different microbial species is described as microbial food web; where the primary producers and heterotrophic picoplankton making up the first trophic level, assimilate organic and inorganic matter and are grazed by phagotrophic protists for energy, which are then consumed by larger sized protists or zooplankton (Carrillo *et al*., 2006; Weisse *et al*., 2016). Thus, their function in food webs is channelling energy among diverse groups of organisms, thereby contributing substantially to the biogeochemical cycle in aquatic ecosystems.

Bacterivorous protists from both freshwater and marine environments have been extensively studied. They are unicellular eukaryotes, and their predation activity dominates aquatic ecosystems (Caron *et al*., 2009). It has long been established that they are an important source of mortality for bacteria in aquatic environments and have received much interest due to their grazing activity in microbial food webs (Sherr and Sherr, 2002; Thomas *et al*., 2012). A study by Šimek *et al*. (2001) revealed that protists were the main predators in aquatic environments and consumed about 50% of bacterioplankton present in freshwater environments, while in marine environments, protist grazing was responsible for 75% of primary particulate production (Landry and Hassett, 1982; Sanders *et al.,* 1992; Foissner, 1999; Wootton *et al*., 2007). This predator-prey relationship, depicted as a microbial loop, regulates bacterial density and shapes the genotypic and phenotypic composition of microbial communities (Azam *et al.,* 1983; Pernthaler, 2005). Therefore, it is important to maintain the structure and the function of ecological communities in aquatic ecosystems (Hahn and Höfle, 2001).

Although bacterivorous protists have long been recognized as the main bacterial predator and regulator of the bacterial population in aquatic environments, it is only now that they are receiving additional attention as potential host or reservoir, enabling protection and survival of ingested bacteria if these are not digested (Barbaree *et al.,* 1986; Barker and Brown, 1994). The ability of bacteria to survive within protozoa was first reported by Rowbotham (1980), who observed intracellular replication of *Legionella pneumophila* within amoebae cells. This predator-prey interaction increased the resistance to harsh conditions (high temperature and acidic conditions) and chemical treatments (Brandl *et al*., 2005). Since then, thirteen species of amoebae have been shown to be potential hosts for species of *Legionella* (Kwaik *et al*., 1998; Greub and Raoult, 2004). Transmission of this respiratory disease-causing bacterial genus is associated with its interaction with amoebae that have been isolated in domestic water as well as in water distribution systems in buildings (Barbaree *et al.,* 1986; Bonetta *et al*., 2010; Dimitriadi and Velonakis, 2014).

There is a growing concern regarding the occurrence of pathogenic bacteria not only in freshwater systems but also in domestic water systems that had undergone thorough disinfection and chlorine treatments (Levy *et al*., 1986; King *et al*., 1988; LeChevallier *et al*., 1988). Large numbers of pathogenic and non-pathogenic bacteria have been regularly isolated from unchlorinated, chlorinated, and drinking water systems (Ridgway and Olson, 1981; Edge *et al.,* 2013). Freshwater systems and sources of drinking water are subject to contamination by pathogenic bacteria. The effluent from wastewater, if not properly treated, represents the main source of pathogenic bacteria as the treated effluent is released into rivers, oceans, and reservoirs (Toze, 2006; Gemmell and Schmidt, 2013; Edokpayi *et al.,* 2017; Mbanga *et al.,* 2020). In the United States of America, contaminated groundwater causes half of the outbreaks of the waterborne diseases reported each year, and the most prominent source of pathogenic bacteria was reported to be septic tank effluent (Stevik *et al*., 2004). Several studies have demonstrated that the survival of bacterial pathogens was related to the interaction of bacteria with protozoa isolated from the wastewater effluent and drinking water reservoir. King *et al*. (1988) and Backer *et al*. (1999) reported the multiplication of coliform bacteria within protozoa. Moreover, these studies demonstrated that bacterial pathogens, when present within protozoa, are

protected from chlorine treatment at levels accepted for use in water treatment systems (Barbaree *et al*., 1986).

Bacterivorous protists are a diverse and ubiquitous group of organisms. They are present in all types of environments, including artificial water systems. The greatest numbers of protozoa can be found in nutrient-rich environments where their grazing activities are of great benefit, and their presence relates to the quality and quantity of their available food. They are a typical component in wastewater treatment systems, with cell numbers typically ranging from 10^4 to 10^6 cells per litre (Pauli *et al*., 2001; Madoni, 2011). Bacterivorous protists were found to be present in 53 of 56 British activated sludge plants studied by Curds and Cockburn (1970) and in all 10 activated sludge plants of South Africa that were studied by Bux and Kasan (1994). Their presence and activity are essential in clearing effluent during wastewater treatment by eliminating bacteria and promoting flocculation, thereby lowering the organic load with a 97% mean reduction of viable bacteria reported (Curds *et al*., 1968). A large fraction of these predators feeding on bacteria are subsequently released with the treated effluent and ultimately end up in a wide variety of freshwater and marine environments or may be present in reused water for domestic purposes and as irrigation water in agricultural practices (Jovanovic, 2008; Jia and Zhang, 2020).

The potential role of protozoa to act as a reservoir for pathogens is well documented. Bacterivorous protists have now received the “infamous” term Trojan horses as they allow survival, replication, and transmission of disease-causing bacteria that they do not digest after uptake in natural and artificial ecosystems, consequently exposing the public to disease-causing bacteria (Fritsche *et al.,* 1993; Kiss *et al*., 2009; Thomas *et al*., 2012; Denoncourt *et al.,* 2014). Although there are ample studies on the predator-prey relationship between protozoa and bacteria (Sherr and Sherr, 2002; Corno and Jürgens, 2008; Bell *et al.,* 2010), the role of protozoa as host and reservoir of pathogenic bacteria - with the exception of the subphylum *Sarcodine* (amoebae) – has received less attention.

1.2. Ciliates

The interaction of bacteria with protists has been extensively studied in both freshwater and marine environments (Porter *et al.,* 1985). Bacterivorous protists consist mainly of flagellates (phylum *Zoomastigophora*) and the monophyletic clade of the ciliates (phylum *Ciliophora*) (Sherr and Sherr, 1987; Šimek *et al.,* 2000; Gao *et al.,* 2016). Although their grazing activities have been extensively studied in aquatic environments, ciliates have received lesser attention as bacterial predators. They were previously regarded as less effective bacterial grazers in the aqueous environment that are more likely to consume larger, filamentous microorganisms such as certain cyanobacteria and nanoplankton (Beaver and Crisman, 1989; Šimek *et al.,* 1995; Kisand and Zingel, 2000). Thus, there is still a need for studies on the interaction of bacteria with the species of phylum *Ciliophora*. Ciliates are described as voracious bacterial feeders; hence they dominate productive ecosystems (Porter *et al.,* 1985; Stabell, 1996). Studies have demonstrated that they represent more than 50% of bacterial grazers and thus account for the major reduction of bacterial populations (Fenchel, 1980; Sherr and Sherr, 1987). Ciliate species were also found to dominate wastewater treatment systems, with over 160 out of 228 protist species identified in wastewater belonging to the phylum *Ciliophora* (Curds and Cockburn, 1970; Madoni, 2011; Pajdak-Stós *et al.,* 2017). Out of 85 protists detected in a wastewater treatment plant in Beijing, China, a total of 57 species were identified as ciliates, and 14 species were identified as amoebae and flagellates (Hu *et al.,* 2013). A study by Sibewu *et al.* (2008) revealed that ciliates were the most dominant group in aeration tanks of the three wastewater treatment plants in the province of Eastern Cape, South Africa, with 60% of protists detected identified as ciliates.

Ciliophora is the largest and most distinct group of eukaryotic subkingdom *Alveolata,* comprising of two main groups, the subphyla *Intramacronucleata* and *Postciliodesmatophora*, and currently subdivided into 11 classes. Of the 8000 species that have been described, 79% are free-living ciliates and are common inhabitants of aquatic environments. They are characterized by the presence of short hair-like organelles called cilia, usually arranged either in spirals around the body or longitudinal rows of the body of the organism. The cilia have specialized feeding and locomotory functions. They have two different nuclei, which is a unique feature in

protists; the small nucleus, which contains normal diploid chromosome, and a large polyploid macronucleus derived from a micronucleus through replication of the genome, being responsible for cell function (Bick, 1972; Corliss, 1975; Finlay *et al.,* 1996; Foissner *et al.,* 2008; Lynn, 2008; Gao *et al.,* 2016).

Bacterivorous ciliates are classified into three ecological groups based on the feeding strategy and the type of locomotion employed. *Holotrichia* is a group of free-swimming ciliates that largely feed on suspended bacteria. The cells are typically covered uniformly by short cilia arranged in rows called kineties, and the cilia are used for swimming and sweeping food particles into their mouth. Because they are fast swimmers, they require much energy and thus dominate the aquatic ecosystem when their food source is in abundance (Madoni, 2011). The most common holotrich ciliates in freshwater environments are species in the class *Oligohymenophorea,* such as *Paramecium* and *Tetrahymena* (Bardele, 1989). *Hypotrichia* is a group of crawling ciliates, their cell bodies are flattened, and their cilia are fused together to form tufts called cirri and are sparsely distributed on the ventral side of the cell. The cirri enable the cell to crawl on solid substrates, debris, and algal filaments to feed on flocculating bacteria and biofilms (Zhou *et al.,* 2008). Well-known hypotrich ciliates include species of *Euplotes*, *Stylonychia,* and *Oxytricha* (Grimes, 1982; Çapar, 2007). Finally, the *Peritrichia* are sessile or stalked ciliates that attach to a plant, animal, or material surface. They either occur singly or as colonies of cells. They have an enlarged anterior, which has cilia around the oral groove that creates a current to move food particles into their mouth region while their slender posterior end or a tail attaches to the surface (Small, 1973). Species of the genera *Vorticella* and *Ophrydium* are well-known peritrich ciliates (Lui and Gong, 2012). Both crawling ciliates and stalked ciliates do not require much energy to actively search for food but are rather efficient food gatherers; thus, they can survive in an environment where the food source is more limited (Pauli *et al.,* 2001; Kexin *et al.,* 2008).

All three groups of bacterivorous ciliates are typically present in wastewater treatment systems; however, each group dominates at different stages of the treatment. Free swimming ciliates arrive in the plant with the sewage sludge and increase in numbers with the increase in bacterial density, thus dominating at the early stages of sludge treatment (Pauli *et al.,* 2001). Once the number of bacteria becomes limited and

flocculation starts to occur, the number of free-swimming ciliates starts to decrease while that of crawling ciliates increases. Sessile ciliates usually dominate at the end of sludge treatment when harsh conditions such as lack of oxygen and toxicants are prevailing. Because sessile and crawling ciliates are bound to the surface of the treatment plant or sediments, they are mostly retained within the treatment plant while free-swimming ciliates are flushed out of the system with the effluent consequently being introduced into receiving water bodies (Madoni, 2011).

1.3. Phagotrophy of ciliates

Ciliates are predominately heterotrophic organisms that acquire their energy through the consumption of food particles (Sherr *et al.,* 1986). Most ciliate species in aquatic environments are mainly bacterivorous; some species, however, have been described to adopt more than one feeding strategy. Ciliates such as *Tetrahymena* spp. have been reported to directly take up nutrients from the surrounding environment through the plasma membrane, thus making them osmotrophic (Rasmussen, 1976). Some ciliates such as *Paramecium bursaria* have been described as mixotrophic; they derive their energy from phagocytosis as well as photosynthesis (Beaver and Chrisman, 1989; Stoecker *et al*., 1989). They harbour endocytobiotic algae such as *Chlorella* spp. forming a symbiotic relationship; the algae provide the host with photosynthetically produced carbohydrates and oxygen while the host cell provides algae with optimal conditions for photosynthesis, thus making ciliates less dependent on food sources from their environment (Tonooka and Watanabe, 2007; Dziallas *et al*., 2012). About 25% of the known ciliate species have been described to be mixotrophs (Foissner *et al*., 1999).

The mechanism by which most heterotrophic ciliates acquire their food prey is well documented. It is a highly specialized mechanism and has been described as a filter-feeding where the oral cavity and the cilia play a central role in the uptake of food particles (Fenchel, 1980; Foissner and Al-Rashaid, 2006). The oral cavity consists of two membranes, the adoral and paroral, that are densely packed with cilia. The undulating cilia on the adoral membranelle generate water current, drawing suspended food particles to the paroral membrane that acts as a sieve retaining food

particles. The food particles retained on the paroral membranelle are then concentrated and directed to the cytostome (a well-developed feeding groove), subsequently being phagocytized by enclosing food particles in a membrane-bound food vacuole (phagosome) where digestion occurs (Fenchel, 1980). This enables the ciliates to select their prey according to the size, food particles that are too small pass through the membrane while particles that are large enough are retained and ingested. However, particles that are too large to be ingested are pumped out of the oral cavity with water from a water vacuole (Fenchel, 1986). The formation of food vacuoles is induced by the presence of food particles on the cytostome and is pinched off once the vacuole is filled with ingested particles (Rasmussen, 1976; Fok *et al.,* 1988).

The process of food digestion in ciliates is well described for species of the hymenostome ciliates of genera such as *Tetrahymena* and *Paramecium* (Rasmussen, 1976; Sherr *et al.,* 1988). This process involves four distinct steps that occur within the cell cytoplasm; formation of a digestive vacuole, acidification of the vacuole, digestion, and then egestion of the undigested materials (Fok *et al.,* 1988; Thurman *et al.,* 2010). The food vacuole containing ingested food particles is transported through the endocytic pathway to fuse with single-membraned lysosome vesicles, transforming the food vacuole into a phagolysosome (digestive vacuole). Once the digestive vacuole is formed, the content in the vacuole is acidified by the release of acid phosphatase from the lysosome. It hydrolyzes peptidoglycan, a structural polymer characteristic of bacterial cell walls (González *et al.,* 1993). Acidification in *Paramecium* was reported to occur as soon as the food vacuole is released from the cytostome, dropping the pH from 7 to 3 in five minutes (Fok *et al.,* 1982; Allen, 1984). The products of digestion are then absorbed into the cytoplasm while the waste materials are ejected from the lysosome vesicles via the cytoproct (fusion pore). The food vacuole containing waste material is released from the lysosome, its membrane fuses with the plasma membrane at the fusion pore, and the vacuole content is released as the fusion pore widens. The spent vacuole membrane is transported back from the cytoproct to the cytopharynx to be reused through a process of membrane recycling (Allen, 1984; Fok *et al.,*1988; Hausmann, 2002; Gray *et al.,* 2012).

Ciliate filter-feeding is the most efficient method in clearing bacteria from wastewater. This mechanism allows ciliates to collect and consume large amounts of bacteria via their vacuoles, thus achieving feeding rates of more than 10^3 bacteria per hour. In contrast, flagellates and amoebae typically consume bacteria as individual cells (Taylor and Sanders, 2010). Ciliates can shift considerable volumes of water, and the volume of water cleared by ciliates is determined by the size of the organism (Curds and Cockburn, 1968). The volume of water cleared by an individual ciliate can range from 12 to 156 nL water per hour for small ciliates such as *Halteria*, *Vorticella,* and *Tetrahymena*, while larger ciliates such as *Paramecium* and *Euplotes* have been reported to clear up to 1000 nL water per hour (Sherr *et al.,* 1988; Pauli *et al.,* 2001). In comparison with ciliates, minute clearance rates of less than 1 nL of water per hour have been reported for flagellates (Sherr *et al.,* 1988; Kinner *et al.,* 1998). A study by González *et al.* (1990) showed that clearance rates of the estuarine ciliate *Strobilidium* sp. ranging in size from 10 to 20 µm width by 15 to 50 µm length was 116 nL x ciliate^{-1} x hr^{-1} when grazing on *Escherichia coli* and 206 nL x ciliate^{-1} x hr^{-1} when grazing on *Enterococcus faecalis*, while choanoflagellate species ranging from 2 to 10 µm diameter had a clearance rate of 1.23 nL x flagellate^{-1} x hr^{-1} and 1.30 nL x flagellate^{-1} x hr^{-1} for the same bacterial organisms respectively.

Curds *et al.* (1968) demonstrated that in the absence of ciliates in sludge treatment, the effluent appears turbid with a high density of bacteria, which disappeared when ciliates were added to the system. The study by Curds and Fey (1969) also revealed that when ciliates are present in the sludge treatment, the half-life of *E. coli* is reduced to 1.8 hours, while the half-life of *E. coli* is 16 hours in the absence of ciliates. Comparative studies showed that ciliates exhibited higher maximum growth rates than other bacterivorous protists. They, therefore, have the potential to increase population density in response to prey concentration, thus, enabling them to dominate and making them better at controlling bacterial biomass (Strom and Morello, 1998).

1.4. Factors influencing the impact of ciliate predation

Bacterivorous protists have been extensively studied in both freshwater and marine environments (Weisse *et al.,* 2016). Their grazing activities play a significant role in regulating bacterial population density and productivity as well as shaping the phenotypic and genotypic characteristics of microbial communities (Jürgens and Matz, 2002; Corno and Jürgens, 2006; Saleem *et al.,* 2013). Bacteria have a crucial ecological function in many biogeochemical cycles and nutrient recycling. Therefore, loss of the bacterial population by protist grazing, referred to as the top-down regulation, may influence many bacterial ecological processes, as bacterivorous ciliates control the proportion of active bacteria and microbial net production (Saleem *et al.,* 2013; Chow *et al.,* 2014).

Ciliates were initially considered as consumers of larger plankton and thus less effective at eliminating bacteria (Fenchel, 1980). However, studies have shown that ciliates are the major bacterial predator in aquatic systems and account for more than 50% of total bacterial mortality in aquatic environments (Sherr *et al.,* 1986; Sherr and Sherr, 1987). Not only are they consumers of bacteria, but they also stimulate bacterial growth and activities by releasing waste products of digested prey as dissolved organic and inorganic matter that can accelerate organic matter cycling (Caron and Goldman 1990; Dolan, 1997; Sherr and Sherr, 2002). The impact of protist grazing on bacterial prey in aquatic ecosystems is influenced by a number of factors, which can be direct (prey response) or indirect effects (prey availability, prey morphology and physiology, and environmental conditions). Thus, ciliate grazing can affect the composition of the bacterial community present (Hahn and Höfle, 2001; Pernthaler, 2005).

1.4.1. Growth characteristics of prey organisms

When the predator feeds upon its prey, its population density increases while that of the prey declines until prey becomes limited. Bacterial populations subjected to predation exhibit different patterns of decline. While some bacterial species populations can be completely eliminated, some persist at concentrations that are

below the threshold level required to sustain the growth of the predator (deLeo and Baveye, 1997). This is determined by the bacterial growth characteristics; fast-growing bacterial species can replace bacterial biomass lost to predation faster, thus survive at higher density, while slow-growing bacteria may be eliminated from the system by predation. The results of a study of six bacterial species suggest that the slow-growing or non-growing species in sewage are eliminated, whereas the fast-growing species persist during protozoan predation (Mallory *et al*., 1983; Gurijala and Alexander, 1990). This, in turn, might be beneficial for the wastewater treatment process if protists completely eradicate some undesirable species of bacteria during sludge treatment.

1.4.2. Prey concentration

The impact of ciliate grazing pressure on the bacterial community is primarily important in nutrient-rich environments where bacteria, as well as protists, are present in large numbers (Caron *et al.,* 1982). Prey concentration determines the rate of ingestion, digestion and consequently determines the growth rate of ciliates. The minimum or threshold concentration required to maintain growth varies according to the size of ciliates. The minimum concentration that is required for the growth of ciliates has been reported to vary between 10^6 and 10^7 bacteria per mL, although concentrations of 10^5 per mL have been reported for some smaller ciliates (Fenchel, 1980; Mallory *et al.,* 1983). Larger ciliates such as *Cyclidium*, *Glaucoma,* and *Paramecium* as well as an estuarine ciliate, *Uronema*, were reported to require a bacterial concentration of 10^6 to 10^8 cells x mL^{-1} to grow while the growth of small ciliates such as *Tetrahymena* and *Halteria* was observed at bacterial concentrations of 10^5 cells x mL^{-1} and higher (Beaver and Chrisman, 1989; Ali and Saleh, 2014).

Studies have shown that the growth of ciliates is concentration-dependent and that their ingestion rate is a function of prey concentration (Šimek *et al.,* 2000). Ingestion rates of ciliates usually increase with the increase in prey concentrations; this was demonstrated in two ciliate species, which had higher ingestion rates at high bacterial concentrations. Thus, the ingestion rates of *Euplotes vannus* and *Euplotes plicatum* were 10x and 20x higher at a bacterial concentration of 10^9 cells x mL^{-1} than at 10^6 cells x mL^{-1}, while *Uronema marinum* had higher ingestion rates at a concentration of

10^9 cells x mL^{-1} than at 10^7 cells x mL^{-1} of attached bacteria (Tuorto and Taghon, 2014). This was also demonstrated using non-living food particles fed to *Euplotes mutabilis*, where the ingestion rates were found to be low (16 microspheres x $ciliate^{-1}$ x h^{-1}) at a low concentration (10^3 microspheres x mL^{-1}), increasing to 128 microspheres x $ciliate^{-1}$ x h^{-1} at a higher concentration of 10^6 microspheres x mL^{-1} (Wilks and Sleigh, 1998).

When the prey concentration is below the predator threshold level, the ciliate cell does not produce food vacuoles as the food vacuole formation is induced by the presence of food particles (Curds and Cockburn, 1968; Fok *et al.,* 1988). The formation rate and the food vacuole size depend on the food particle concentration in the medium; the rate of food vacuole formation is higher when the food particle concentration is high, while at too low concentrations of food particles, the cell does not form food vacuoles (Ramoino *et al.,* 2012). This was observed in *Paramecium* species, which did not form food vacuoles at low bacterial concentrations, while the number of vacuoles increased with an increase in prey concentrations. At a concentration of 1.5 x 10^6 particles x mL^{-1}, the rate of vacuole formation in *Tetrahymena pyriformis* was 0.12 vacuoles x $cell^{-1}$ x min^{-1}, which increased to 0.64 vacuoles x $cell^{-1}$ x min^{-1} at a higher concentration of 2.1 x 10^8 particles x mL^{-1} (Thurman *et al.,* 2010). For *Tetrahymena,* which can absorb nutrients through its plasma membrane, slow rates of vacuole formation and slow growth rates were observed when growing in a protein-rich medium (Fok *et al.,* 1988; Ramoino, 1996; Taylor and Sanders, 2010). Ciliates do not start to multiply until a sufficiently high bacterial density is available. Thus, they typically dominate productive systems where bacteria occur in high concentrations to meet their energy demand (Taylor, 1977; 1978).

The effect of prey concentration on protist grazing is usually studied over a narrow range of prey concentrations, such as 10^5 to 10^8 bacteria x mL^{-1} to mimic oligotrophic conditions. However, this range of prey concentrations does not encompass higher bacterial concentrations found in polysaprobic and, therefore, productive ecosystems or wastewater treatment plants, where bacteria are the most abundant organisms with concentrations ranging from 10^6 to 10^{10} bacteria x mL^{-1} (Tuorto and Taghon, 2014).

1.4.3. Prey morphology

Most phagotrophic protists are known to be selective feeders, which consequently influences the size and structure of the bacterial population (Gonzàlez *et al.,*1990). Bacterial characteristics such as size, motility, and surface-specific properties of bacteria play crucial roles in the selection of bacterial prey; thus, the protists consume and digest various bacterial prey species with different efficiency (Šimek *et al.,* 1997).

Bacterivorous ciliates feed mainly on suspended bacteria by using a filter-feeding mechanism, which fails to distinguish different kinds of prey except for their mechanical properties (Bautista-Reyes and Macek, 2012). Therefore, feeding selectivity is mainly based on the size of the food prey and is regulated by the feeding mechanism employed. The phylum *Ciliophora* consists of species with a cell size that can range from 10 µm to 3 mm; thus, the size range of particles that can be ingested by the predator is limited by the morphology of the oral apparatus. While the maximum prey size is set by the width of the cytostome, the minimum prey size is controlled by the paroral membranelle, which retains food particles to be ingested. Thus, prey particles that are too small pass through the membranelles, and particles that are too large to be ingested are pumped out with water (Fenchel, 1986; Posch *et al.,* 2001). Grazing efficiency, which is determined by the size of the food particle, is high for the preferred prey size and low for smaller and larger prey. Experiments using the ciliate *Euplotes mutabilis* showed that the uptake rate is dependent on the food particle size; though this organism was able to ingest microsphere beads of varied sizes ranging from 0.57 to 10 µm in diameter, its highest uptake rates were detected for particle size between 1.90 and 3.06 µm in diameter (Wilks and Sleigh, 1998). Consequently, size-selective grazing shapes the bacterial community structure as it allows smaller and larger sized bacteria to dominate the ecosystem while the medium-sized bacterial population selected for decreases (Hahn and Höfle, 2001; Rønn *et al*., 2002).

Bacterial motility may also influence the interaction of bacterial prey with protozoa. Motility allows the motile prey to escape predation; this reduces prey contact with the predator, thereby reducing the ingestion rate of protozoa (Jürgens and Matz, 2002). This has been described for heterotrophic flagellates, which had higher clearance and ingestion rates for non-motile to moderately motile bacteria (Matz and Jürgens, 2005). The average swimming speed of bacteria in the aquatic environment has been

reported to be 45 µm x s^{-1} with maximum speed recorded as 200 µm x s^{-1} (Mitchell *et al.,* 1995; Johansen *et al.,* 2002). Free swimming ciliates swim faster than flagellates with a swimming speed of 400 to 7000 µm x s^{-1}, while flagellates can only achieve a swimming speed of 20 to 200 µm x s^{-1} and amoebae both naked and testates, crawl slowly at speed up to 5 µm x s^{-1} (McNeill, 1979; Boenigk and Arndt, 2002; Claußen and Schmidt, 2017; Lisicki *et al.,* 2019). Based on the swimming speed of free-swimming ciliates and the mechanism of capturing food prey, which creates water currents that draw food particles towards the oral groove, the motility of bacterial prey should not have a significant influence on the ingestion of prey particles.

The surface properties of a bacterial cell may determine the degree of susceptibility of the prey cell to protist grazing. There is, however, limited evidence suggesting how such characteristics influence protist grazing (Jürgens and Matz, 2002). Gram-positive bacteria are apparently less suitable food prey for bacterivorous protozoa than Gram-negative bacteria. Heterotrophic flagellates and amoebae showed growth when grazing on Gram-negative *Pseudomonas chlororaphis* but did not show an increase in biomass when grazing on a Gram-positive *Mycobacterium chlorophenolicum*, which also remained viable in the presence of protozoa (Rønn *et al.,* 2001). Though Gram-positive bacteria cannot escape phagocytosis once captured, their cell wall properties are less susceptible to digestion within the food vacuole (Plante, 2000; Rønn *et al.,* 2002). This may enable the survival of bacteria during the digestion cycle in the protist cell (Gonzàlez *et al*.,1990).

Studies have demonstrated that surface properties such as hydrophobicity strongly affect the contact probability between predator and prey and, in turn, the ingestion process (Jürgens and Matz, 2002). Bacteria such as *Streptococcus* and *Pseudomonas* spp. with high surface hydrophobicity tend to adhere to biotic and abiotic surfaces due to strong Van der Waals forces; this may result in bacteria forming biofilms, aggregates, and flocculation, which reduces the susceptibility of bacteria to protist grazing (Krasowska and Sigler, 2014). These forces equally so increase interaction between the prey and predator as the prey is attracted towards and adheres to its host cell. The cell surface properties of bacteria have been implicated in the resistance of the organisms to phagocytes of mammalian immune systems (Krasowska and Sigler, 2014). It has been demonstrated that hydrophilic bacteria

resist ingestion and that the efficiency of phagocytosis increases with the hydrophobicity of bacterial cells by phagocytes. A study by Gurijala and Alexander (1990) reported that bacteria with a highly hydrophobic surface survived grazing by *Tetrahymena thermophila* at high density. However, the role of hydrophobicity in bacterial resistance to protist predation is still not understood. When flagellates were grazing on suspended bacteria with a hydrophobic or a hydrophilic surface, no significant difference in grazing rates were observed (Hahn and Höfle, 2001; Matz and Jürgens, 2001). In addition, it was shown that surface biochemistry could govern the digestibility of ingested particles in food vacuole (Dürichen *et al.,* 2016). This was demonstrated with *E. coli* XL1-blue, expressing the red fluorescing protein DsRed2 and modified with oligoamines which were not acidified in food vacuoles of *T. pyriformis*. The study attributed the impaired digestion to the interaction of biomolecules with either the membrane proteins or phagosome membrane (Siegmunda *et al.,* 2018).

1.4.4. Defense mechanisms of the prey

Protist grazing on bacteria can alter the composition and diversity of bacterial communities due to the selective feeding of protists on a limited size range of bacterial prey cells (Glücksman *et al.,* 2010; Jousset, 2012). Similarly, the grazing pressure of bacterivorous protists can select behavioural traits that are enhancing resistance against protist grazing (Güde, 1989; Jürgens *et al.,* 1999). Bacteria have developed various defense strategies to avoid and survive protozoa predation by either avoiding ingestion or resisting digestion. These include changes in morphology, biofilm formation, secretion of toxins, or evolving symbiotic relationships with protists (Matz and Kjelleberg; 2005; Jousset, 2012). The ability of bacteria to resist and survive predation can affect the impact of protist grazing on bacterial communities. The interaction of bacterivorous protists and their prey has been well documented; however, little is known about how different species of protists influence the diversity and composition of bacterial communities.

1.4.4.1. Morphological change to resist ingestion

The impact of protist grazing on bacteria is influenced by the size characteristics of bacterial cells. Ciliates are morphologically diverse organisms; thus, each ciliate species has a distinct range of preferred particle size determined by oral morphology (Pernthaler *et al*., 1996; Posch *et al.,* 2001). In general, ciliates selectively feed on a limited range of medium-sized bacteria, while smaller-sized bacteria are ingested at lower rates, thus getting a selective advantage. Strong grazing pressure by protists can cause shifts in bacterial populations from a freely dispersed single cell to complex growth forms such as filaments or aggregation in biofilm and flocs that are more resistant to protist grazing (Güde, 1979; Jürgens and Güde, 1994).

Studies have shown that under strong grazing pressure by protists, filamentous bacteria dominate the aquatic ecosystems. Filamentous bacteria are widespread in marine and freshwater environments, and their occurrence is correlated with the presence of high protozoa biomass (Jürgens *et al.,* 1999). Shikano *et al.* (1990) observed bacterial cells that persisted in a filamentous form of up to 20 µm in length in the presence of *Cyclidium* sp., a bacterivorous ciliate. Filamentous morphotype represents an ecologically important bacterial defense strategy against protist predation as the size of bacteria can exceed the cell size range that can be ingested by most bacterivorous protists. (Hahn *et al.,* 1999). Only ciliates with specialized feeding mechanism can feed on filamentous bacteria, e.g., Hausmann and Peck (1979) reported a *Pseudomicrothorax dubius* that feeds on filamentous blue-green algae at rates of up to 15 µm per second, by means of a cytopharyngeal basket.

In their natural environment, bacteria predominately live in microcolonies. This is a result of the interaction between bacterial cells adhering to each other and the surface while being embedded in an extracellular polymeric matrix. Bacteria living in a biofilm have different properties than free-living planktonic bacteria due to the exopolymeric substance that surrounds the microcolonies and provides protection against harsh environmental conditions (Matz *et al.,* 2005; Böhme *et al.,* 2009). The extracellular polymeric matrix present in biofilms also protects bacteria from protist predation (Blom *et al.,* 2010). Cell aggregation in microcolonies favors intercellular communication with increased bacterial activities when compared to activity in suspended bacteria; thus, a potential increase in the production of secondary metabolites that may be active

against predators (Böhme *et al.,* 2009). A study by Matz *et al.* (2004) reported a wild-type strain of *Pseudomonas aeruginosa* that formed microcolonies in the presence of the flagellate *Rhynchomonas* but remained unattached in the absence of the predator. This study demonstrated that protist grazing might stimulate the formation and growth of biofilms, and its effect may vary according to bacterial strains (Dopheide *et al.,* 2011). Grazing of *Tetrahymena pyriformis* on the biofilm-forming *Acinetobacter* sp. strain C6 led to an increase of biofilm biovolume with larger irregular and elongated microcolonies, which were generally rounder and more regular in the absence of a grazer (Scherwass *et al.,* 2016). The presence of predators on biofilm may further stimulate the growth of bacteria in microcolonies by releasing substances from live and dead cells due to the decline of predator numbers, and movement of predators on biofilm may create patches creating ventilation; thus, biofilms have an additional supply of nutrients and oxygen that stimulate bacterial growth.

Aggregation is usually high for bacteria with a highly hydrophobic surface. The strong intercellular Van der Waals forces are a result of extracellular polysaccharides on the bacterial cell wall, driving similar cells to adhere to each other, leading to aggregation or clumps that settle out of solution (Pavoni *et al.,* 1972). This phenomenon of flocculation is exploited in the wastewater treatment process as it allows easy removal of microorganisms from treated sludge (Friedman *et al.,* 1969). For a South African wastewater treatment plant, over 30 bacterial isolates have been described with high bio-flocculation ability, including members of the genus *Bacillus*, as well as pathogenic species of genera the *Klebsiella*, *Staphylococcus,* and *Pseudomonas* (Buthelezi *et al.,* 2010). Protist grazing has been reported to promote bacterial flocculation by enhancing the secretion of extracellular polysaccharides. Jürgens *et al.* (1997) observed the presence of bacterial clumps at maximal diameters of 20 to 40 µm in cultures, with a bacterial density of about 10^6 cells x mL^{-1} within 24 hours of protists grazing, while in the absence of protists bacterial cells remained freely dispensed. Such aggregates, when analyzed, consist of bacterial cells and extracellular polymers (Alber and Valiela,1994).

Studies on bacterial resistance to protist grazing demonstrated that bacterial defense morphotypes are induced by protist predation. Defensive traits evolved in response to protist grazing and production of metabolites are energetically costly, thus are induced

specifically in response to predation (Steiner, 2007). Defense traits in bacteria are regulated by various sensors, which sense chemical cues associated with protist predator interaction stimulating the formation of microcolonies or inedible morphotypes (Jousset *et al.,* 2010). Jousset *et al.* (2010) reported that *Pseudomonas fluorescens* responded to such chemical cues within minutes of addition of bacterivorous amoebae and that the genes that regulate the synthesis of extracellular toxins were induced and up-regulated in the presence of amoebae. Expression of defense traits is dependent on the availability of nutrients, i.e., they are expressed when the nutrient concentration is high, and high bacterial density allows quorum sensing (Queck *et al.,* 2006). At high nutrient concentrations, the growth rate is high, and energy production is sufficient for both maintaining the functioning of the cells and producing defensive traits so that cells can attain the defense morphotype rapidly. While the energy synthesized at low nutrient concentration is only sufficient for maintaining the basic functioning of the cell (Steiner, 2007; Friman *et al.,* 2008). Protist grazing promotes bacterial cell to cell interaction leading to the formation of microcolonies of high cell density that can produce large quantities of metabolites that effectively act against predation. Hence, dense biofilms of *Pseudomonas aeruginosa* were more resistant to predation than planktonic cells (Friman *et al.,* 2013).

Morphological change in response to protist predation does not provide bacterial communities with complete protection but rather reduces susceptibility to grazing. The grazing effect of protists on bacterial microcolonies depends on the predator feeding mode. Feeding on attached bacterial communities requires physical contact and longer contact time. Unlike free-swimming ciliates that feed on suspended bacteria, crawling ciliates such as *Stylonychia* spp. move over and through bacterial flocs, whereas attached ciliates such as *Stentor* spp. are fixed to flocs while feeding on planktonic bacteria, thus dominating during the flocculation phase of sludge treatment (Kexin *et al.,* 2008; Madoni, 2011). In biofilms, amoebae are considered more important as a predator of bacteria than flagellates and ciliates due to their mostly surface-associated lifestyle, with the exception being heliozoa (Parry, 2004; Thomas *et al.,* 2008). Because they attach to the surface of the biofilm and form pseudopodia as temporary cell extensions as a means to feed, it allows them to have longer contact time with prey in biofilm (Weitere *et al.,* 2005).

1.4.4.2. Production of metabolites/ endotoxins to resist digestion

Bacterivorous ciliates ingest prey using the process of phagocytosis, where prey cells concentrated on the cytostome are engulfed and enclosed in the food vacuole. This is where the prey comes in direct contact with the predator cell; the prey cells are localized within the phagosome, where digestion occurs. The phagosomes of the predator are typically inhospitable environments as they are nutritionally deprived and contain bactericidal compounds that are released to kill and digest the prey (Ray *et al.,* 2009; Smith and May, 2013). Similarly, ingested prey may secrete metabolites that are toxic to predator cells. Such effect was found with Shiga toxin-producing strains of *E. coli*, which killed its *Tetrahymena* host. It was reported that after *E. coli* has been ingested, it can sense phagosome conditions and secrete metabolites that are toxic to the host cell (Lainhart *et al.,* 2009). The fate of ingested bacteria is, therefore, dependent on the susceptibility of the host cell and bacterial virulence (Vaerewijck *et al.,* 2014). This was demonstrated using the bacterial strains *E. coli* O157: H7 and *E. coli* K-12 with the Shiga-toxin producing gene possessing strain showing increased survival within phagosomes of *Tetrahymena pyriformis* (Steinberg and Levin, 2007).

Many bacterial species have been reported to evade digestion within phagosomes (Brown and Barker, 1999). The resistance of bacteria to protozoa digestion is attributed to the secretion of proteins, which are important for their virulence (Tseng *et al.,* 2009). The production of toxins is the most powerful defense mechanism against protist predation (Jousset *et al.,* 2010). Rehfuss *et al.* (2011) reported that the genes responsible for the pathogenesis of *Salmonella enterica* were expressed during the food vacuole passage through the food vacuole of *Tetrahymena*. A gene that expresses a Type III Secretion System (T3SS) was highly induced during *Salmonella enterica* infection in *Acanthamoeba polyphaga* (Bleasdale *et al.,* 2009). The ability of bacteria to resist digestion within the phagosome may aid pathogenic bacteria to survive and persist in the environment. For example, the pathogenic bacterium *Campylobacter jejuni* remained viable after being ingested by the freshwater ciliate *Colpoda* sp. (Lamothe *et al.,* 2007; First *et al.,* 2012)

In addition to intracellular survival within the host cell, bacteria have evolved a mechanism to exploit their host cell for intracellular replication, thus maintaining a high abundance within the protozoa (King *et al.,* 1988). They achieve this by manipulating

the pathway of phagosome maturation (Becken *et al.,* 2010; Smith and May, 2013). This has been well documented for *L. pneumophila,* which can invade and multiply in free-living amoebae and remain viable when released as a faecal pellet (Rowbotham, 1980; 1986; Harb *et al.,* 2000). To achieve successful digestion, a newly formed vacuole undergoes a series of maturation processes through an endocytic pathway to form a fully functional digestive food vacuole. However, bacteria can disrupt the endocytic pathway at any point in the food vacuole maturation pathway (Fokin, 2004; Becken *et al.,* 2010; Smith and May, 2013).

Bacteria have developed several strategies to evade digestion within the food vacuole of their host cell by either preventing fusion of the food vacuole with a lysosome to form a digestive vacuole or by modulating the environment within the phagolysosome (Ray *et al.,* 2009). *L. pneumophila* uses Type IV Secretion System (T4SS) to evade digestion by blocking the phagosome with ingested bacteria from fusing with a lysosome vesicle. The bacteria divert the phagosome away from the normal route of maturation by preventing it from fusing with the lysosome vesicle, subsequently making the phagosome less acidic. Once the bacteria have modified the phagosome, it forms a replicative vacuole. Therefore, such bacteria exploit host cell organelles to support their replication using either T2SS, T3SS, or T4SS (Kwaik *et al.,* 1998). Bacteria may disrupt phagosome maturation and escape the phagosome to settle in cell compartments that are not associated with lytic organelles, such as the cytoplasm or the nucleus. Intracellular replication in the protozoan cytoplasm is the most common niche as it is rich in nutrients and is isolated from digestive enzymes (Ray *et al.,* 2009). *Mycobacterium tuberculosis* invading amoebae can use a T7SS protein to permeabilize the phagosome membrane, thereby enabling their escape to the cytoplasm, where replication takes place (Strassmann and Shu, 2017).

Bacterial pathogenicity for higher organisms originally evolved as a defense strategy against protist predation. The first line of defense against bacterial infection comprises of phagocytosis of pathogenic bacteria by macrophages (Brüssow, 2007; Rehfuss *et al.,* 2011; Strassmann and Shu, 2017). The process of digestion after the ingestion of bacterial cells within the macrophage is similar to that of unicellular protists. Therefore, a bacterial response to phagocytosis should somewhat be the same. *Salmonella enterica* serovar Typhimurium stimulates phagocytosis via T3SS and establishes a

replicative niche by secreting proteins within the macrophage (Sillo *et al.,* 2011). The transcription of the gene encoding T3SS of this bacterium was highly induced during the grazing of amoebae (Bleasdale *et al.,* 2009).

Intracellular bacteria in ciliates were first described in *Paramecium*, which was infested by *Holospora* bacteria. *Holospora* species show micro- and macro-nucleus specificity in their host *Paramecium*. However, their mechanism of evading lysosome digestion in the food vacuole and migration to the nucleus is still not understood (Fokin, 2004; Lanzoni *et al.,* 2016). Bacterial endosymbiosis is widely spread amongst the species of *Ciliophora*, with over 200 species of ciliates recorded to host different intracellular bacteria (Fujishima and Görtz, 1983; Fokin *et al.,* 2005). Additionally, anaerobic ciliates such as *Trimyema* spp. harbour methanogenic archaea as symbionts (Lewis *et al.,* 2018). Because ciliates are usually large cells, they can harbour a large number of bacteria. Endosymbionts in ciliates have been found to inhabit the cytoplasm enclosed in a vesicle or as a free cell, the micro- and macro-nucleus, and the perinuclear space (Görtz, 2001).

1.4.5. Environmental factors impacting grazing

Ciliates are mainly limited to environments where an adequate amount of moisture is present. They dominate aquatic environments and are therefore subjected to a substantial wide fluctuation of various environmental factors, particularly in freshwater systems (Weisse and Stadler, 2006; Bock *et al.,* 2020). Despite their ubiquitous presence in many environments, environmental factors such as temperature, oxygen availability, and pH are recognized as fundamental factors governing their distribution and productivity; a particular habitat is likely to be characterized by a certain group of protists. These physicochemical factors impact the physiological processes of heterotrophic protists, such as grazing rates, growth rates, respiration, and nutrient regeneration in aquatic ecosystems (Noland, 1925; Lackey, 1938). Ciliates are adapted to a range of physical conditions and are found in diverse environments; however, their response to environmental conditions varies with different species. Any change that is outside the range suitable for physiological processes may affect their population composition and abundance and ultimately alter the aquatic food chain

(Hiltunen *et al.,* 2015). However, the influence of these physical characteristics on protists has not been extensively studied (Caron *et al.,* 1986).

Temperature

The response of protists to temperature has been generally studied by observing the growth rates. As expected on microbiological grounds, the growth rates increase with an increase in temperature within the physiologically suitable ranges. An increase in temperature results in a significant increase in the rate of formation of food vacuoles, which ultimately increases the growth rate by increasing the efficiency of food uptake (Caron *et al.,* 1986; Luan *et al.,* 2012). A strain of *Tetrahymena pyriformis,* which grows at a temperature range of 5ºC to 35ºC, had a maximum growth rate at 32.5ºC, with a doubling time of 2.25 hours, which increased to a doubling time of 3.70 hours at 29ºC (Prescott, 1957; Thormar, 1962). Furthermore, temperature also influences the cell size of ciliates due to an increased rate of food vacuole formation. The growth of *Tetrahymena pyriformis* at temperatures of 10, 20, and 30ºC resulted in cells with significantly different volumes of 16.250, 12.350, and 9.375 μm^3 respectively (James and Read, 1957), While Finlay (1977) reported cell volumes of 178.255, 266.025 and 253.998 μm^3 and generation times of 111.50, 30.41 and 17.30 hours of *Paramecium aurelia* at temperatures of 8.5, 15.0 and 20ºC**.** When the temperature is outside the range that allows the functioning of physiological processes, some ciliates may form survival structures such as cysts, which can remain dormant for long periods (Corliss and Esser, 1974; Foissner, 2009). These cysts can withstand extreme temperatures; cysts of *Colpoda* species germinated even after heat treatment at 100ºC for three hours (Verni and Rosati, 2011).

Oxygen

Oxygen deficiency is becoming a threat in many aquatic ecosystems due to the accumulation of chemical pollutants and eutrophication. All aquatic ecosystems are characterized by an oxygen gradient, which plays a significant role in the distribution of protists within the ecosystem. Ciliates are sensitive to changes in oxygen and show a dramatic response in view of community composition. Although a great majority of ciliate species require oxygen for their metabolism, there are some species that have

adopted the anaerobic lifestyle (Kovalchuk and Babko, 1997; Bręk-Laitinen *et al.,* 2012). The concentration of oxygen varies with temperature and photosynthetic and respiratory processes of aquatic microbiota; organisms that inhabit these systems are therefore usually well adapted to the seasonal and daily fluctuation of dissolved O_2 (Noland, 1925; Lackey, 1938). Aerobic ciliates show preference to a particular oxygen concentration and are usually limited to the water column and the top 1 to 2 cm of sediment. Oxygen, when used as an electron acceptor, provides the highest energy yield and therefore is the primary electron acceptor used by protists during aerobic energy metabolism (Fenchel, 2014; Rocke and Liu, 2014). The oxygen requirements of protists are a function of their body size (Finlay and Esteban, 2009). Large ciliates need access to elevated concentrations of oxygen, while small aerobic ciliates can approach their maximum O_2 uptake even at low oxygen concentrations. Many studies have demonstrated that aerobic ciliates can persist even in a low concentration of dissolved oxygen. Studies on the response of ciliates to dissolved oxygen demonstrated that the O_2 uptake by the ciliate *Euplotes* sp. increased with an increase in O_2 concentration up to 100% O_2 saturation; the strain tested showed maximum growth at 4-5% O_2 saturation, but its growth rate decreased by about 30% with an increase of dissolved O_2. Grazing rates of a *Euplotes* sp. decreased from its maximum of 1000 bacteria x ciliate^{-1} x hour^{-1} to 200 bacteria x ciliate^{-1} x hour^{-1} when exposed to a lower concentration of dissolved oxygen (1.25 mg O_2 L^{-1}) (Rocke and Liu, 2014). Large ciliates such as *Spirostomum*, *Loxodes,* and *Frontonia* are confined to deep-water sediments in winter but become planktonic during summer due to the warming of the lake sediments and depletion of oxygen close to the sediments (Finlay, 1981; Finlay and Esteban, 2009).

pH

The pondus hydrogenii (pH) is a major environmental factor of aquatic ecosystems that regulate both biological and physicochemical processes. The hydrogen ion concentration affects metabolic functions and transport processes across cellular membranes taking place in the cytoplasm and cellular organelles. pH values in freshwater ecosystems usually range from pH 6 – 8 but can be extremely acidic (pH 2) due to the release of humic acids or extremely alkaline (pH 12) and may fluctuate due to the absence of buffering systems in such environments (Lackey, 1938). The

change in pH is inversely correlated with the amount of carbon dioxide; pH is therefore usually higher during the day when photosynthesis by aquatic plants and algae is at its peak and drops at night when carbon dioxide is produced in large amounts by aquatic organisms' respiration forming carbonic acids with water (Noland, 1925). Although the pH tolerance of planktonic ciliates is poorly documented in the literature, studies have shown that ciliates tolerate a wide range of pH. Ciliates of the *Prostomatida*, *Hypotricha,* and *Peritricha* groups were found to dominate in acidic environments of less than pH 5 (Weisse and Stadler, 2006). Though they perform better at moderately high pH values, ciliates such as *Oxytricha* species with an optimum pH of 8.0 and *Urotricha* species with an optimum pH of 7.0 were found to have high acid tolerance, surviving even at pH values of below 3. The growth of *Paramecium* species, which usually have an optimum pH close to 6.8, was limited to pH values between 4 and 8 (Humphrey and Humphrey, 1948). pH-sensitive ciliates, such as *Spirostonum ambiguum,* avoided the change in pH by retreating to decaying leaves when pH values rise (Weisse *et al.,* 2011).

1.4.6. Consequences of bacteria resisting protozoa predation

Protozoan predation plays a vital role in regulating and controlling bacterial populations in aquatic environments. The impact of protozoa grazing on bacteria relies on the success of the phagocytosis process, which involves ingestion and digestion of prey. Thus, the ability of bacteria to resist protozoan predation has given rise to bacteria that can persist in such environments (Greub and Raoult, 2004; Denoncourt *et al.,* 2014).

The morphological changes of bacteria are a frequent mechanism to resist protist predation and have been reported for many bacterial species (Hahn and Höfle, 2001). Biofilms allow bacteria to adhere to surfaces and are increasing access to trace elements and various organic compounds by accumulating these at the water biofilm surface interface. This allows bacteria to survive in nutrient-limited environments where they would not grow as single-cell bacteria (Ridgway and Olson, 1981). Moreover, bacteria in the biofilm are less susceptible to chemical disinfection and antimicrobial agents due to the extracellular polymeric matrix that protects the

microcolonies. *E. coli* resistance to ampicillin increased in biofilms, requiring 500 times higher Minimum Inhibitory Concentrations to provide a 3 log reduction than was required for planktonic cells (Ceri *et al.,* 1999). The innate resistance of bacteria in biofilms to antimicrobial agents and chemical treatments is a serious challenge as these are difficult to eradicate and may present a reservoir for pathogenic bacteria.

Since the discovery that bacteria can survive within the cells of protozoa, studies have revealed that bacteria can manipulate a hostile environment to ensure survival in an otherwise inhospitable environment (Rowbotham, 1980; 1986). Undigested bacterial cells within the food vacuole can affect their host cells by either surviving without killing the host cell, multiplying within the host without killing the host cell, or multiplying within the host vacuole and causing host cell lysis. Such effects have been reported for pathogenic bacterial species of genera such as *Mycobacterium*, *Burkholderia,* and *Listeria,* which can multiply within and can cause lysis of their host cell, *Vibrio*, which can multiply without killing the host cell, and *Salmonella* species which survived without multiplying and killing the host cell, and were found to survive in *Acanthamoeba* and *Tetrahymena* (Brandl *et al.,* 2005; Matz and Kjelleberg, 2005; Gong *et al.,* 2016). The pathogenic bacterium *Campylobacter jejuni* remained viable in cells of the freshwater ciliate *Colpoda* sp. for 5 hours after ingestion (First *et al.,* 2012). Therefore, intracellular survival or even replication within protists allows bacteria to be transmitted and persist in environments.

Moreover, protozoa may aid in the survival and transmission of bacteria by providing protection under harsh conditions. Waste products from the protists are packaged and egested out of the cell in the form of a pellet called a vesicle. These faecal pellets can be a source of pathogenic bacteria as undigested prey cells are expelled through such vesicles at the cytoproct. Viable bacteria have been detected in these vesicles, and they have been shown to protect and enhance the survival of bacteria present therein. This was demonstrated by Brandl *et al.* (2005), who observed that undigested bacterial cells in expelled vesicles survived for periods of up to 10 weeks, while free cells survived for only 10 days. In addition, *Salmonella enterica* in *Tetrahymena* vesicles even survived treatment with the biocide calcium hypochlorite (Gourabathini *et al.,* 2008). These vesicles are protecting enclosed bacteria from harsh conditions such as

freeze-thaw cycles with temperatures ranging between 70ºC and 35ºC (Berk *et al.,* 1998).

In addition, bacteria internalized by protozoa may be protected from harsh conditions when the host cell forms a cyst. Some species of ciliates can undergo encystment when environmental conditions become adverse and excyst and proliferate when conditions are favorable. These cysts are resistant to harsh conditions such as desiccation, osmotic stress, high temperatures, and chemical treatments (Verni and Rosati, 2011). Ciliate cysts can be major dispersal vehicles of bacteria since they can survive and remain viable in these cysts under harsh conditions for long periods (Foissner, 2006). Funatani *et al.* (2010) observed that dry cysts of *Colpoda cucullus* survived 3 to 4 years and were highly resistant to UV light (254 nm) (Matsuoka *et al.,* 2017). *Amoebae* cysts containing *L. pneumophila* protected the bacterium from chlorine treatments and desiccation (Gourabathini *et al.,* 2008). King *et al.* (1988) reported that bacterial species ingested by *Tetrahymena* and amoebae had a 30- and 120-fold increased resistance to 2 to 10 mg/L chlorine treatments, which is above the lethal concentration (1 mg/L) for most freely suspended bacterial cells.

There is a growing concern regarding the occurrence of bacterial pathogens in freshwater systems and drinking water. These pathogens can survive chlorine disinfection during wastewater treatments, especially when residing within protozoan cells or cysts (King *et al.,* 1988; Smith *et al.,* 2012). Waterborne diseases are a worldwide concern, and according to the WHO (2018), 26% of the world's population drink water that is at least contaminated with faecal indicator bacteria, and 502 000 diarrhoeal deaths are due to drinking contaminated water each year. In New Zealand, about 18 000 to 34 000 of gastrointestinal disease cases reported per year are due to drinking water containing waterborne pathogens (Hambling and Bandaranayak, 2012). From 2013 to 2014, 42 outbreaks due to drinking contaminated water were reported in the USA, and of the 1006 cases reported, 75% were linked to community water systems (Benedict *et al.,* 2017). In South Africa, nearly 50% of gastrointestinal diseases and 3.1% of deaths are attributed to drinking unsafe water (Norman *et al.,* 2006; Govender *et al.,* 2011). A severe cholera epidemic and waterborne disease outbreak that occurred in 2000 to 2001 in rural communities of KwaZulu-Natal, Limpopo, Eastern Cape, and Mpumalanga in South Africa resulted in 114 000 cases

reported and 260 resulting deaths (Hemson and Dube, 2004). The lack of well-managed water treatment resulted in 400 cases of typhoid fever, and 2500 cases of diarrhoea were reported during the outbreak in 2005 in a small town of Delmas, Mpumalanga (DoH, 2005; Sidley, 2005).

Similarly, fresh produce is increasingly linked to outbreaks of foodborne diseases; pathogenic bacteria have been frequently detected in fresh produce, which may persist despite thorough disinfection treatments due to protozoan predation, especially when pathogenic bacteria are residing in protozoan cells or cysts (Lambrecht *et al.,* 2015). Over 75% of illnesses from 905 foodborne outbreaks *of Salmonella* spp. and 255 outbreaks due to *E. coli* O157 reported between 2014 and 2018 in the USA were attributed to consumption of vegetables (IFSAC, 2020). Foodborne illnesses caused by Shiga toxin-producing *E. coli* O157:H7 and *Salmonella* species were traced to contaminated fresh spinach, lettuce, and tomatoes (Gourabathini *et al.,* 2008; Ravva *et al.,* 2010; IFSAC, 2020). A study by Akoachere *et al.* (2018) detected faecal coliforms in vegetables sold in markets in Fako Division, Cameroon, with carrots and lettuce having the highest faecal coliform counts (248.9 and 165.03 MPN/g). However, this study also reported pathogenic bacterial species such as *Enterobacter cloacae*, *Shigella* sp., *Klebsiella pneumoniae*, and *Staphylococcus aureus* present on the vegetables. Irrigation water was frequently reported as a source of pathogens leading to the contamination of fruits and vegetables. While *E. coli*, a general hygiene indicator, was detected on cabbage seedlings that were irrigated with wastewater, whereas *E. coli* was not detected on seedlings irrigated with municipal water (Steele and Odumery, 2004). Survival of bacterial pathogens in drinking water and on fresh produce was associated with their interaction with protozoa, which have also been frequently detected in the same environment. Guaíbia lake in Brazil, which is used for water supply, irrigation, and recreational activities, is highly impacted by wastewater effluent discharge. The lake had a high density of pathogenic bacteria and was colonized by mostly *Peritrich* ciliates, with 22 genera of ciliates, including *Vorticella* and *Epistylis,* recorded to occur in the lake (Safi *et al*., 2014). Gourabathini *et al.* (2008) isolated protozoa from a bundle of spinach and lettuce, of which two belonged to the phylum *Ciliophora* (*Colpoda steinii* and *Glaucoma* sp.).

One of the biggest challenges that South Africa faces is water quality, as unsafe levels of potentially pathogenic bacteria have been detected in water resources in many provinces. In 2010, the level of bacteria detected in surface waters in Limpopo was more than 5 times the amount that the WHO recommends for safe consumption (Mellor *et al.,* 2013). *Escherichia coli*, *Shigella* spp., *Aeromonas* spp., *Salmonella* spp., *Pseudomonas* spp., and *Vibrio* spp. have been detected in Berg and Plankenburg Rivers (Western Cape, South Africa), and the source of contamination is mainly the effluent from wastewater treatment facilities (Paulse *et al.,* 2012). The wastewater treatment plants in South Africa are frequently unable to completely remove pathogens, and the consequences are the microbial contamination of receiving water bodies used for consumption and irrigation. The effluents discharged from four wastewater treatment plants in the province of Eastern Cape of South Africa were identified as pollution point sources for their respective receiving water bodies (Nahoon river, Tembisa Dam, and Eastern Beach, which is part of the Indian Ocean; the Kat River and the Tyume River) (Momba *et al.,* 2006). Sundram *et al.* (2002) reported a maximum *E. coli* counts of 6.1 x 10^5 per 100 mL Umngeni river in Pietermaritzburg in KwaZulu-Natal, South Africa, which receives effluent from Darvill wastewater treatment plants, which had a maximum *E. coli* counts of 1.1 x 10^6 per 100 mL. A report by Pretorius and Pretorius (1999) revealed that only 33% of the total effluent released from the wastewater treatment plants into receiving water bodies in South Africa met the bacteriological standards (e.g., faecal counts). Chlorine treated wastewater effluents from four wastewater treatment plants (Baviaanspoort Rayton, Refilwe Water Care Works, and Zeekoegat) in Pretoria, South Africa, did not comply with the bacterial standard of the South African Water Services Authority and had a high occurrence of *E. coli*, *Vibrio cholerae* and *S.* Typhimurium (Dungeni *et al.,* 2010). The Msunduzi River, which is a key source of water for agricultural, domestic, and industrial use to the community in Pietermaritzburg, KwaZulu-Natal, South Africa, is highly impacted by runoff from rural communities and wastewater treatment effluent and had high levels of faecal coliforms (63 000 MPN/ 100mL) and *E. coli* (7 900 MPN/ 100mL), exceeding the safe threshold value for domestic and irrigation use (1000 MPN/ 100mL) (Gemmell and Schmidt, 2013). Faecal coliform bacteria have also been detected in household tap water from small towns of Pietermaritzburg (KwaZulu-Natal, South Africa) (September *et al.,* 2007), and *E. coli* was detected in treated drinking water in Fort Beaufort in the province of Eastern Cape, South Africa (Momba *et al.,*

2008). In 2010, water containers used to supply drinking water to households in Limpopo, South Africa, had excessive counts for biofilm-associated coliforms (Mellor *et al.,* 2013).

Agriculture in South Africa largely relies on surface water for irrigation for the production of fruits and vegetables on a large scale. The quality of surface water has immensely decreased to the point that water is now regarded as unsafe for irrigation due to the presence of a high level of pathogenic bacteria in water (CSIR, 2010; Griffin *et al.,* 2014; Sigge and Lamprecht, 2016). The water from Plankenburg River in Stellenbosch, Western Cape of South Africa, utilized for irrigation is deemed as unsafe for irrigation due to the high levels of *E. coli* detected (>1000 to 10^6 MPN/ 100mL), which exceeds the limit recommended by the WHO for irrigation water used for fresh produce (1000 faecal coliforms/ 100 mL) (Britz *et al.,* 2013). Iwu *et al.* (2021) reported the prevalent occurrence of pathogenic *E. coli* O157:H7 (37% of samples) and high *E. coli* O157:H7 counts up to 4.11 Log_{10} CFU/ 100 mL in irrigation water from Amathole and Chris Hani District Municipalities in the Eastern Cape Province of South Africa. Therefore, the use of such water presents a risk to public health as it can lead to the contamination of fresh produce. For example, *E. coli* O157:H7 was present on cabbage, carrots, cucumbers, onions, and spinach from farmers' markets and retail stores in the Eastern Cape (Abong'o *et al.,* 2008). The assessment of the Baynespruit river, commonly used for irrigation by the community in Pietermaritzburg, KwaZulu-Natal, revealed that the river had high levels of faecal coliforms (up to 5.17 Log MPN/ 100 mL) and is not safe to use for irrigation. Moreover, fresh produce irrigated with the river water were contaminated with faecal coliform, and *E. coli* detected on spinach and parsley ranged from <1.26 to 4.92 Log MPN/g (Gemmell and Schmidt, 2012). In a period of 5 years, from 2013 to 2017, 327 foodborne disease outbreaks were reported, with 11 155 cases reported (Shonhiwa *et al.,* 2018).

The role of protists in the persistence of pathogenic bacteria in freshwater environments is now receiving more attention due to the potential risk they pose to public health (Kiss *et al.,* 2009; Thomas *et al.,* 2012). Studying the interaction of protists with bacteria is relevant not only in determining their ecological function in aquatic environments but also in view of public health and microbial pollution control since protists have been described to harbour and protect pathogenic bacteria. In

addition, some studies demonstrated that ciliates might enable the spread of antibiotic resistance genes (Schlimme *et al.,* 1997). This also provides insight into the evolution of bacterial pathogenicity and the mechanisms underlying the interaction with their host (Schulz *et al*., 2014). Though the interaction of protists with bacteria is one of the oldest forms of predator-prey relationships that have been studied, it appears that the interaction of protists and their bacterial prey in rivers have received lesser attention than that in oceans and lakes (Jürgens and Güde, 1994). Amongst the most important factors controlling bacterial communities in polluted surface waters is predation by ciliates (Curds and Cockburn, 1968; Posch *et al.,* 2001). Although a few studies have reported on the abundance and diversity of protists in South African wastewater and freshwater environments, studies on predator-prey interaction between protists and bacteria are limited.

Therefore, the aim of this study was to determine the predator-prey relationship between various bacterial species and local ciliate isolates from river water in KwaZulu-Natal.

Objectives of the study:

- Isolation and characterization of bacterivorous ciliates from river water in Pietermaritzburg, KwaZulu-Natal, South Africa
- Determining the feeding/ grazing rates and growth kinetics of the isolated ciliates using various species of bacteria
- Determine feeding selectivity using various microorganisms and microparticles
- Determining the uptake and vacuole formation rates using fluorescently labelled bacteria and microparticles
- Evaluating the uptake of plastic microparticles
- Interaction between isolated ciliates and bacteriophages
- Determining the response of tested bacterial species to ciliate grazing

1.5. References

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CHAPTER 2

Isolation and characterization of ciliates from freshwater samples

2.1. Introduction

Protist predation constitutes one of the major mortality factors for bacteria in aquatic environments (Gonzàlez *et al.,* 1992; Hahn and Höfle, 2001; Sherr and Sherr, 2002). Predation activity is important in the control of bacterial biomass in aquatic environments, specifically in freshwater environments which are subjected to constant microbial contamination, and the main source is the effluent from wastewater treatment plants (CSIR, 2010; Jung *et al.,* 2014; Gemmell and Schmidt, 2013; Mbanga *et al.,* 2020). It is estimated that only 60 out of the 824 treatment plants in South Africa meet the set quality standards for sewage systems; thus, the effluent released from most wastewater treatments plants carries bacteria into receiving water bodies (Herbig, 2019). These pose a risk to public health as freshwater is heavily utilized for domestic and agricultural purposes. Particularly in South Africa, where 19% of the rural population lacks access to potable water, thus use untreated surface water directly for drinking and domestic purposes (Gemmell and Schmidt*,* 2012; Mpenyana-Monyatsi *et al.,* 2012; Odiyo and Makungo, 2012; Gemmell and Schmidt, 2013; Mulamattathil *et al.,* 2014).

Among bacterivorous protists, ciliates are considered major consumers of bacteria, representing 50% of all bacterial grazers in aquatic environments (Sherr and Sherr, 1987; Stabell, 1996; Ali and Saleh, 2014). The study of ciliate population dynamics and ecological impact is, however, less in comparison with other groups of protists. Ciliates are a diverse group of protists belonging to the phylum *Ciliophora* that inhabit a great variety of environments (Corliss, 1979; Foissner *et al.,* 2008; Lynn, 2008; Gao *et al*., 2016). Their predation on bacteria is also essential in wastewater treatment plants and dominates during sludge treatments (Pauli *et al.,* 2001; Madoni, 2011). Ciliates have been detected in water intended for domestic and agricultural processes, building distribution systems, tap water, and on the surface of fruits and vegetables (Sibille *et al.,* 1998; Valster *et al.,* 2009; Gourabathini *et al.,* 2008; Vaerewijck *et al.,* 2014).

Quantifying the rates of bacterial consumption by protists is essential for determining the regeneration efficiency of nutrients, predation impact on the prey population, and also the fate of bacterial production, thus establishing a functional role of these grazers, which is a central issue in aquatic ecosystems (Šolić and Krstulović, 1994; Neuer and Cowles, 1995; Eisenmann *et al.,* 1998). Given the generally greater biomass of microorganisms in freshwater environments and their productivity, such measurements of grazing activity are imperative (Bott and Kaplan, 1990; Berninger *et al.,* 1991). This predator-prey interaction is often studied by monitoring the change in both predator and prey numbers during a period of grazing or by tracing the fate of ingested particles (fluorescently or radiolabelled) within the cell of predators (Kemp, 1988; Batani *et al.,* 2016). Ciliate growth and grazing largely depend on the type and quantity of available food, and their response, which is species-specific, is still not well understood (Posch *et al.,* 2015). To better understand this important trophic relationship, investigations on a large variety of predator-prey relationships are therefore needed (Stabell, 1996). Thus, the cultivation of an axenic culture of ciliates isolated from aquatic environments allows for these organisms to be studied in vitro to determine their nutritional and physiological requirements (Gold, 1970; Gifford, 1985).

Therefore, in this study, ciliates were isolated from water samples collected from a local stream in Pietermaritzburg, KwaZulu-Natal, and identified based on their morphology and molecular characteristics. Their growth and grazing kinetics and their prey selectivity were determined using various bacterial and eukaryotic organisms.

2.2. Materials and methods

2.2.1. Enrichment and isolation

Ciliates were isolated from water samples collected from the Blackborough Spruit stream in Pietermaritzburg in KwaZulu-Natal, South Africa (29°38'00.0"S 30°24'26.5"E to 29°37'14.3"S 30°24'23.1"E) using sterile Schott bottles and transported back to the lab. The collected water samples were used immediately.

Ciliate species were isolated by enrichment in sterile Chalkley's medium containing 3 – 5 rice grains to support bacterial growth. The medium was prepared by dissolving 100 mg NaCl, 4 mg KCl, and 6 mg $CaCl_2$ per 1 L of distilled water, pH 7. 5 mL of the water sample was added to 15 mL of Chalkley's medium. The inoculated medium was then incubated at room temperature (25 ± 2ºC) in the dark. After 7 days of incubation, individual cells showing typical morphology of ciliates were drawn out of the culture under a light microscope to inoculate a 20 mL fresh medium. This was repeated until mono-clonal cultures were established (i.e., until only one type of ciliated protozoa was present in the culture). The clonal isolates were maintained in the medium by transferring to a fresh medium when required.

2.2.2. Characterization of the isolated ciliates

The microscopic examination of cells was carried out to determine the size, shape, and pattern of ciliary lining and locomotion.

Light microscopy

Live cells of the isolated ciliates were observed on wet mount slides for morphological details using bright field and phase-contrast microscopy at an appropriate magnification. Prior to viewing, the cells were left standing for a period of time to allow the cells to slow down. Cell images and cell dimensions were captured using a Carl Zeiss Axio Scope A1 microscope fitted with a Zeiss AxioCam ICc3 digital camera.

Ciliates were dry-stained with 1% silver nitrate according to the method described by Klein (1958). The cells were harvested by gentle centrifugation at 500x *g* for 5 minutes and air-dried on a slide. Silver nitrate was added to the air-dried film of the ciliates for

about 5 minutes and washed gently with distilled water. The stained slides were exposed to illuminating light until the smear of cells turned lightly brown. Cell images were captured from appropriate preparations with a microscope fitted with a digital camera as specified above.

Scanning electron microscopy (SEM)

Scanning electron microscopy preparations were obtained as described by Foissner (2014). Isolated ciliates were fixed with Parducz's or Barry Wicklow's reagent in a ratio of sample to fixative 3:1. After 30 minutes of fixing, ciliates were washed three times with distilled water and then dehydrated in a series of ethanol (30, 50, 70, 90, and 100%) for 5 minutes each. After the samples were dried using critical point drying, they were mounted on aluminium stubs, coated using gold, and then examined by a Zeiss EVOLS15 Scanning Electron Microscope. Parducz's reagent was prepared by mixing 4 mL of 2% Osmium tetroxide and 1 mL 6% Mercuric chloride solution. Barry Wicklow's reagent was prepared by mixing equal amounts of 2% Osmium tetroxide and 3% glutaraldehyde.

2.2.3. Analysis of the 18S rRNA gene sequence

DNA extraction

About 1000 cells of isolated ciliates were collected by centrifugation at 500x *g* for 5 minutes and washed three times. Genomic DNA was extracted from the cells using the GeneJET genomic DNA purification kit (Thermo Fisher Scientific).

Amplification

Genomic DNA was amplified using two sets of primers: P-SSU 342f (5' - CTTTCGATGGTAGTGTATTGGACTAC - 3') and Medlin B (5' - TGATCCTTCTGCAGGTTCACCTAC - 3') designed to amplify 1.36 kbp fragment of DNA encoding 18S small subunit ribosomal RNA (18S rRNA) (Medlin *et al.,* 1988; Karnati *et al.,* 2003) and EukFor (5' - AATATGGTTGATCCTGCCAGT - 3') and EukRev (5' - TGATCCTTCTGCAGGTTCACCTAC - 3') designed to amplify the 1.8

kbp fragment of the 18S rRNA gene (Regensbogenova *et al.,* 2004). The primers used were purchased from Inqaba Biotechnologies Industries, South Africa.

PCR amplification was performed with 12.5 µL of 2x concentrated one Taq standard master mix with standard buffer (Biolabs, Inc), 0.5 µL of each primer (10 µM), 1 µL of extracted DNA, and the volume was made up to 25 µL with Milliq H_2O. The PCR was performed using Labnet Multi Gene II thermocycler with the following cycling conditions; initial denaturation at 94°C for 2 minutes, followed by 35 repetition cycles of denaturation at 94°C for 60 seconds, annealing at 50°C for 60 seconds, extension at 72°C for 3 minutes, and the final elongation at 72°C for 7 minutes.

Analysis of PCR products

PCR amplicons were analyzed using 1.5% agarose gel electrophoresis in Tris-Acetate-EDTA buffer at 80V for 45 minutes. Prior to loading the gel, 5 µL PCR amplification products were mixed with 3 µL of 6x loading dye (Thermo Fischer Scientific). A 1 Kb DNA ladder (Biolabs Inc) was used to establish the amplicon size. For visualization of the DNA bands, the gel was stained with 2% Ethidium bromide for 10 minutes and then de-stained in distilled H_2O for about 5 minutes. The agarose gel was viewed on a UV transilluminator.

Sequencing of the obtained amplicons

Amplified products were sent for direct sequencing to the Central Analytical Facility of Stellenbosch University (South Africa).

Phylogenetic analysis of the resulting sequences

The sequences obtained were assembled into contigs using Geneious prime (version 2020.2), and the resulting sequences were compared against the sequences available in the NCBI GenBank database (www.ncbi.nim.nih.gov). The sequence alignment was done using ClustalW, and phylogenetic trees were constructed using Mega X (Kumar *et al.,* 2018) based on the maximum likelihood and neighbourhood joining methods.

The 18S rRNA gene sequences for isolates of closely related ciliates were obtained from NCBI.

2.2.4. Growth and grazing studies of isolated ciliate species

All growth and grazing experiments of the isolated ciliates were carried out in 5 mL Chalkley's medium with various prey organisms listed in Table 2.1. over a period of 5 days. All experiments were performed in duplicate at room temperature (25 ± 2ºC) in the dark with shaking every 12 hours. Two sets of controls were employed, control 1 contained a predator organism without the prey, and control 2 was a prey organism in the medium without the predator to account for non-predatory changes. The data generated was used to determine growth rates (μ), grazing rates (bacteria x $pred^{-1}$ x hr^{-1}), clearance rates (nL x $pred^{-1}$ x hr^{-1}), and feeding selectivity of isolated ciliates on various prey organisms.

Preparation of predator organisms

Isolated ciliates were harvested by gentle centrifugation at 500x *g* for 5 minutes and washed thrice with fresh medium. The harvested cells were then re-suspended in 5 mL medium to a final concentration of 1 x 10^3 cells x mL^{-1}. Subsamples were taken every 24 hours in replicates, and the change in predator cells was measured microscopically by total cell counts in 50 μL volume. To immobilize cells, they were fixed with 0.5% *v/v* Formaldehyde.

Ciliates' growth rates were calculated from changes in their cell numbers ($\mu = \ln N_t - \ln N_0$) / hr) assuming exponential growth. Clearance and grazing rates per ciliate were calculated as estimates of protozoan bacterivory by monitoring the change in prey cell numbers. Average grazing (bacteria x $ciliate^{-1}$ x hr^{-1}) and clearance rates (nL x $ciliate^{-1}$ x hr^{-1}) were calculated after 24 hours as previously reported (Zimmermann-Timm and Barkmann, 2000).

Preparation of prey organisms

Bacterial prey organisms listed in Table 2.1. were grown overnight in nutrient broth. All cultures were grown at 25ºC in a rotary shaker at 100 rpm. The cultures were harvested by centrifugation at 10 000x *g* for 10 minutes and washed twice in a 0.85% saline solution. The washed culture was then added to the culture of ciliates in Chalkley's medium to a final concentration of 1 x 10^8 bacteria x mL^{-1} (*Tetrahymena* sp.) and 1 x 10^{10} bacteria x mL^{-1} (*Coleps* sp. and *Paramecium* sp.) to initiate growth and grazing of isolated ciliates.

Two algal species (*Parachlorella* sp. strain AA1 and *Haematococcus* sp. strain AA3) were used to assess the grazing of isolated ciliates. The algal prey organisms were grown in 50 mL Bristol medium under light at static conditions for 2 weeks. The cultures were harvested by centrifugation at 10 000x *g* for 10 minutes and washed twice with fresh medium. The washed cells were then used to inoculate medium containing isolated ciliates to a concentration of 1 x 10^8 cells x mL^{-1}.

Spores of *Fusarium* sp. V3 were harvested from growing cultures on nutrient agar by adding 10 mL of 0.85% saline solution and scrapping off the agar surface with a hockey stick. The collected spores were then transferred to Eppendorf tubes, centrifuged at 8000x *g* for 10 minutes, and washed twice with the same solution. The washed suspension was used to inoculate the culture of ciliates to a final concentration of 1 x 10^8 cells x mL^{-1}.

Saccharomyces cerevisiae cells were harvested from yeast cultures growing in nutrient broth with 2% glucose and washed twice with 0.85% saline solution. The washed suspension was then used to inoculate the culture of ciliates to a final concentration of 1 x 10^8 cells x mL^{-1}.

To determine grazing kinetics of ciliates on prey, the change in prey concentrations was monitored every 24 hours by direct cell counts using a bacterial counting chamber (Improved Neubauer; Marienfeld, Germany).

Influence of prey concentrations on growth and grazing of isolated ciliates

The effect of prey concentrations on growth rates of isolated ciliates was observed in 5 mL Chalkley's medium using *E. coli* ATCC 8739 at a concentration ranging from 1 x 10^6 to 1 x 10^{12} bacteria x mL^{-1}. To initiate growth and grazing, washed bacterial culture was added to the culture of ciliate (1 x 10^3 ciliates x mL^{-1}) in 5 mL Chalkley's medium to appropriate concentrations (10^6, 10^7, 10^8, 10^9, 10^{10}, and 10^{12} bacteria x mL^{-1}).

The growth of ciliates was observed every 24 hours for 5 days by monitoring the change in cell concentrations of the predator. In addition, the number of viable cells (CFU x mL^{-1}) were determined by the plate count method using nutrient agar to determine the change in viable bacteria during predator grazing.

Influence of particle size on grazing rates of isolated ciliates

Polystyrene microparticles of density 1.05 gcm^{-3} (SPI supplies and Sigma-Aldrich) of different sizes were used as artificial food particles. Microbeads of size 2, 5, and 10 µm diameter were washed with 0.85% saline solution and centrifuged at 10 000x *g* for 10 minutes and then added to a washed culture of ciliates (10^3 ciliates x mL^{-1}) to a final concentration of 10^8 particles x mL^{-1} in 5 mL Chalkley's medium. After 24 hours of incubation in the dark at room temperature (25 ± 2ºC), the grazing rates of microbeads were measured in replicates by direct counts of particles using the counting chamber (Improved Neubauer; Marienfeld, Germany). To verify the uptake of microbeads, a total of 10 individual ciliate cells were analyzed per replicate slide by phase-contrast microscopy.

Effect of temperature on growth and grazing of isolate ciliates

The influence of temperature on isolated ciliates was studied in 5 mL Chalkley's medium incubated in the dark at 5, 25, and 35ºC. *E. coli* ATCC 8739 was inoculated in Chalkley's medium containing ciliates (1 x10^3 cells x mL^{-1}) to a final concentration of 10^8 bacteria x mL^{-1} for *Tetrahymena* sp. and 10^{10} bacteria x mL^{-1} for *Paramecium* sp. and *Coleps* sp. The growth of ciliates at different temperatures was observed every

24 hours for 5 days by monitoring the change in cell numbers of both the prey and the predator.

The response of the isolated ciliates to prey availability fluctuations

The change in the growth pattern of isolated ciliates in response to prey availability was observed when the culture of growing ciliates was continuously fed with prey cells. A culture of ciliates (1 x 10^3 cells x mL^{-1}) was initially fed with *E. coli* ATCC 8739 at a concentration of 1 x 10^8 bacteria x mL^{-1} for *Tetrahymena* sp. and 1 x 10^{10} bacteria x mL^{-1} for *Coleps* sp. and *Paramecium* sp. and were continuously fed with fresh *E. coli* ATCC 8739 culture once the prey concentration was below the threshold for ciliate growth (i.e., at 96 and 192 hours). The change in ciliate and prey was observed every 24 hours by monitoring the change in cell numbers of both the prey and predator.

Prey selectivity by isolated ciliates and nutritional quality of various microorganisms

Growth of the isolated ciliates was observed in Chalkley's medium with various prokaryotic bacterial prey (Gram-positive and Gram-negative bacteria) and eukaryotes (algae and fungi) listed in Table 2.1. to determine prey selectivity of the isolated ciliates as well as the nutritional quality of various prey.

2.2.5. Respiration rates of isolated ciliates

Oxygen uptake of ciliates was measured in a Clark-type oxygen electrode (Oxytherm, Hansatech instruments LTD) at 25ºC. Isolated ciliates were harvested by centrifugation at 500x *g* for 5 minutes and washed three times. The cells were re-suspended in medium to a final concentration of 5 x 10^3 cells x mL^{-1}. 1 mL of ciliate suspension was placed in the electrode chamber with the magnetic stirrer bar adjusted to 20% rpm. Respiration rates were measured for ciliates without prey (endogenous respiration) for 30 minutes and further 30 minutes with bacterial prey by the addition of 10 µL of prey to a final concentration of 1 x 10^{10} bacteria x mL^{-1}. Oxygen uptake

rates for individual cells were determined in duplicate and expressed as nmol O_2 x $pred^{-1}$ x hr^{-1}.

Table 2.1. Microorganisms tested for isolated ciliates grazing

Prey	Morphology
Gram-negative bacteria	
Escherichia coli ATCC 8739	Rod, motile (0.25 – 1.0 x 2.0 µm)
Escherichia coli isolate EC33 (ARB: GEN-TOB)[a]	Rod, motile (0.25 – 1.0 x 2.0 µm)
Salmonella Typhimurium ATCC 14028	Rod, motile (0.7 – 1.5 x 2.0 – 5.0 µm)
Pseudomonas sp. strain B12[b]	Rod, motile (0.5 – 0.8 x 1.5 – 3.0 µm)
Vibrio sp. strain KM1[b]	Curved-rod, motile (0.5 x 1.5 – 3.0 µm)
Acinetobacter sp. strain S21[b]	Coccobacilli, non-motile (1.0 – 1.5 x 1.5 – 2.5 µm)
Gram-positive bacteria:	
Staphylococcus sp. CPS23 (ARB: E-CLM-FD)[a]	Coccus, non-motile (0.6 µm diameter)
Staphylococcus aureus ATCC 6053	Coccus, non-motile (0.6 µm diameter)
Enterococcus faecalis ATCC 29212	Ovoid coccus, non-motile (0.6 x 2.0 – 2.5 µm)
Enterococcus durans ATCC 6056	Coccus, non-motile (0.6 x 2.0 – 2.5 µm)
Lyophilized *Micrococcus lysodeikticus* ATCC 4698	Coccus, non-motile (0.5 – 3.5 µm)
Bacillus subtilis (BGA)	Rod, motile (4 – 10 x 0.25 – 1.0 µm)
Algae:	
Parachlorella sp. strain AA1[c]	Oval (2.0 – 5.0 µm diameter)
Haematococcus sp. strain AA3[c]	Spherical (12 – 15 µm diameter)
Fungi:	
Fusarium sp. V3 conidia[d]	Curved rod (3.0 – 4.5 x 23 – 54 µm)
Saccharomyces cerevisiae	Spherical (4.0 – 6.0 µm diameter)

All species, except American Type Culture Collection (ATCC) and BGA strains, are wild-type strains provided by the Department of Microbiology, University of KwaZulu-Natal Pietermaritzburg campus. a – antibiotic-resistant isolates (ARB) from pit latrine faecal sludge and their antibiotic resistance profile (GEN - gentamycin, TOB - tobramycin, E - erythromycin, CLM - clindamycin, FD - fusidic acid); b – isolates from estuaries water samples; c – isolates from Msunduzi river in Pietermaritzburg; d – isolate from soil samples collected from Crafty Duck Farm in Pietermaritzburg, KwaZulu-Natal.

2.2.6. Measurements of uptake rates of fluorescently labelled bacteria

Preparation of fluorescently labelled bacteria (FLB)

E. coli ATCC 8739 was stained with 2 mg of 5-([4,6-Dichlorotriazin-2-yl] amino) fluorescein hydrochloride (DTFA) (Sigma-Aldrich), which has maximum excitation at 492nm and emission at 516nm following the method described by Sherr *et al.* (1987). The bacterial culture was harvested in the mid to late logarithmic phase by centrifugation at 22 000x *g* for 12 minutes. The pellet was then suspended in 10 mL of 0.05 M NaH_2PO_4-0.85% NaCl (pH 9) to a final concentration of 10^{10} bacteria x mL^{-1}. 2 mg of 5-([4,6-Dichlorotriazin-2-yl] amino) fluorescein hydrochloride was added to the bacterial suspension and incubated in a water bath at 60ºC for 2 hours. After incubation, cells were washed three times with 0.05M NaH_2PO_4-0.85% NaCl (pH 9) and then resuspended in 0.02M NaH_2PO_4-0.85% NaCl (pH 9). The stained cells were stored in small aliquots (1 mL) at -20ºC. The cells were thawed and sonicated to disperse any clumps before use. A long-term grazing experiment was carried out in 5 mL Chalkley's medium with 1 x 10^3 cells x mL^{-1} of ciliate and 1 x 10^8 of FLB x mL^{-1} to determine the nutritional quality and potential toxicity of the stained cells.

Uptake of fluorescently labelled bacteria (FLB)

For bacterial uptake and food vacuole formation measurements, experiments were carried out in 5 mL Chalkley's medium at 25ºC in the dark in duplicates. FLB was added to 10^3 ciliate cells x mL^{-1} to a final concentration of 10^8 FLB cells x mL^{-1}. To determine the rates, ingested cells were enumerated by monitoring the decrease in FLB concentration microscopically in the medium after 0, 5, 10 minutes, and then every 30 minutes for a period of 3 hours incubation using a bacterial counting chamber (Improved Neubauer; Marienfeld, Germany). The rates of food vacuole formation were determined by direct counts of food vacuoles containing FLB bacteria within the cells of ciliates using a Zeiss Universal Epifluorescence Microscope with a 75-W xenon lamp, at least 10 individual ciliate cells were counted per slide. For imaging of the cells with food vacuoles containing FLB cells, the ciliate cells were analyzed under the ZEISS Confocal Laser Scanning Microscope (LSM710, Germany).

The uptake of FLB by isolated ciliates was compared with the uptake of fluorescent latex microbeads. Carboxylate-modified microbeads of size 2 µm diameter containing a red fluorescent dye with an excitation maximum of 576nm and an emission maximum of 610nm (Sigma-Aldrich) were used to simulate nutrient particles in the feeding studies. The concentration of fluorescent microbeads in suspension was determined microscopically using a bacterial counting chamber (Improved Neubauer; Marienfeld, Germany). Prior to use in uptake experiments, microbeads were centrifuged at 10 000x *g* for 10 minutes and washed twice with 0.85% saline solution.

The uptake experiments of the polystyrene microbeads were carried out in 5 mL Chalkley's medium at 25ºC in the dark. An appropriate volume of microbeads suspension was added to a culture of 10^3 ciliate cells x mL^{-1} to a final concentration of 10^8 particles x mL^{-1}. The uptake of particles and the number of food vacuoles formed was measured microscopically by direct counts of particles ingested within the ciliate cell after 0, 5, 10 minutes, and then every 30 minutes for 3 hours of feeding. 20 µL was withdrawn and viewed immediately under the Olympus AX70 Fluorescent microscope with a 75-W xenon lamp. An equal volume of 0.01% formaldehyde was added to slow down motile cells. The uptake rates (particles x $ciliate^{-1}$ x hr^{-1}), rates of food vacuole formation, and particles per vacuole were determined from the data generated. In addition, ZEISS Confocal Laser Scanning Microscopy (LSM710, Germany) was used for imaging of the ciliate cells containing fluorescently labelled microparticles.

2.3. Results

2.3.1. Morphological characterization of the isolated ciliates

All isolated ciliates were selected based on their motility and morphological characteristics of ciliates, which is mainly the presence of cilia used for feeding and locomotion. Thus, three different ciliates were successfully isolated from freshwater samples collected from a stream in Pietermaritzburg, KwaZulu-Natal (South Africa). Microscopic characterizations were carried out using light and scanning electron microscopy techniques to determine the shape, size, pattern of ciliature, and swimming pattern of the isolated ciliates.

The first isolate (Figure 2.1. and 2.2.) resembles the phenotype of species of genus *Coleps*. It is a barrel-shaped ciliate with an average size of 40 – 65 x 20 µm. The oral cavity (OC) occupies the whole anterior end, while the posterior end has four prominent spikes (PS). This isolate has an external skeleton or calcified plate with longitudinal rows that appear to be like armour plates on its surface. Cilia are projected through the kineties (K) between the plates. The arrangement of cilia on the cell is uniform but sparse except at the oral cavity that is densely packed with cilia. This ciliate swims by rotation of the main body in an anticlockwise direction.

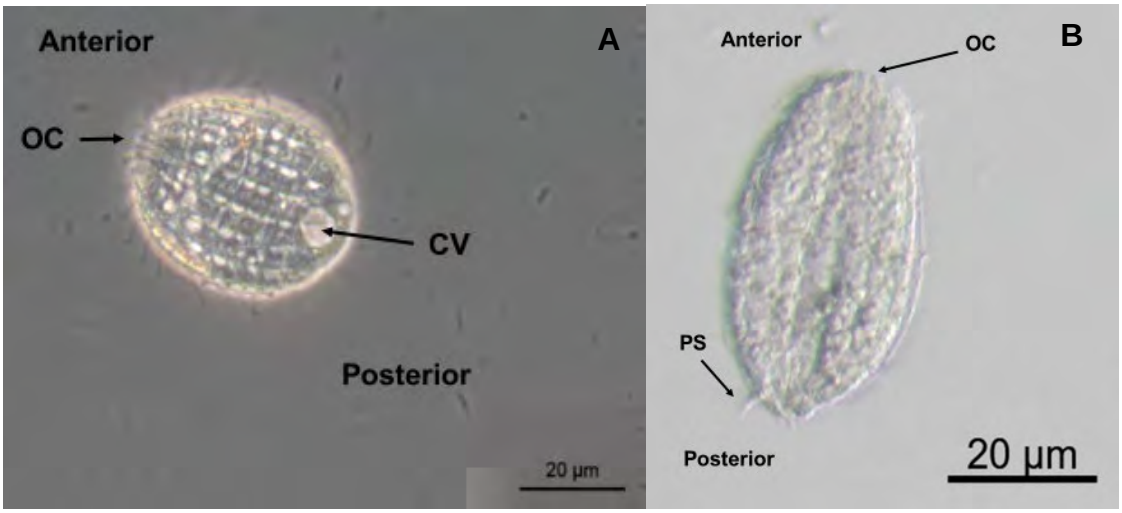

Figure 2.1. Micrographs of typical live cells of isolate RB3 under Phase contrast. OC – oral cavity, CV – contractile vacuole, PS – prominent spikes.

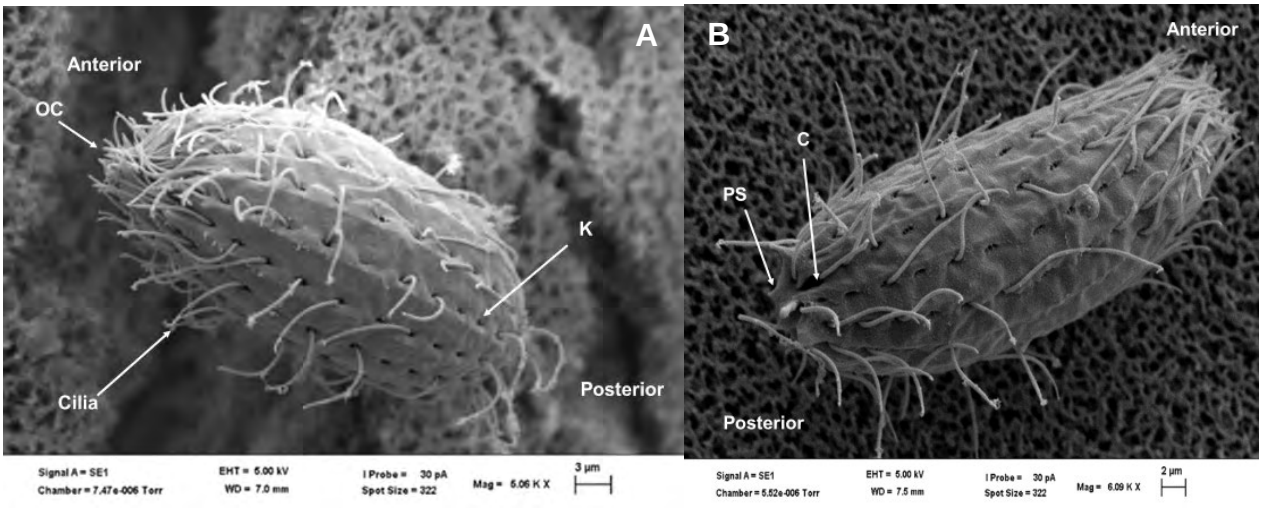

Figure 2.2. SEM micrographs of typical cells of isolate RB3 with a focus on the lateral (A) and the dorsal (B) side of the cell. OC – oral cavity, CV – contractile vacuole, K – kinetosome, PS – prominent spikes, C – cytoproct.

The second isolate resembles the shape of species of the genus *Paramecium* (Figure 2.3. and 2.4.). The cells are elongated ovoid, asymmetrical with an average size of 180 x 80 µm. The whole-body surface is covered with short cilia (C) that occur in longitudinal rows of kinetosome (K) that are closely packed over the cell surface. A shallow depression of its oral cavity (OC) runs from the anterior end of the cell on the ventrolateral side to the middle of the cell on the ventral side. Whilst the anterior end is more rounded, the posterior end is more pointed with few longer cilia, thus giving the cell an asymmetrical appearance. The cytoproct (C) is located on the dorsal side at the posterior end. The macronucleus (MA) is ovoid and is localized in the middle of the cell with two visible contractile vacuoles surrounded by water canals (CV) on both ends of the cell. The cilia beat in synchronized rhythm, creating a metachronal wave, and the cells move by rotation on its long axis in both directions

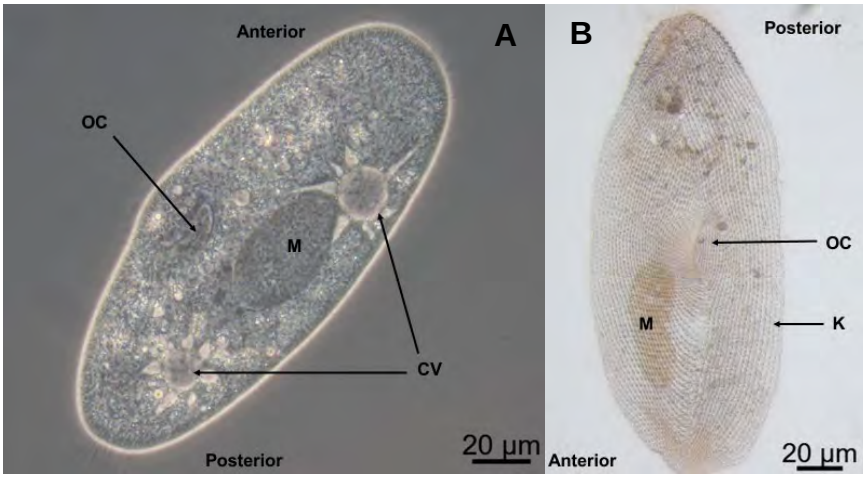

Figure 2.3. Micrographs of typical cells of isolate RB1. Phase-contrast image of a live cell (A) and silver-stained cell (B) of isolate RB1. OC – oral cavity, M – micronucleus, CV – contractile vacuole, K – kinetosome.

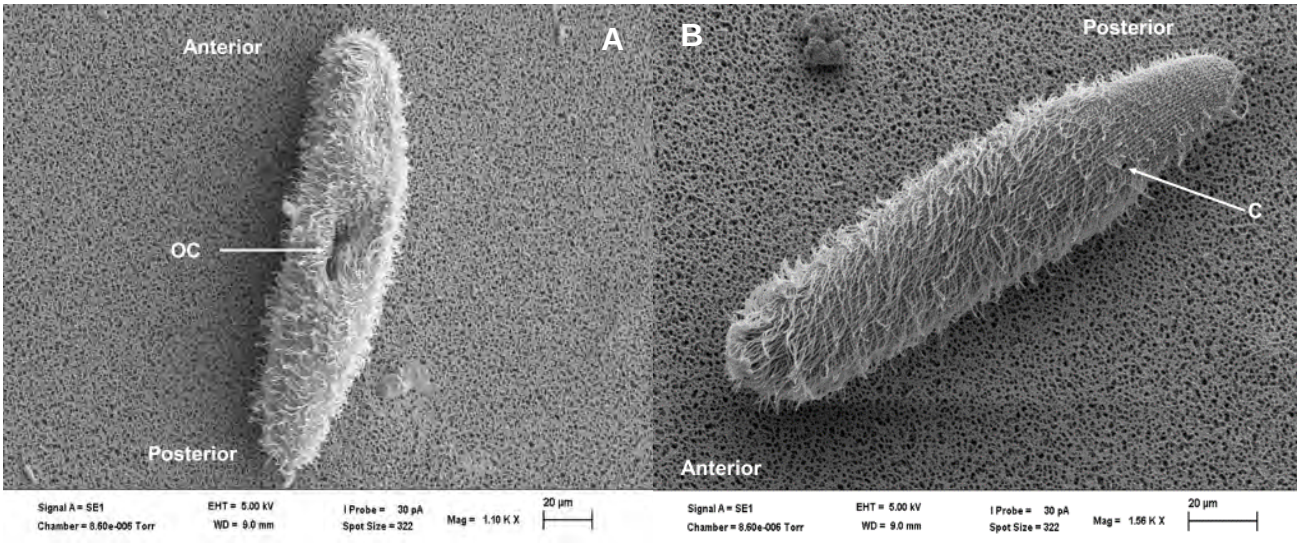

Figure 2.4. SEM micrographs of typical cells of isolate RB1 with a focus on the lateral (A) and the dorsal (B) side of the cell. OC – oral cavity, C – cytoproct.

The third isolate (Figure 2.5. and 2.6.) is a pear- to an ovoid-shaped cell with an average size of 50 x 30 µm. Both the silver staining and SEM revealed a pronounced oral morphology that is typical of species of the genus *Tetrahymena*; that is a shallow oral cavity (OC) that lies toward the anterior end and is composed of three rows of ciliated basal bodies making up the adoral membranelles (AM), which are parallel to each other and lies on the left and undulating membranelle (UM) on the right of the

cell. While the anterior end of the cell is bluntly pointed, the posterior end is more rounded. The body is covered with short hair-like cilia that are evenly spaced over the cell surface with rows of kinetosomes (K) that lie parallel to the longitudinal of the cell. The cytoproct (C) is located at the posterior end of the ventral body surface. This isolate is highly motile and moves by rotation in an anticlockwise direction.

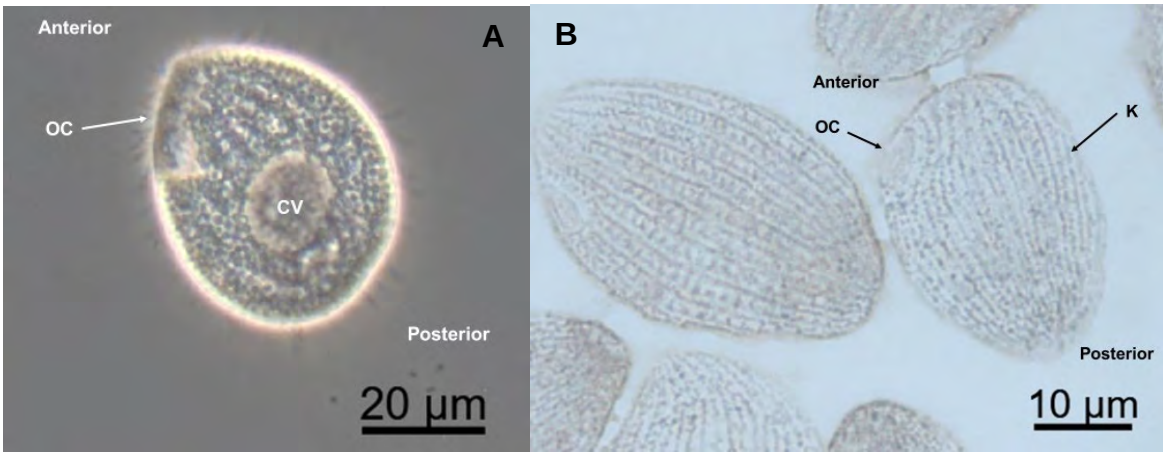

Figure 2.5. Micrographs of typical cells of isolate RB2. Phase-contrast image of a live cell (A) and silver-stained cells (B) of isolate RB2. OC – oral cavity, CV – contractile vacuole, K – kinetosome.

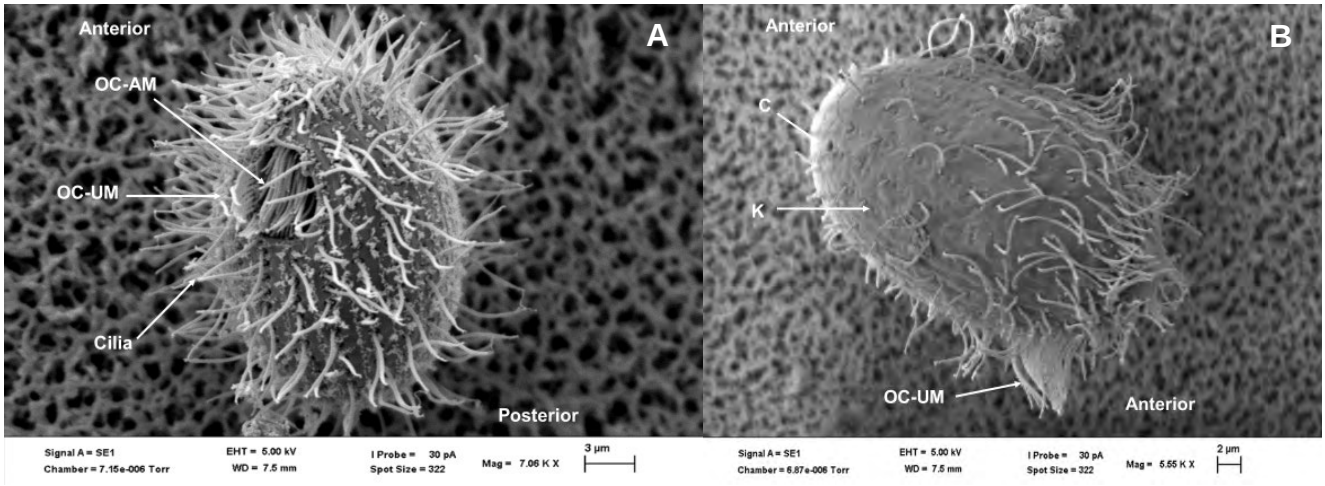

Figure 2.6. SEM micrographs of cells of isolate RB2 with focus on the ventral (A) and dorsal lateral side (B). OC-AM – oral cavity - adoral membranelle, OC-UM – undulating membranelles, K – kinetosome, C – cytoproct.

2.3.2. 18S rRNA gene sequence analysis of the isolated ciliate species

The eukaryotic small subunit ribosomal RNA gene is the most frequently used in the phylogenetic studies of eukaryotes. It is an excellent marker as it is sufficiently large and has both conserved and variable regions, which are useful for classification and identification. Therefore, the DNA extracted from ciliates was amplified using primers that amplify the fragment of DNA encoding the 18S rRNA gene.

The analysis of the PCR products obtained on agarose gel revealed that the size of the amplicons was the expected size of ~1,3 kbp (primer set 1 - P-SSU 342f and Medlin B) and ~1.8kbp (primer set - EukFor and EukRev) for the two ciliates analyzed (Figure 2.7.).

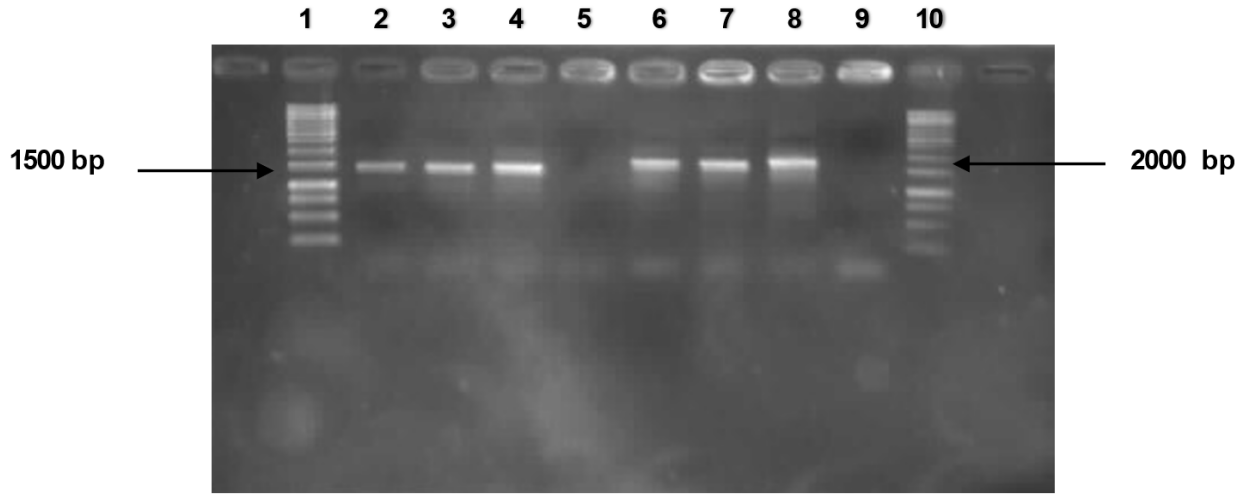

Figure 2.7. Agarose gel electrophoresis of amplified 18S rRNA gene from isolated ciliates. Lane 1 – 1 kb ladder, Lane 2 – RB1, Lane 3 - RB2, Lane 4 – RB1, Lane 5 – negative control amplified with primer set 1 (PSSU-342F and Medlin B), Lane 6 – RB1, Lane 7 - RB2, Lane 8 – RB2, Lane 9 – negative control amplified with primer set 2 (EukFor and EukRev), Lane 10 – 1 kb ladder.

The resulting sequences were compared with the 18S rRNA gene sequence of closely related species of the phylum *Ciliophora* published in NCBI GenBank using the Basic Local Alignment Search Tool (Blast, www.ncbi.nim.nih.gov). The best matches based on the sequence and the E-value from the database are listed in Table 2.2. and 2.3.

Table 2.2. Best 4 matching sequences for isolate RB1 obtained from NCBI blast, accessed on 31 May 2021

Accession Number	Organism	E - value	Max score	Identity
AB252007.1	*Paramecium multimicronucleatum* gene for 18S ribosomal RNA, partial sequence, strain: YM25	0.0	3011	100%
MK595741.1	*Paramecium multimicronucleatum* strain US_Bl 16I1 small subunit ribosomal RNA gene, partial sequence	0.0	2983	99.69%
AB252006.1	*Paramecium multimicronucleatum* gene for 18S ribosomal RNA, partial sequence, strain: TH105	0.0	2983	99.69%
MN198111.1	*Paramecium multimicronucleatum* isolate RJ1 small subunit ribosomal RNA gene, partial sequence	0.0	2968	99.51%

Table 2.3. Best 4 matching sequences for isolate RB2 obtained from NCBI blast, accessed on 31 May 2021

Accession number	Organism	E - value	Max score	Identity
AF364041.1	*Tetrahymena setosa* 18S small subunit ribosomal RNA gene, complete sequence	0.0	3009	100%
X56171.1	*T. pyriformis* gene for small subunit ribosomal RNA (16S like rRNA)	0.0	3009	100%
MT012299.1	*Tetrahymena* sp. isolate 41 small subunit ribosomal RNA gene, partial sequence	0.0	3003	100%
AF330216.1	*Tetrahymena* aff. *pyriformis* small subunit ribosomal RNA gene, complete sequence	0.0	2988	100%

Comparison of the resulting 18S rRNA gene sequences of the isolated ciliates RB1 and RB2 to 18S rRNA gene sequences of members of the phylum *Ciliophora* published in NCBI GenBank gave a similarity level of more than 99%, which is regarded as sufficient to assign isolates to genus level. Therefore, isolate RB1 is a member of the genus *Paramecium* with 100% similarity to *Paramecium multimicronucleatum* (Table 2.2.), and isolate RB2 is a member of the genus *Tetrahymena* with 100% similarity to *Tetrahymena setosa* (Table 2.3.).

Due to the Covid-19 pandemic and campus access restriction, the isolate RB3 was lost and was therefore not analyzed. However, the morphological characteristics were sufficient to identify and classify the isolate to the genus level of *Coleps*.

The 18S rRNA gene sequences for strain RB1 and strain RB2 were deposited in GenBank (www.ncbi.nlm.nih.gov) under accession no. MT975687 and MT975644, respectively.

To determine the genetic relatedness of the isolated ciliates with other ciliate species, phylogenetic trees were constructed with MEGA X (Kumar *et al.,* 2018) using the 18S rRNA gene sequences of the isolated ciliates and 18S rRNA gene sequence of the ciliates available in the NCBI database.

The phylogenetic tree revealed that the isolated ciliates are closely related with the species of class *Oligohymenophorea*, phylum *Ciliophora.* While strain RB1 was grouped with species of the genus *Paramecium* and formed a monophyletic unit with *Paramecium multimicronucleatum* (Figure 2.8.), strain RB2 was grouped with species of the member "*Tetrahymena borealis"* group and was closely related to *Tetrahymena pyriformis* (Figure 2.9.).

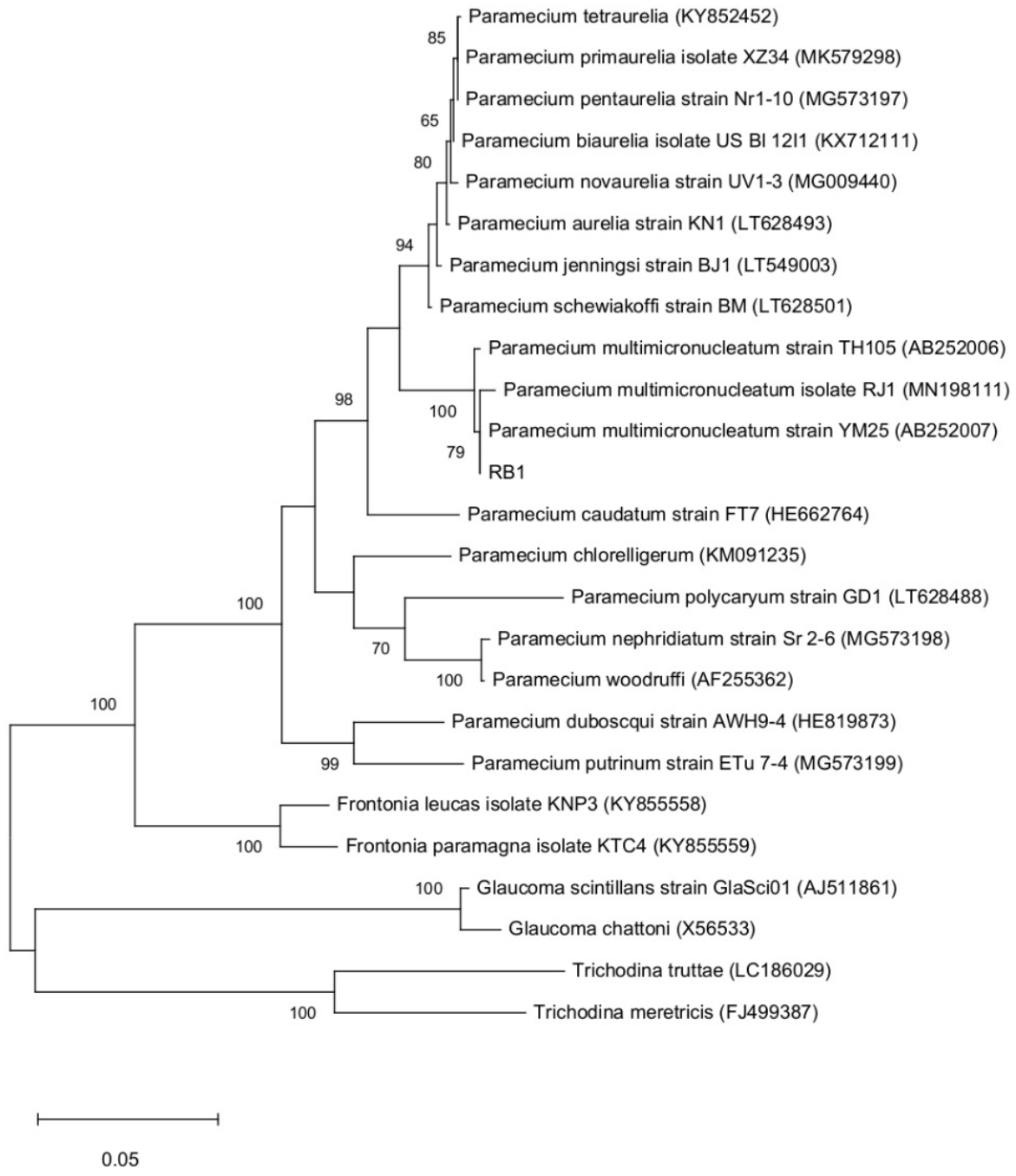

Figure 2.8. Phylogenetic assignment of *Paramecium* sp. RB1 using a maximum likelihood model based on the 18S rRNA gene sequence of the two isolates in comparison with 18S rRNA gene sequences of selected species of the genus *Paramecium* published in GenBank (www.ncbi.nlm.nih.gov). Only bootstrap values of ≥50% of 500 replicates are shown. *Frontonia* spp., *Glaucoma* spp., and *Trichodina* spp. were used as outgroups. The scale bar corresponds to 5 changes per 100 nucleotides.

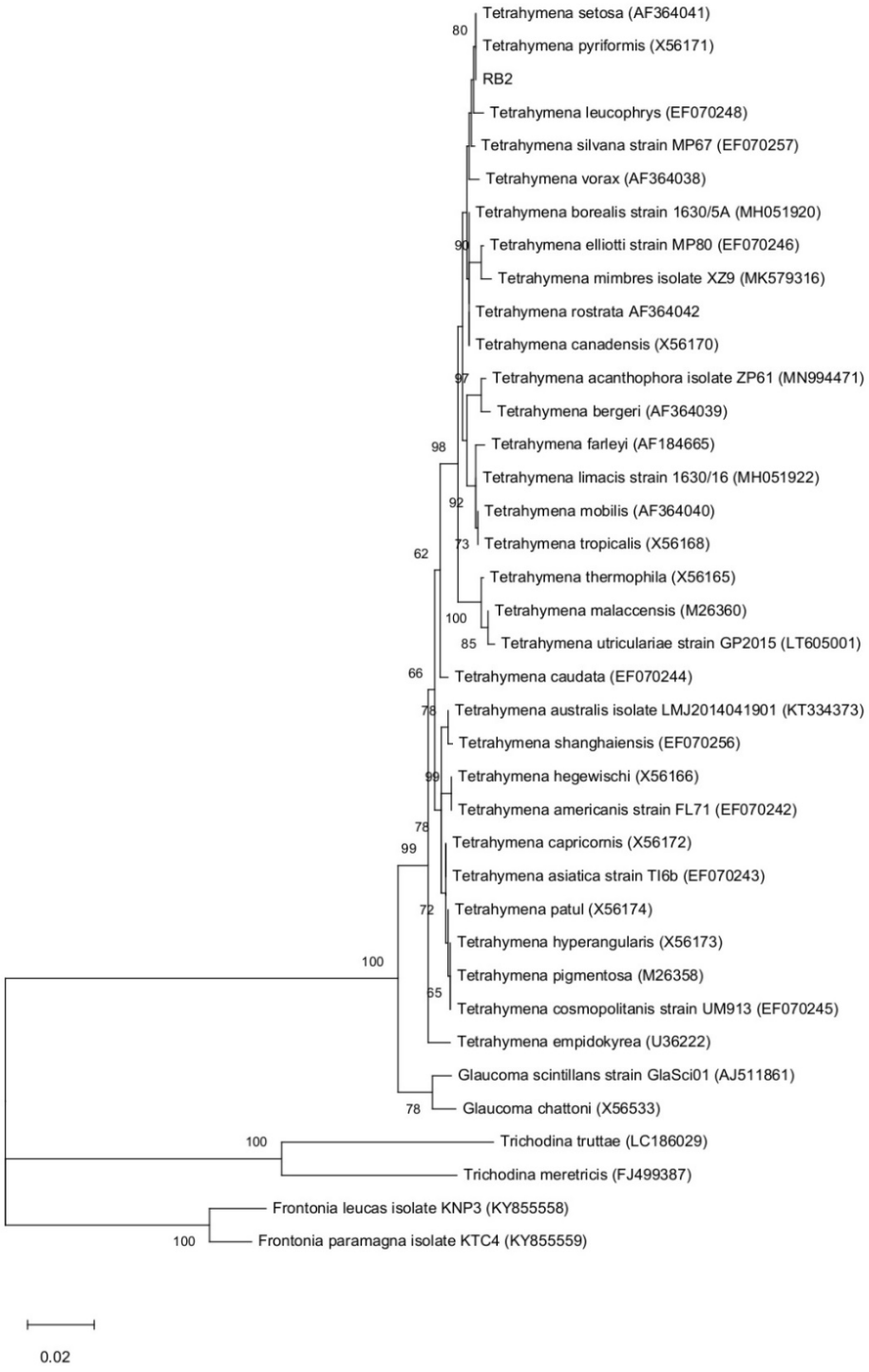

Figure 2.9. Phylogenetic assignment of *Tetrahymena* sp. RB2 using a maximum likelihood model based on the 18S rRNA gene sequence of the two isolates in comparison with 18S rRNA gene sequences of selected species of the genus *Tetrahymena* published in GenBank (www.ncbi.nlm.nih.gov). Only bootstrap values of ≥50% of 500 replicates are shown. *Frontonia* spp., *Glaucoma* spp., and *Trichodina* spp. were used as outgroups. The scale bar corresponds to 2 changes per 100 nucleotides.

2.3.3. Growth and grazing studies of the isolated ciliates

Bacterivorous protists play a significant role in regulating and controlling bacterial biomass in aquatic ecosystems. Ciliates are considered important bacterial grazers and account for more than half of bacterial mortality in freshwater environments (Weisse, 2017). Therefore, predator-prey interaction of isolated ciliates was studied with various microbial species to determine their growth and grazing characteristics.

2.3.3.1. Growth and grazing studies of *Coleps* sp. RB3

The response of *Coleps* sp. RB3 to prey concentrations

Prey concentrations determine the ingestion rates of ciliates, which ultimately determines their growth rate. There is a minimum prey cell concentration where ingestion rates do not offset metabolism; therefore, growth is less or equal to zero. This threshold concentration varies from species to species. Thus, the influence of bacterial prey concentrations on *Coleps* sp. RB3 was studied with *E. coli* ATCC 8739.

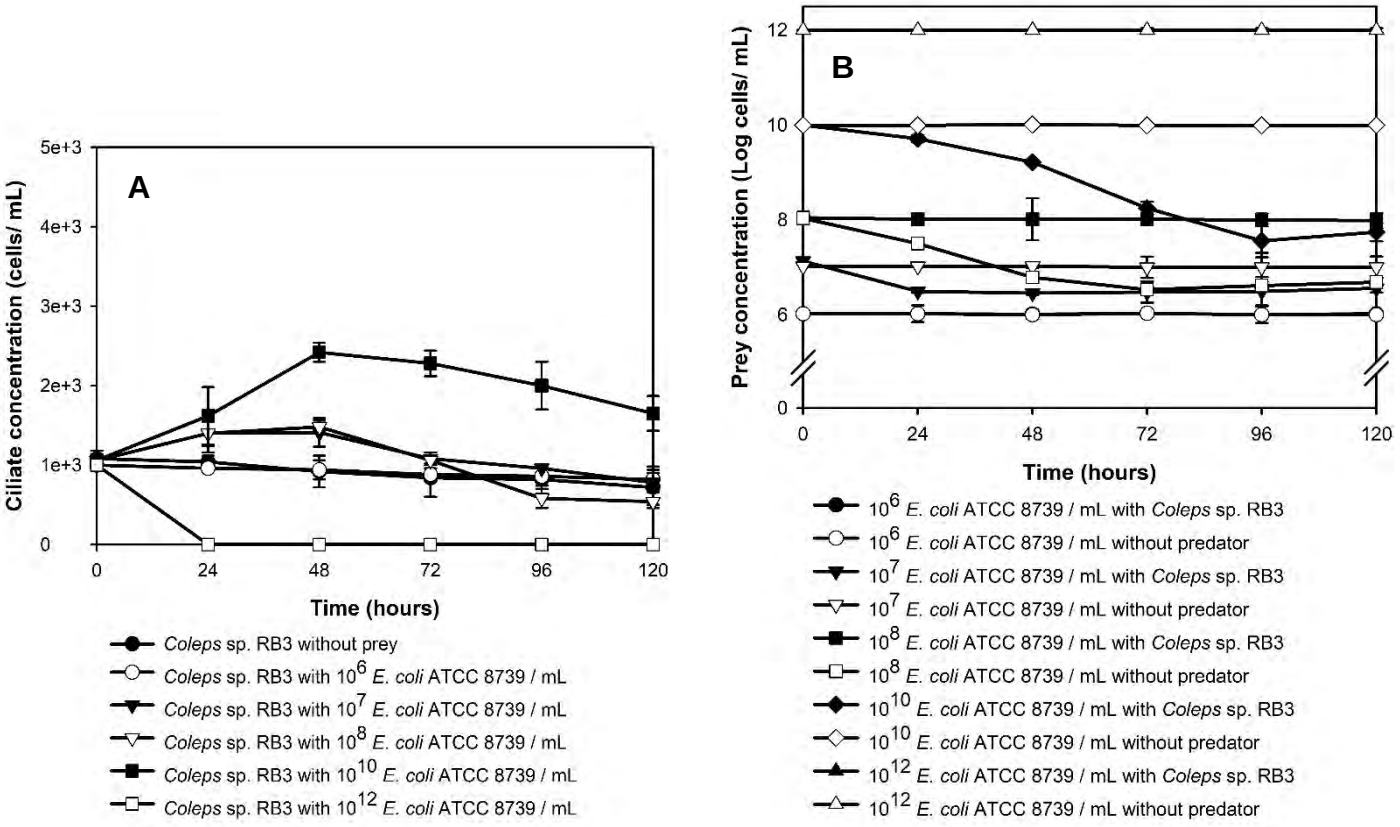

Figure 2.10. Growth of *Coleps* sp. RB3 with *E. coli* ATCC 8739 (A) and change of *E. coli* ATCC 8739 in the presence and absence of predator (B) at different prey concentrations of *E. coli* ATCC 8739 in Chalkley's medium incubated at 25 ± 2ºC in the dark. All data are the means of two independently performed experiments. Error bars represent the difference between the means.

Growth of *Coleps* sp. RB3 was observed at *E. coli* ATCC 8739 concentrations from 10^7 to 10^{10} bacteria x mL^{-1}. Maximum growth was achieved at 10^{10} bacteria x mL^{-1}, and ciliate growth increased with a decrease in prey concentrations over time. A prey concentration of 10^{12} bacteria x mL^{-1} caused the death of the ciliate cells. *Coleps* sp. RB3 had an exponential growth that lasted for 48 hours at 10^8 and 10^{10} bacteria x mL^{-1}, declining once the bacterial concentration had reached 10^7 bacteria x mL^{-1}. The reduction of *E. coli* ATCC 8739 cells was solely due to the presence of *Coleps* sp. RB3 and was observed from a concentration of 10^7 to 10^{10} bacteria x mL^{-1}; thus, these concentrations were required to induce ingestion. Therefore, the threshold concentration to support ingestion and growth for *Coleps* sp. RB3 was 10^7 bacteria x mL^{-1} under the experimental conditions used.

The experimental data generated were used to establish the growth kinetics and grazing characteristics of *Coleps* sp. RB3 when feeding on *E. coli* ATCC 8739 at different concentrations.

Table 2.4. Growth and grazing kinetics of *Coleps* sp. RB3 growing with *E. coli* ATCC 8739 at different concentrations in Chalkley's medium incubated at 25 ± 2ºC in the dark

Bacteria x mL^{-1}	Specific growth rate (hr^{-1})	Generation time (hr)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
10^6	0	0	0	0
10^7	0.0060	141.38	$2.10 \times 10^2 – 1.58 \times 10^2$	15.53
10^8	0.0070	102.98	$2.00 \times 10^3 – 1.43 \times 10^3$	18.57
10^{10}	0.0172	40.61	$1.65 \times 10^5 – 7.20 \times 10^4$	16.44
10^{12}	-	-	0	0

The values presented are the means of two independently performed experiments. (-) indicate that ciliate cells were not detected in the cultures after 24 hours of incubation. Grazing rates were corrected for the increased number of individual cells during exponential growth.

Growth and the grazing rates of the isolated ciliate of *Coleps* sp. RB3 were detected from *E. coli* ATCC 8739 concentrations of 10^7 to 10^{10} bacteria x mL^{-1}, which increased with an increase in prey concentrations. The highest growth and grazing rates were detected at 10^{10} bacteria x mL^{-1}, while no growth and grazing were detected at 10^6 and 10^{12} bacteria x mL^{-1}.

In addition to the change in total cell counts, the impact of predation on the viability of bacteria in the presence of *Coleps* sp. RB3 was determined by the plate count method.

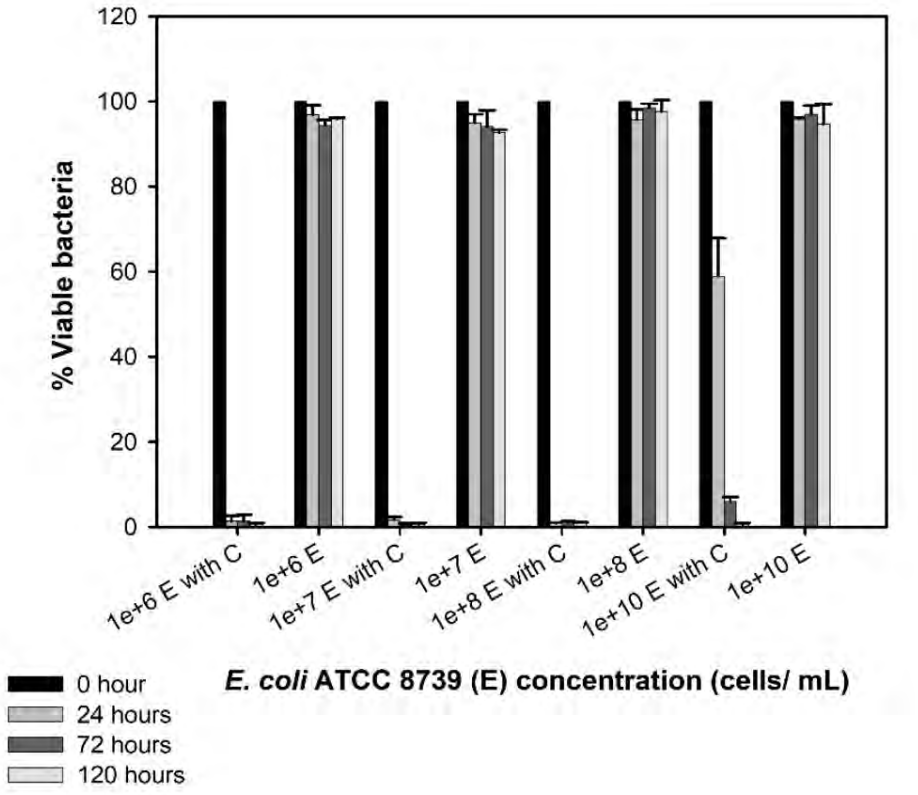

Figure 2.11. The change in viable counts (% CFU x mL^{-1}) of *E. coli* ATCC 8739 (E) at different bacterial concentrations in the presence of *Coleps* sp. RB3 (10^3 cells x mL^{-1}) (C) and absence of predator in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between the means.

The grazing activity of *Coleps* sp. RB3 resulted in more than 90% reduction of viable *E. coli* ATCC 8739 from the concentration of 10^6 to 10^{10} bacteria x mL$^{-1,}$ while in the absence of this ciliate, no reduction of CFU x mL^{-1} was observed. As with the total bacterial counts in Figure 2.10., the decrease of viable *E. coli* ATCC 8739 was solely due to the grazing of the *Coleps* ciliate isolate.

Growth of *Coleps* sp. RB3 on various bacterial species

Different species of ciliates consume different bacterial species at different efficiency; while some bacterial species are preferred food prey, some species are rejected and may not serve as a food source for the growth of ciliates. Similarly, some bacteria have the ability to resist ingestion as well as digestion and may even cause the death of ciliates. Therefore, the grazing of *Coleps* sp. RB3 on various bacterial species was studied to determine the grazing rate and nutritional quality of various bacteria.

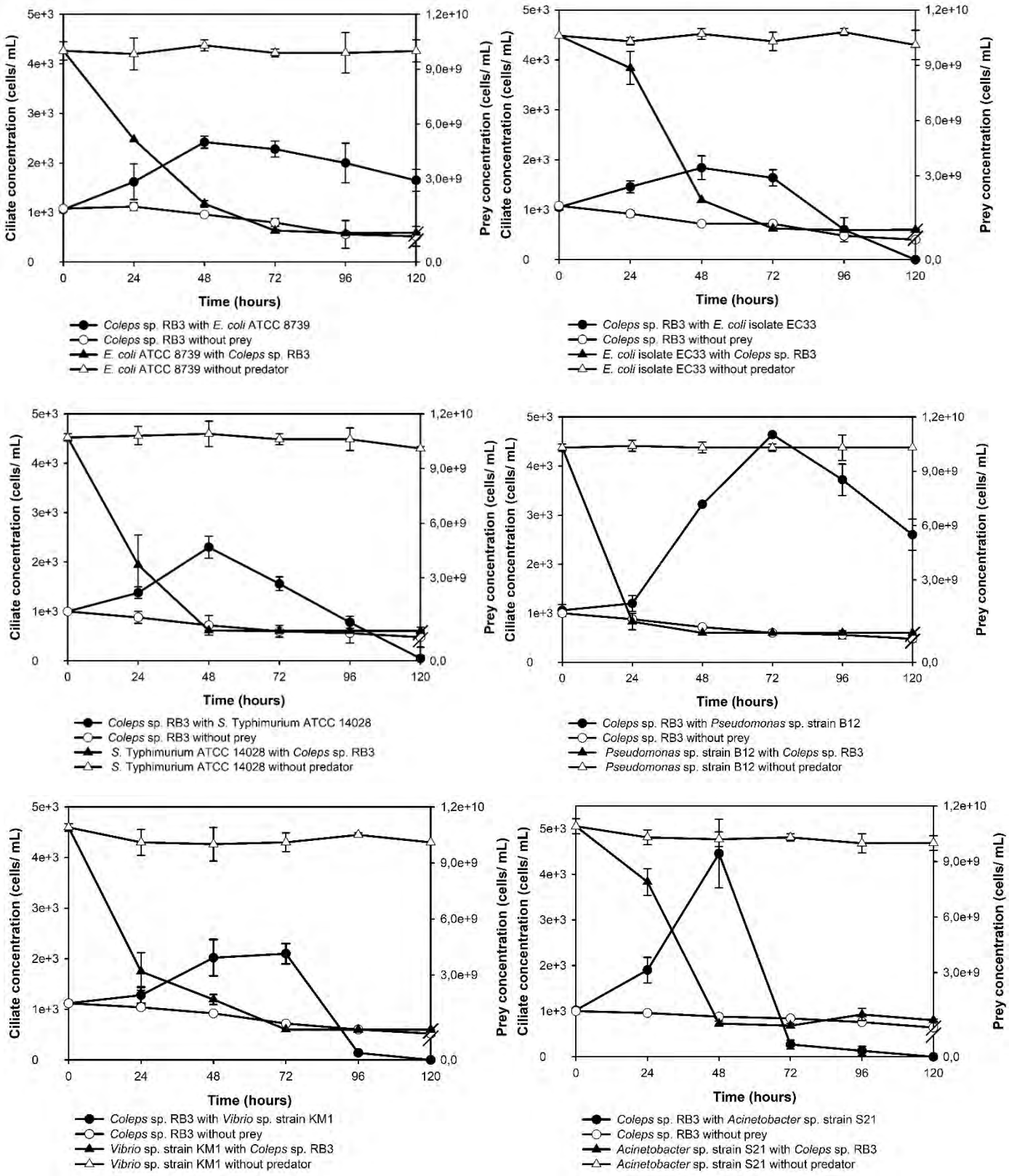

Figure 2.12. Growth of *Coleps* sp. RB3 grazing on various Gram-negative bacteria (10^{10} bacteria x mL^{-1}) in Chalkley's medium incubated at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between the means.

Growth of the isolated ciliate *Coleps* sp. RB3 was solely due to the presence of bacterial prey cells, which increased with a concomitant decrease in prey cell numbers when feeding on selected Gram-negative bacteria. Growth of the ciliate was not detected in the absence of bacterial prey. Though *Coleps* sp. RB3 was able to grow with all Gram-negative bacteria tested, the population increase of this ciliate varied with each bacterial species. The highest growth was observed with *Pseudomonas* sp. strain B12 and *Acinetobacter* sp. strain S21. The growth of *Coleps* sp. RB3 was lower *E. coli* isolate EC33, *Salmonella* Typhimurium ATCC 14028, *Vibrio* sp. strain KM1, and *Acinetobacter* sp. strain S21 causing a complete disappearance of *Coleps* sp. RB3 cells from the culture once the concentration of bacterial prey was below 10^8 bacteria x mL^{-1}. The grazing of *Coleps* sp. RB3 resulted in a more than 90% reduction of bacterial prey cells in the culture.

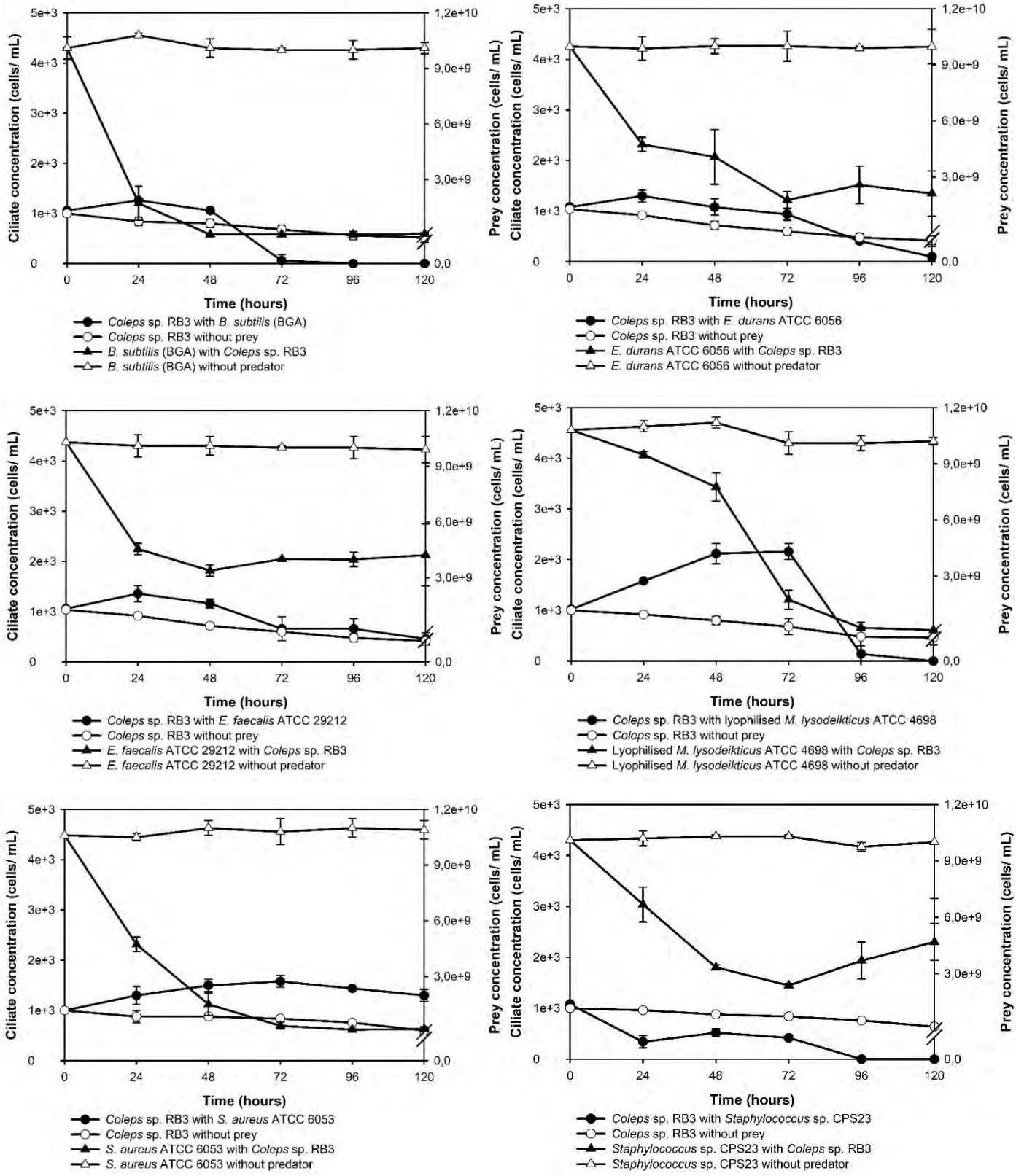

Figure 2.13. Growth of *Coleps* sp. RB3 grazing on various Gram-positive bacteria (10^{10} bacteria x mL^{-1}) in Chalkley's medium incubated at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between the means.

Growth of the isolated ciliate *Coleps* sp. RB3 was observed even though to a lesser degree than with Gram-negative bacterial prey when provided with selected Gram-positive bacteria except with the antibiotic-resistant strain *Staphylococcus* sp. CPS23, where the population of *Coleps* sp. RB3 declined. Prey concentrations for all bacterial species tested declined in the presence of ciliate, with the highest growth observed with lyophilized cells of *Micrococcus lysodeikticus* ATCC 4698. Consumption of bacteria by *Coleps* sp. RB3 resulted in more than 90% reduction of bacterial cells except for *Staphylococcus* sp. CPS23 and *E. faecalis* ATCC 29212 cells were reduced to about 50%, and *E. durans* ATCC 6056 reduced to about 70%. The decline in the *Coleps* sp. RB3 numbers resulted in the increase in *Staphylococcus* sp. CPS23 cell numbers this probably due to the utilization of cellular content from lysed ciliate cells by bacteria.

Growth of *Coleps* sp. RB3 on various eukaryote species

Ciliate species in aquatic ecosystems are mainly bacterivorous; however, some have been described to possess more than one feeding strategy as they can feed additionally on other microscopic microbial organisms. Therefore, the ability of *Coleps* sp. RB3 to feed on selected eukaryotes (i.e., algal and fungal species) was determined.

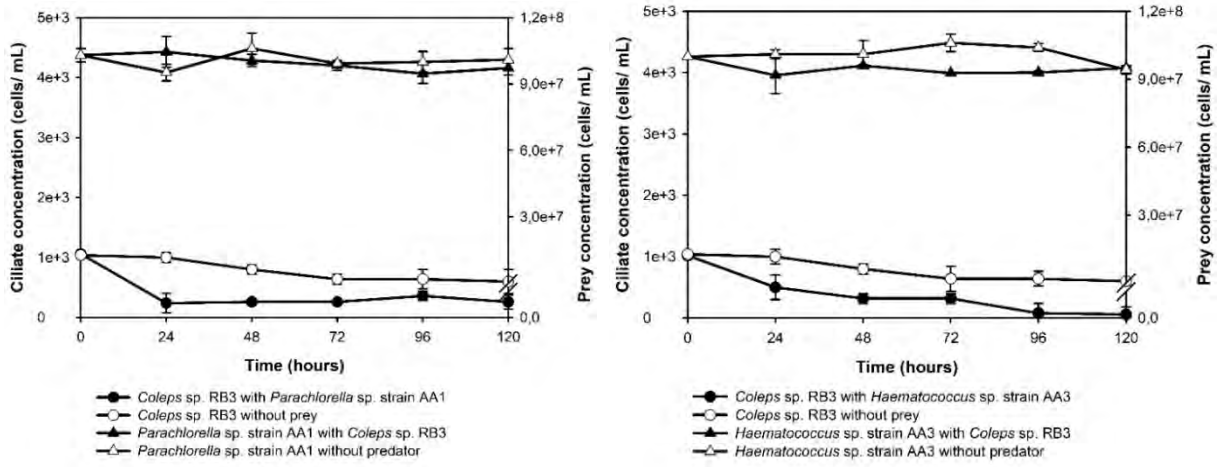

Figure 2.14. Growth of *Coleps* sp. RB3 with two algal species, *Parachlorella* sp. strain AA1 and *Haematococcus* sp. strain AA3 (10^8 cells x mL^{-1}) in Chalkley's medium incubated at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between the means.

Grazing on the two algal species tested did not result in the growth of *Coleps* sp. RB3 but rather resulted in a decline in the ciliate cell numbers. The decline of ciliate cells in the presence of algal cells was more rapid than in the absence of algal cells. The concentrations of both algal cells were unchanged throughout the incubation period, both in the presence and absence of the ciliate predator.

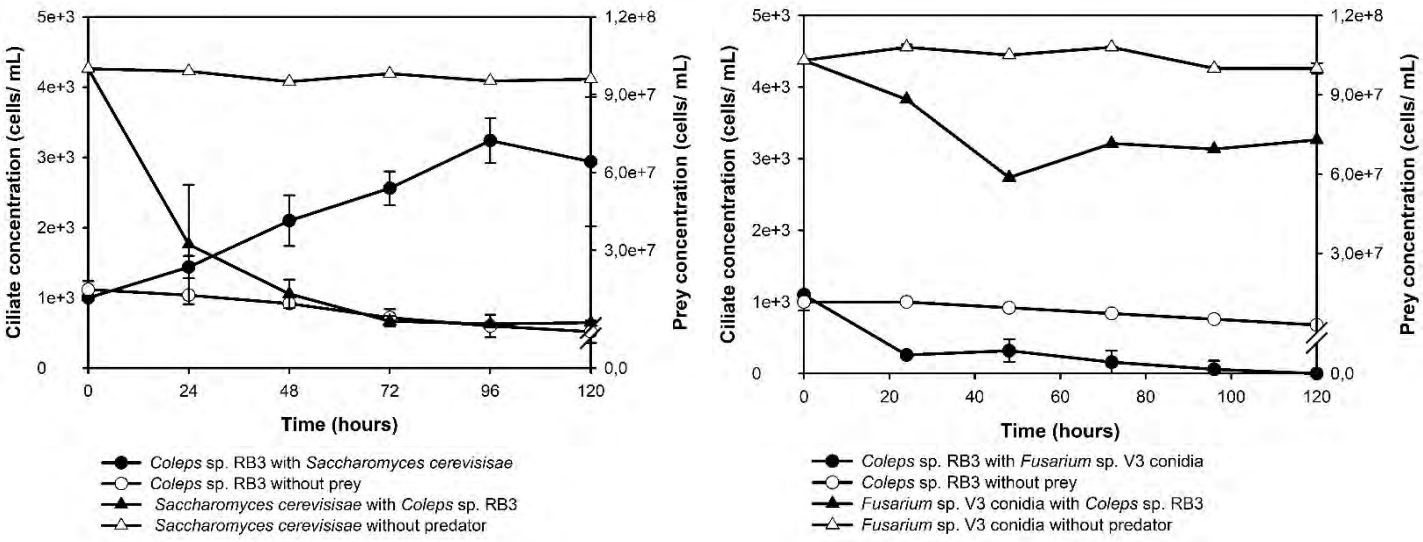

Figure 2.15. Growth of *Coleps* sp. RB3 with two fungal species, *Saccharomyces cerevisiae* and spores of *Fusarium* sp. V3 in Chalkley's medium incubated at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between the means.

The isolate *Coleps* sp. RB3 consumed yeast cells, and its growth resulted in a 98% reduction of yeast cells. Although the presence of *Coleps* sp. RB3 resulted in a 24% reduction of *Fusarium* sp. V3 conidia, which was not observed in the absence of the ciliate. The presence of fungal spores did not cause the growth of the ciliate but rather a decline in the ciliate cell numbers.

The experimental data generated were used to establish the growth kinetics and grazing characteristics of *Coleps* sp. RB3 when feeding on various microbial organisms.

Table 2.5. Growth and grazing kinetics of *Coleps* sp. RB3 with various microbial prey species in Chalkley's medium incubated at 25 ± 2ºC in the dark

Prey	Specific Growth rate (hr^{-1})	Generation time (hr)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
Gram-negative bacteria (10^{10} cells x mL^{-1}):				
E. coli ATCC 8739	0.0172	40.61	$1.65 \times 10^5 – 7.20 \times 10^4$	16.44
E. coli isolate EC33	0.0117	61.51	$1.77 \times 10^5 – 9.97 \times 10^4$	16.69
Pseudomonas sp. strain B12	0.0282	23.89	$1.35 \times 10^5 – 3.06 \times 10^4$	13.05
S. Typhimurium ATCC 14028	0.0172	41.31	$2.20 \times 10^5 – 9.71 \times 10^4$	20.65
Vibrio sp. strain KM1	0.0122	58.55	$1.71 \times 10^5 – 9.60 \times 10^4$	15.73
Acinetobacter sp. strain S21	0.0307	22.58	$2.18 \times 10^5 – 5.02 \times 10^4$	19.97
Gram-positive bacteria (10^{10} cells x mL^{-1}):				
Staphylococcus sp. CPS23	-	-	9.94×10^4	9.83
S. aureus ATCC 6538	0.0064	110.71	$1.43 \times 10^5 – 9.02 \times 10^4$	13.44
E. faecalis ATCC 29212	0.0104	85.77	$2.27 \times 10^5 – 1.77 \times 10^5$	22.07
E. durans ATCC 6056	0.0077	96.97	$1.99 \times 10^5 – 1.66 \times 10^5$	20.09
Lyophilized *Micrococcus lysodeikticus* ATCC 4698	0.0152	45.98	$6.21 \times 10^4 – 3.01 \times 10^4$	5.75
B. subtilis BGA	0.0072	101.64	$3.69 \times 10^5 – 3.11 \times 10^5$	33.27
Algae (10^8 cells x mL^{-1}):				
Haematococcus sp. strain AA3	-	-	4.14×10^1	0.41
Parachlorella sp. strain AA1	-	-	4.50×10^1	0.80
Fungi (10^8 cells x mL^{-1}):				
Saccharomyces cerevisiae	0.0121	57.25	$1.03 \times 10^3 – 3.17 \times 10^2$	10.25
Fusarium sp. V3 spores	-	-	6.48×10^2	4.87

All data are the average of two independently performed experiments. (-) indicates a decline of ciliate cell numbers. Grazing rates were corrected for the increased number of individual cells number during exponential growth.

Coleps sp. RB3 was able to consume all bacterial prey tested with up to a 90% reduction of prey cells for all Gram-negative bacterial species. However, growth was detected with all except the antibiotic-resistant *Staphylococcus* sp. CPS23, and the growth rates of *Coleps* sp. RB3 varied with each bacterial species tested. Growth rates were higher for most Gram-negative bacteria in comparison with Gram-positive bacteria. The highest growth was observed when grazing on a non-motile Gram-negative *Acinetobacter* sp. strain S21, while the Gram-positive non-motile *S. aureus* ATCC 6538 enabled only slow growth of the isolate. Although growth rates varied for each bacterial species tested, the grazing and clearance rates were within a similar range of $>10^4$ to 10^5 bacteria per hour and 12 to 20 nL per hour for individual cells of *Coleps* sp. RB3. Of the eukaryotic organisms selected, only yeast cells supported the growth of *Coleps* sp. RB3 with grazing rates of $<10^3$ prey per hour for individual ciliate cells.

The response of *Coleps* sp. RB3 to prey cell fluctuations

Freshwater environments are characterized by fluctuations of bacterial prey abundance; thus, bacterivorous ciliates inhabiting such environments are subjected to such intermittent changes in prey availability. When the population of prey is abundant, the predator population increases, which then decreases once the prey population becomes limited. The response of *Coleps* sp. RB3 to prey fluctuation and its ability to recover from starvation was determined with *E. coli* ATCC 8739, and the results are presented below.

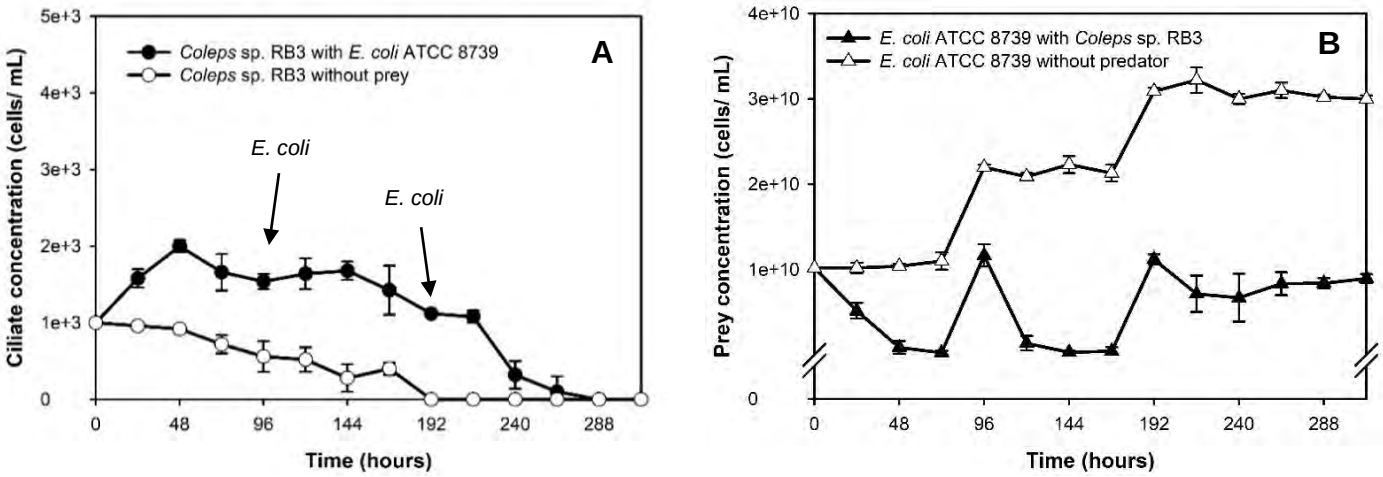

Figure 2.16. Growth of *Coleps* sp. RB3 with *E. coli* ATCC 8739 (A) and the change in *E. coli* ATCC 8739 in the presence and absence of *Coleps* sp. RB3 (B) after the addition of fresh bacterial fodder at 10^{10} bacteria x mL^{-1} at 0, 96, and 192 hours in Chalkley's medium at 25 ± 2ºC in the dark. All data are the means of two independently performed experiments. Error bars represent the difference between the means.

The cell numbers of *Coleps* sp. RB3 increased, causing a decrease in bacterial concentrations until the prey concentration was below 10^8 bacteria x mL^{-1}. Upon the second addition of prey *E. coli* ATCC 8739, *Coleps* sp. RB3 increased slightly and started to decrease once the bacterial concentration had reached less than 10^8 bacteria x mL^{-1}. However, the third addition of *E. coli* ATCC 8739 did not cause an increase in the ciliate cell numbers; instead, the population remained stationary for 24 hours and then declined with a slight decrease in prey cell numbers.

Influence of particle size on the grazing of *Coleps* sp. RB3

Particle size has been shown to play a significant role in determining the ingestion rates of ciliates. Ciliates mainly select their prey based on the particle size, and there is a preferred size range which the ciliate can ingest (Jonsson, 1986). Therefore, the responses of *Coleps* sp. RB3 to varying particle sizes were studied using microspheres of different sizes. The results obtained are presented in Table 2.6.

Table 2.6. Grazing of *Coleps* sp. RB3 on polystyrene microbeads of different sizes in Chalkley's medium at 25 ± 2ºC in the dark

Particle diameter (µm)	Grazing rate (particles x pred^{-1} x hr^{-1})	Clearance rate (nL x pred^{-1} x hr^{-1})
2	1.13 x 10^3	11.30
5	9.49 x 10^2	9.92
10	0	0

All data are the means of two independently performed experiments.

The grazing of *Coleps* sp. RB3 was only detected with microbeads of size 2 and 5 µm diameter. The grazing and clearance rates were highest for smaller-sized microbeads and decreased with increasing particle size.

Influence of temperature on growth and grazing rates of *Coleps* sp. RB3

The temperature has long been recognized as an important factor affecting biological processes, consequently influencing population abundance and productivity. The response of protists to temperature has been generally studied by observing the growth rates. It is assumed that growth rates increase with an increase in temperature within the physiological range. Therefore, the growth response of *Coleps* sp. RB3 was evaluated with *E. coli* ATCC 8739 at varying temperatures.

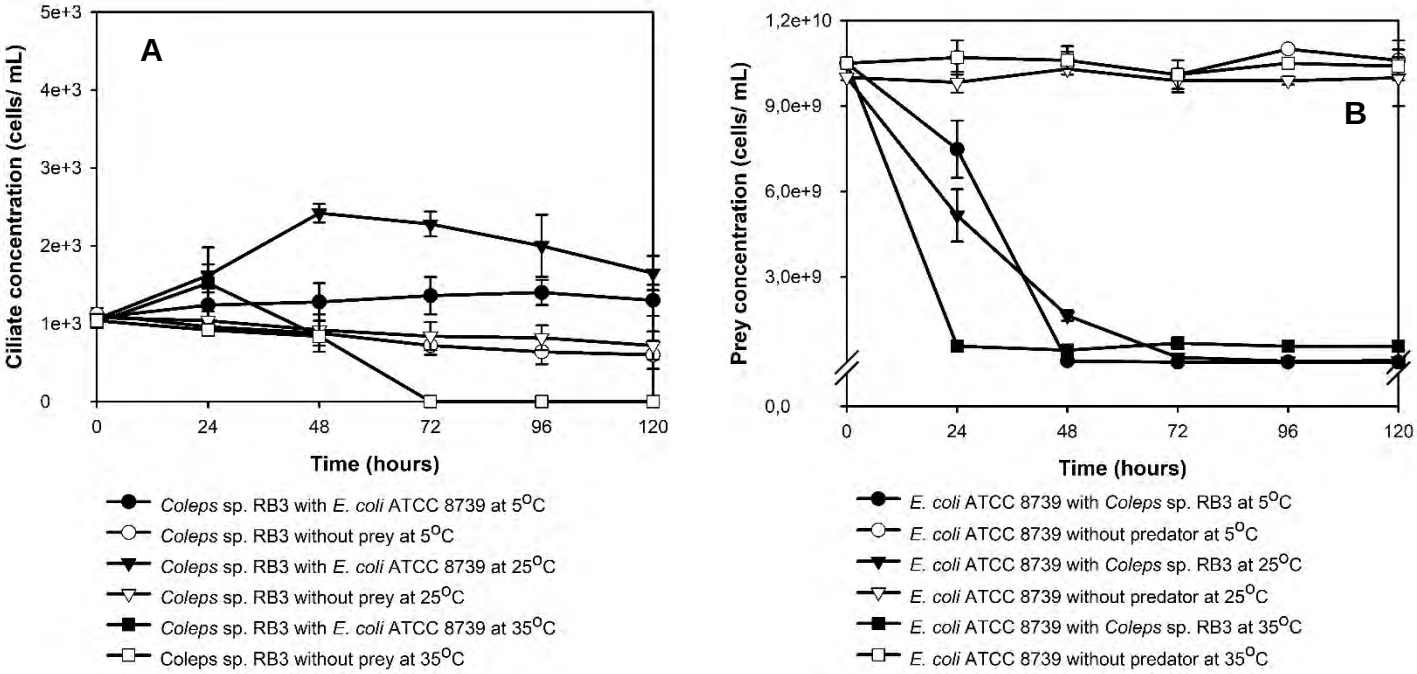

Figure 2.17. Growth of *Coleps* sp. RB3 with *E. coli* ATCC 8739 (A) and the change in *E. coli* ATCC 8739 concentrations in the presence and absence of *Coleps* sp. RB3 (B) at a temperature of 5, 25, and 35ºC in Chalkley's medium in the dark. All data are the means of two independently performed experiments. Error bars represent the difference of the means.

Growth of *Coleps* sp. RB3 was observed at all three temperatures tested, with the highest growth observed at 25ºC. *Coleps* sp. RB3 grew up to 24 hours at 35ºC, with no cells of *Coleps* sp. RB3 present in the culture after 48 hours of incubation. There was a rapid decline in prey concentration at 35ºC in the presence of the ciliate, while at 5 and 25ºC, the decline lasted over 48 hours.

The experimental data generated were used to establish growth and grazing characteristics of *Coleps* sp. RB3 at different temperatures.

Table 2.7. Growth and grazing kinetics of *Coleps* sp. RB3 with *E. coli* ATCC 8739 at different temperatures in Chalkley's medium in the dark

Temperature (ºC)	Specific growth rate (hr^{-1})	Generation time (hr)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
5	0.0048	155.82	$1.01 \times 10^5 – 9.39 \times 10^4$	9.64
25	0.0172	40.61	$1.65 \times 10^5 – 7.20 \times 10^4$	16.44
35	0.0132	46.37	$4.06 \times 10^5 – 2.74 \times 10^5$	38.65

The data presented are the means of two independently performed experiments. Grazing rates were corrected for the increased number of individual cells number during exponential growth.

The growth rates of *Coleps* sp. RB3 clearly increased from 5ºC to 25ºC and decreased between the temperature of 25ºC and 35ºC. While the clearance rates increased by a factor of 2 with an increase in temperature, the grazing rates increased slightly from 5ºC and 25ºC but were substantially higher at 35ºC.

2.3.3.2. Growth and grazing studies of *Paramecium* sp. RB1

The predator-prey interaction between the isolated ciliate *Paramecium* sp. RB1 and various microbial prey were studied to determine the growth and grazing characteristics and prey selectivity of this isolate, as well as the nutritional quality of various microorganisms.

The response of *Paramecium* sp. RB1 to different prey concentrations

The influence of prey concentration on the growth of *Paramecium* sp. RB1 was determined with different concentrations of prey (*E. coli* ATCC 8739).

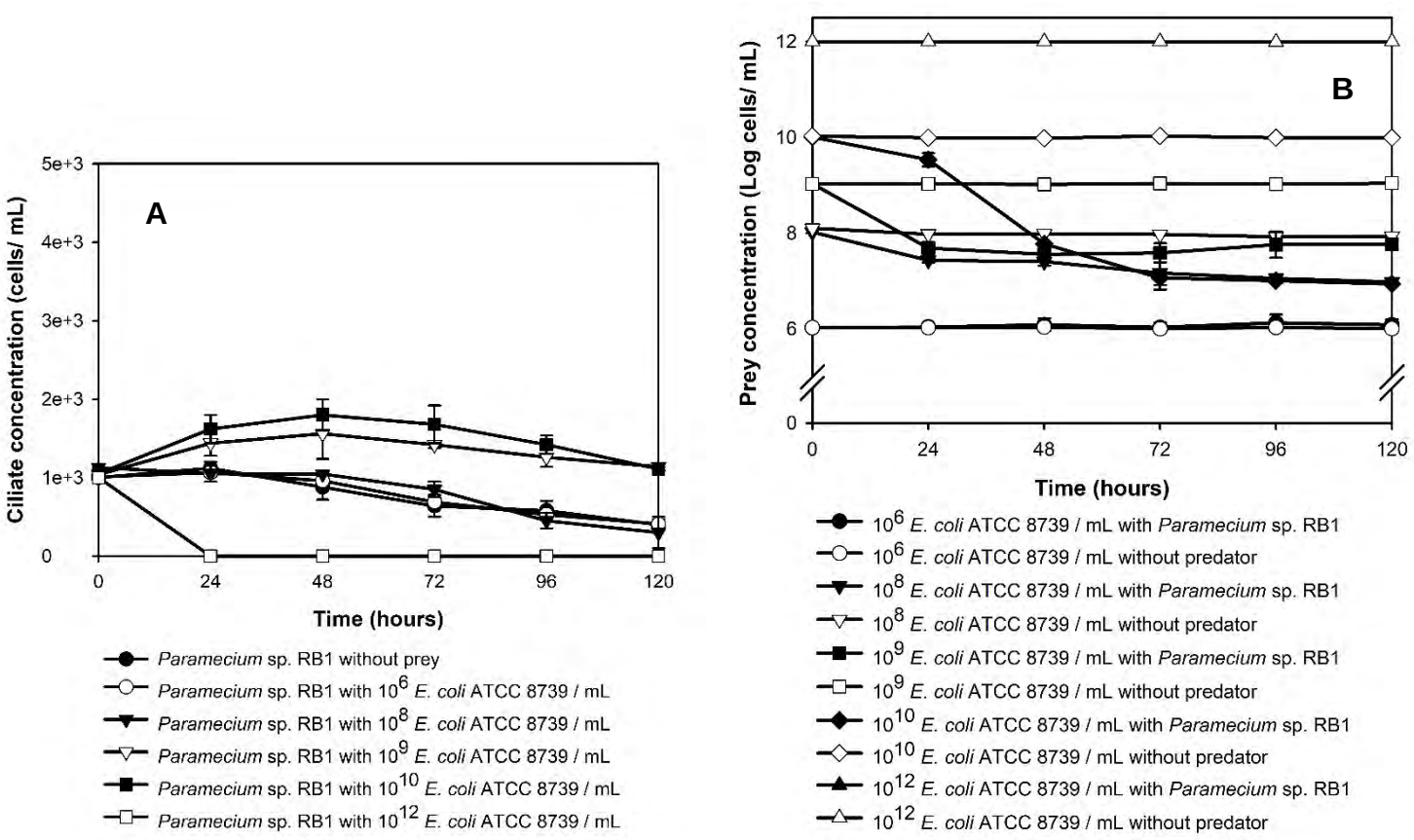

Figure 2.18. Growth of *Paramecium* sp. RB1 with *E. coli* ATCC 8739 (A) and the change of *E. coli* ATCC 8739 in the presence or absence of *Paramecium* sp. RB1 (B) at different prey concentrations of *E. coli* ATCC 8739 in Chalkley's medium at 25 ± 2ºC in the dark. The values are the means of two independently performed experiments. Error bars represent the difference between means.

Growth of *Paramecium* sp. RB1 was observed in the presence of *E. coli* ATCC 8739 from the concentration of 10^9 bacteria x mL^{-1} with the highest growth at 10^{10} cells x mL^{-1}. The addition of bacterial prey at a concentration of 10^{12} bacteria x mL^{-1} caused lysis of *Paramecium* sp. RB1 cells. Concentrations of less than 10^9 bacteria x mL^{-1} were insufficient to enable the growth of *Paramecium* sp. RB1. *Paramecium* sp. RB1 cell numbers ceased to increase once the concentration of *E. coli* ATCC 8739 was reduced to below 10^8 bacteria x mL^{-1}, and more than 90% of prey cells were consumed in the presence of *Paramecium* sp. RB1 at *E. coli* ATCC 8739 concentrations of 10^8, 10^9, and 10^{10} bacteria x mL^{-1}. In the absence of ciliate cells, bacterial cell concentrations remained unchanged.

The experimental data generated were used to establish growth and grazing kinetics of *Paramecium* sp. RB1 feeding on *E. coli* ATCC 8739 at different concentrations.

Table 2.8. Growth and grazing kinetics of *Paramecium* sp. RB1 feeding on *E. coli* ATCC 8739 at different concentrations in Chalkley's medium at 25 ± 2ºC in the dark

Bacteria x mL^{-1}	Specific growth rate (hr^{-1})	Generation time (hr)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
10^6	0	0	0	0
10^8	0	0	1.48×10^3	14.03
10^9	0.0084	85.87	$2.07 \times 10^4 – 1.40 \times 10^4$	19.38
10^{10}	0.0112	63.73	$1.99 \times 10^5 – 1.18 \times 10^5$	19.81
10^{12}	-	-	0	0

The values are the average of two independently performed experiments. (-) indicate that ciliate cell numbers declined throughout incubation. Grazing rates were corrected for the increased number of individual cells during exponential growth.

Growth rates of *Paramecium* sp. RB1 was detected at prey concentrations of 10^9 and 10^{10} bacteria x mL^{-1} with the highest growth rates at 10^{10} bacteria x mL^{-1}. Though *E. coli* ATCC 8739 concentration of 10^8 bacteria x mL^{-1} was not sufficient to cause the growth of *Paramecium* sp. RB1, the grazing rates were detected at this concentration, and it increased by a factor of 10 with an increase in prey concentrations up to 10^{10} bacteria x mL^{-1}.

The impact of *Paramecium* sp. RB1 grazing activity on the viability of *E. coli* ATCC 8739 at different prey concentrations was determined using the plate count method.

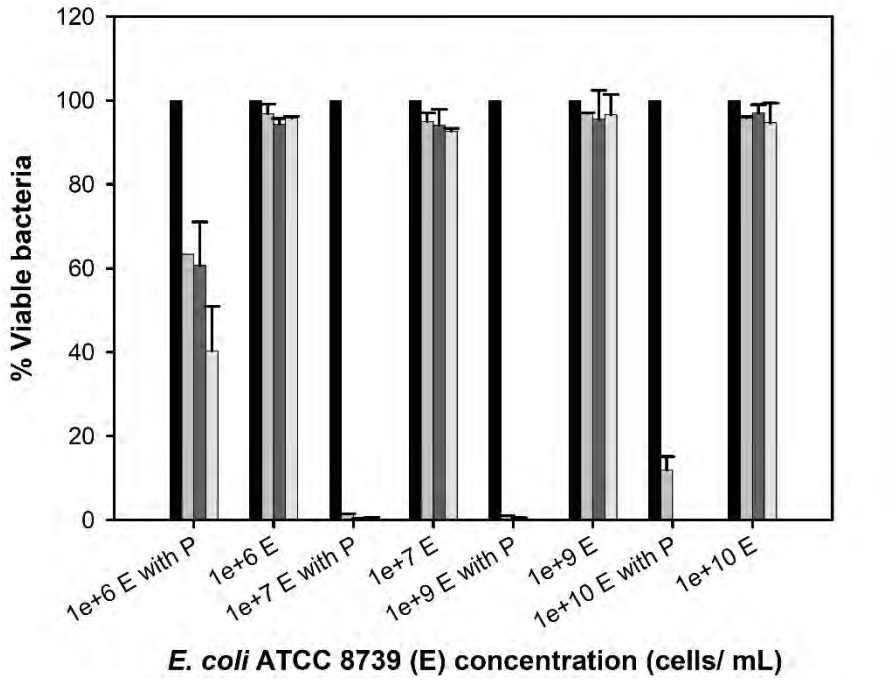

Figure 2.19. The change in viable counts (% CFU/ mL) of *E. coli* ATCC 8739 (E) at different concentrations in the presence of *Paramecium* sp. RB1 (10^3 cells/ mL) (P) and the absence of ciliate in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between means.

In the presence of *Paramecium* sp. RB1, more than 90% of viable *E. coli* ATCC 8739 was reduced from a concentration of 10^7 to 10^{10} bacteria x mL^{-1}, which was not observed in the absence of *Paramecium* sp. RB1. Though growth and grazing of *Paramecium* sp. RB1 was not detected at 10^6 *E. coli* ATCC 8739 per mL, viable bacteria were reduced by about 60% in the presence of ciliate isolate. Thus, a decrease in viable bacteria was due to the grazing activity of *Paramecium* sp. RB1.

Growth of *Paramecium* sp. RB1 with various bacterial species

The grazing of *Paramecium* sp. RB1 on bacteria was studied with various bacterial species to determine grazing efficiency and prey selectivity of the ciliate and the nutritional quality of various microbial species.

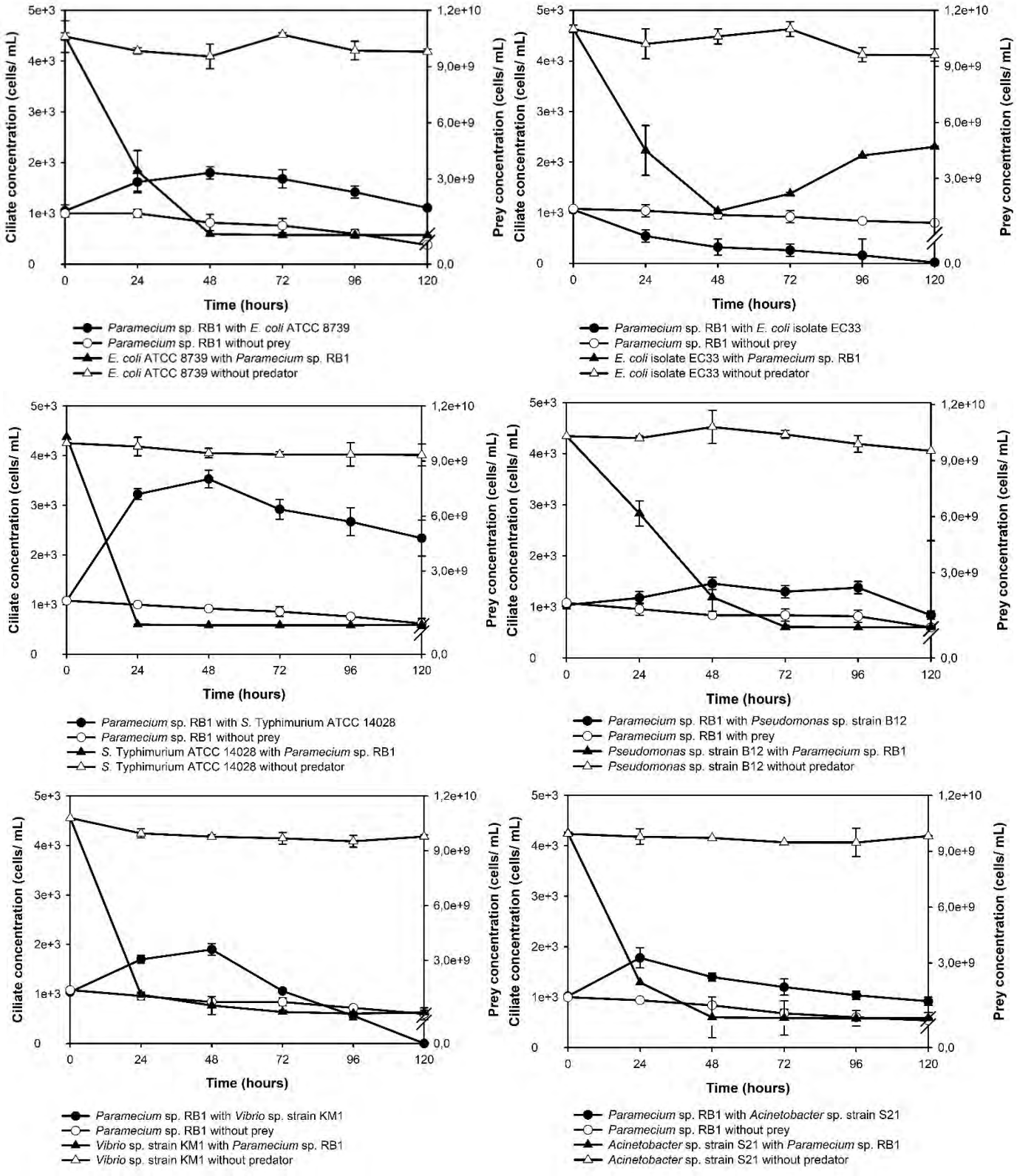

Figure 2.20. Growth of *Paramecium* sp. RB1 grazing on various Gram-negative bacteria (10^{10} bacterial x mL^{-1}) in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between means.

Growth of *Paramecium* sp. RB1 was solely due to consumption of bacteria; ciliate numbers increased mostly with a decrease in bacterial cell numbers. Although *Paramecium* sp. RB1 did not show selective feeding when grazing on Gram-negative bacterial species as there was a decrease in bacterial cell numbers in the presence of the ciliate isolate. Consumption of antibiotic-resistant *E. coli* isolate EC33 caused a decline in *Paramecium* sp. RB1 cell numbers and the lysis of ciliate cells stimulated an increase in bacterial concentrations after 72 hours of incubation. The highest growth was observed with *S.* Typhimurium ATCC 14028. Grazing activity of *Paramecium* sp. RB1 resulted in up to 90% reduction in bacterial prey cells.

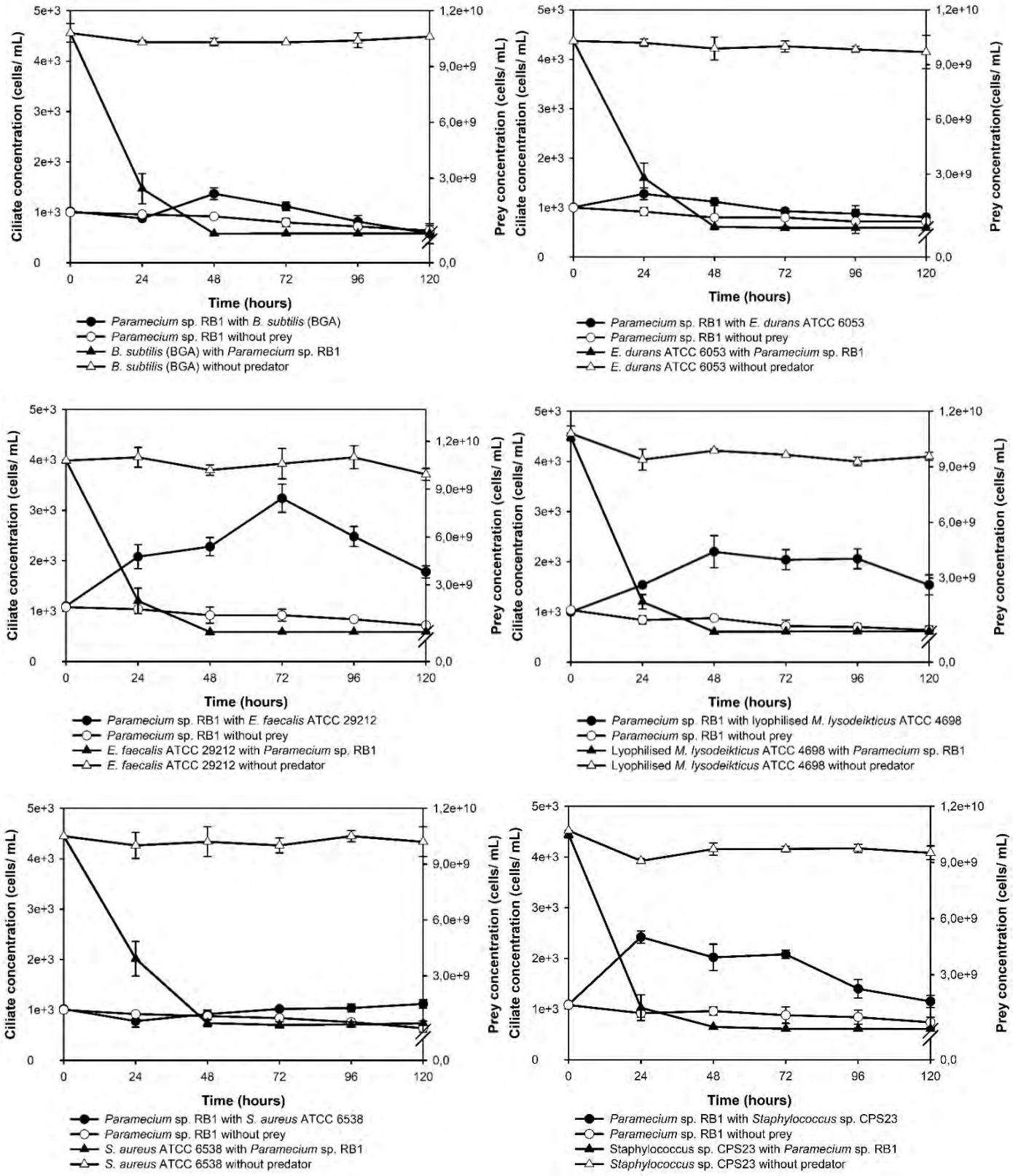

Figure 2.21. Growth of *Paramecium* sp. RB1 grazing on various Gram-positive bacteria (10^{10} bacteria x mL^{-1}) in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between means.

Growth of *Paramecium* sp. RB1 with Gram-positive bacteria increased with the decrease of bacterial cells, as no growth was observed in the absence of bacterial prey. This ciliate did not show selective feeding on any of the six Gram-positive bacteria selected, and its grazing activity resulted in a 90% reduction in bacterial cell numbers. However, growth of the ciliate varied substantially with each bacterial species selected, the highest growth of *Paramecium* sp. RB1 was observed with *E. faecalis* ATCC 29212, while *S. aureus* ATCC 6538 did not enable good growth of this ciliate.

Growth of *Paramecium* sp. RB1 on various eukaryote species

In addition to bacteria, eukaryotic organisms were selected to determine the ability of *Paramecium* sp. RB1 to consume and grow with other microbial eukaryotes as prey.

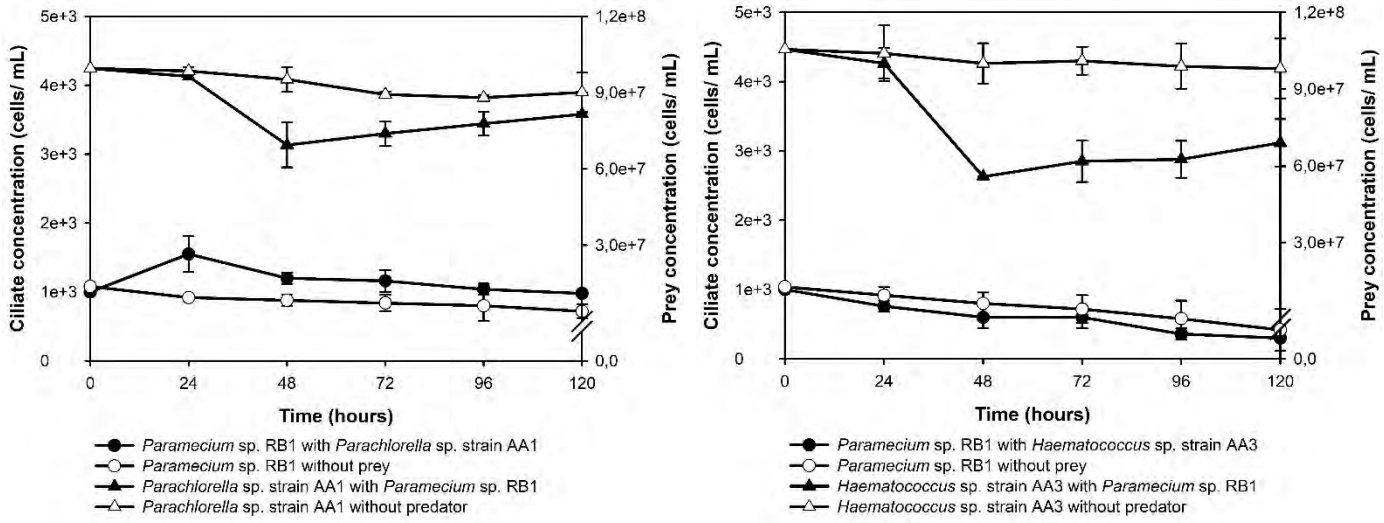

Figure 2.22. Growth of *Paramecium* sp. RB1 grazing on two algal species (*Parachlorella* sp. strain AA1 and *Haematococcus* sp. strain AA3) at a concentration of 10^8 cells x mL^{-1} in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between means.

Only slight growth was observed when *Paramecium* sp. RB1 was grazing on *Parachlorella* sp. strain AA1, while *Haematococcus* sp. strain AA3 caused a decline in the ciliate cell numbers. A decrease in algal cell concentrations was only observed in the presence of *Paramecium* sp. RB1 and the grazing by this ciliate resulted in about 30% reduction of *Parachlorella* sp. AA1 and about 40% reduction of *Haematococcus* sp. strain AA3.

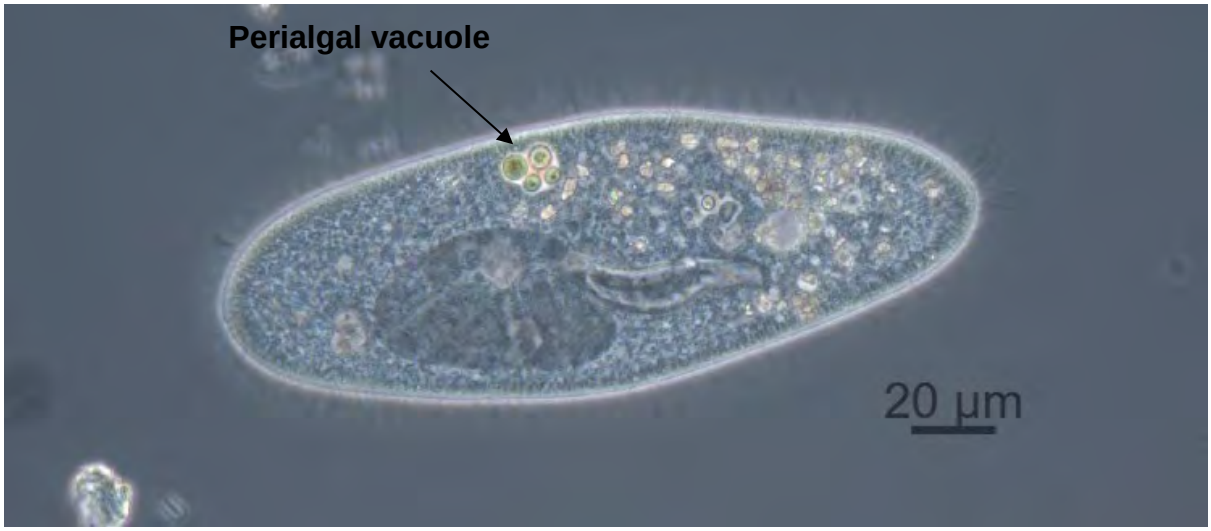

Figure 2.23. A live cell of *Paramecium* sp. RB1 with ingested *Parachlorella* sp. strain AA1 after 24 hours feeding in Chalkley's medium at 25 ± 2ºC in the dark.

Paramecium sp. RB1 ingested cells of *Parachlorella* sp. strain AA1. The algal cells were detected within food vacuoles of the ciliate. The space between the algal cell and the membrane surrounding the cell is indicative of a perialgal vacuole.

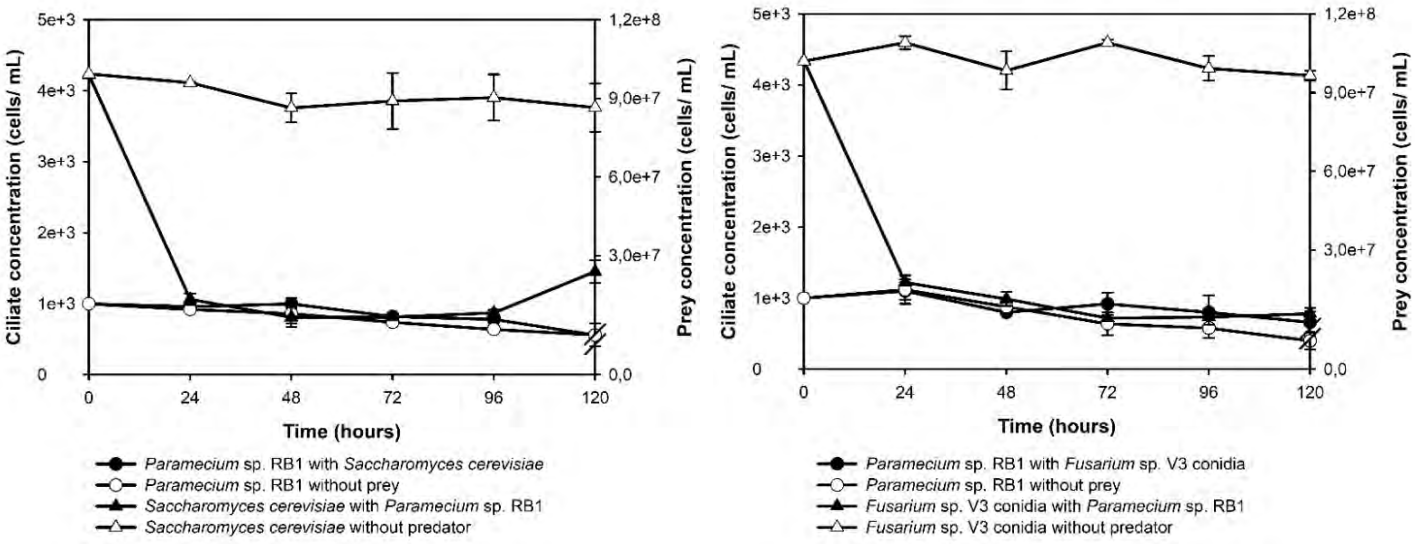

Figure 2.24. Growth of *Paramecium* sp. RB1 grazing on two fungal species, *Saccharomyces cerevisiae* and spores of *Fusarium* sp. V3, at a concentration of 10^8 cells x mL^{-1} in Chalkley's medium in the dark at 25 ± 2ºC. All data are means of two independently performed experiments. Error bars represent the difference between means.

Though there was a decrease in the number of yeast cells and fungal spores in the presence of *Paramecium* sp. RB1, growth of this ciliate was not observed when these prey microbes were present. In the presence of *Paramecium* sp. RB1, prey cells were reduced by 70%, which was not observed in the absence of the ciliate cells.

The experimental data generated from growth experiments were used to establish growth and grazing kinetics of *Paramecium* sp. RB1 with various microbial prey species.

Table 2.9. Growth and grazing kinetics of *Paramecium* sp. RB1 with various microbial prey in Chalkley's medium at 25 ± 2ºC in the dark

Prey	Specific growth rate (hr^{-1})	Generation time (hrs)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
Gram-negative bacteria (10^{10} cells x mL^{-1}):				
E. coli ATCC 8739	0.0112	63.73	$1.99 \times 10^5 – 1.18 \times 10^5$	19.81
E. coli isolate EC33	-	-	6.85×10^4	5.94
Pseudomonas sp. strain B12	0.0071	104.86	$1.74 \times 10^5 – 1.24 \times 10^5$	16.75
S. Typhimurium ATCC 14028	0.0247	28.33	$1.98 \times 10^5 – 6.09 \times 10^4$	19.22
Vibrio sp. strain KM1	0.0126	55.82	$2.66 \times 10^5 – 1.11 \times 10^5$	19.09
Acinetobacter sp. strain S21	0.0232	30.33	$2.68 \times 10^5 – 1.75 \times 10^5$	32.88
Gram-positive bacteria (10^{10} cells x mL-^{1}):				
Staphylococcus sp. CPS23	0.0332	21.10	$3.80 \times 10^5 – 1.71 \times 10^5$	34.00
S. aureus ATCC 6538	-	-	9.85×10^4	7.88
E. faecalis ATCC 29212	0.0150	46.96	$1.36 \times 10^5 – 4.63 \times 10^4$	12.57
E. durans ATCC 6056	0.0066	90.67	$3.11 \times 10^5 – 2.42 \times 10^5$	30.21
Lyophilized *Micrococcus lysodeikticus* ATCC 4698	0.0164	43.04	$2.09 \times 10^5 – 9.61 \times 10^4$	20.65
B. subtilis BGA	0.0096	93.51	$2.20 \times 10^5 – 1.68 \times 10^5$	20.41
Algae (10^8 cells x mL^{-1}):				
Haematococcus sp. strain AA3	-	-	1.24×10^3	10.66
Parachlorella sp. strain AA1	0.0181	40.22	$6.52 \times 10^2 – 4.21 \times 10^2$	5.82
Fungi (10^8 cells x mL^{-1}):				
Saccharomyces cerevisiae	-	-	1.93×10^3	19.53
Fusarium sp. V3 spores	-	-	8.07×10^2	7.89

All values are the average of two independently performed experiments. (-) indicate that no growth of the isolated ciliate was observed. Grazing rates were corrected for the increased number of individual cells number during exponential growth.

Grazing rates of *Paramecium* sp. RB1 were detected with all bacterial prey tested, leading to up to 90% reduction of bacterial cells except with antibiotic-resistant *E. coli* isolate EC33. However, growth of the ciliate was detected with all bacterial species except Gram-negative *E. coli* isolate EC33 and Gram-positive *S. aureus* ATCC 6538. Although growth rates varied with each bacterial species, the highest growth rate was observed with a Gram-positive non-motile *Staphylococcus* sp. CPS23, grazing rates were within a similar range of $>10^4$ to 10^5 bacteria per hour, while clearance rates ranged 5 to 34 nL per hour for individual ciliates. In addition to bacteria, grazing rates were detected with eukaryotes with grazing and clearance rates that were within the range detected with bacteria at the same concentrations.

The response of *Paramecium* sp. RB1 to prey availability fluctuations

Previous growth studies demonstrated that *Paramecium* sp. RB1 population declines once prey concentration is below its threshold. The response of *Paramecium* sp. RB1 to changes in prey availability and its ability to recover from starvation once the prey concentration is sufficient to support growth was studied with *E. coli* ATCC 8739.

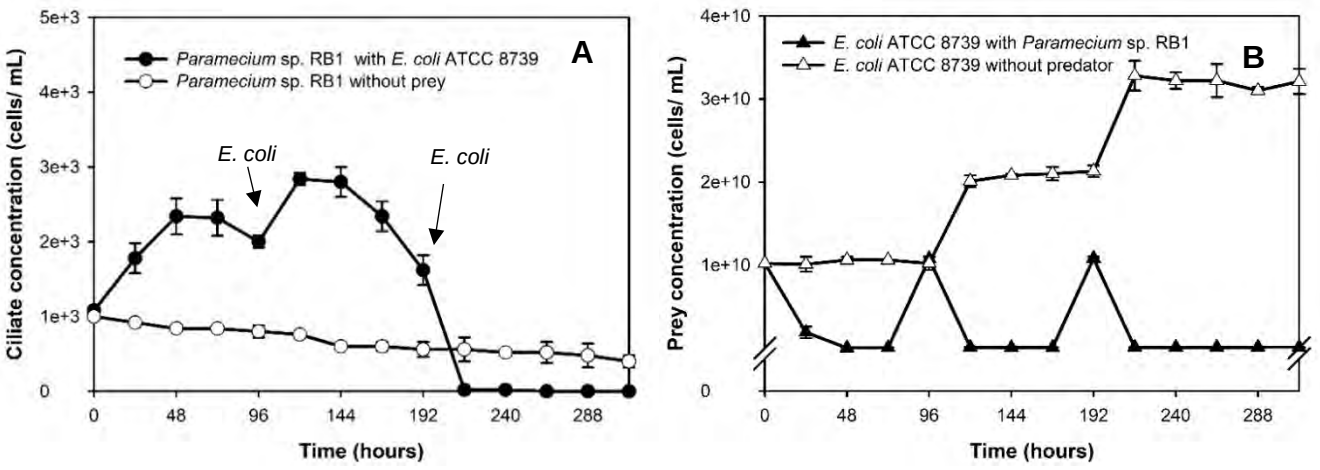

Figure 2.25. Growth of *Paramecium* sp. RB1 with *E. coli* ATCC 8739 (A) and the change of *E. coli* ATCC 8739 concentration in the absence and presence of a *Paramecium* sp. RB1 (B) after the addition of fresh bacterial fodder at 10^{10} *E. coli* ATCC 8739 x mL^{-1} at 0, 96, and 192 hours in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between means**.**

The numbers of *Paramecium* sp. RB1 increased with a decrease in bacterial concentrations, declining once the prey concentration of *E. coli* ATCC 8739 was below 10^8 bacteria x mL^{-1}. Upon the addition of fresh fodder in the form of *E. coli* ATCC 8739 at 92 hours, *Paramecium* sp. RB1 cell numbers increased with a decrease in bacterial cell numbers, again declining after the concentration of *E. coli* ATCC 8739 was below 10^8 bacteria x mL^{-1}. However, the third addition at 192 hours of *E. coli* ATCC 8739 did not cause an increase in ciliate cell numbers; instead, the population continued to decline, and no ciliate cells were detected after 216 hours though prey concentrations were reduced.

Influence of particle size on grazing rates of *Paramecium* sp. RB1

The influence of particle size on the grazing efficiency of *Paramecium* sp. RB1 was determined using microspheres, e.g., latex beads, of different sizes. The grazing and the clearance rates were determined from the change in microparticles concentration after 24 hours.

Table 2.10. Grazing and clearance rates of *Paramecium* sp. RB1 on different sizes of polystyrene microbeads in Chalkley's medium at 25 ± 2ºC in the dark

Microparticle size (µm diameter)	Grazing rate (particle x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
2	3.86×10^3	37.83
5	3.87×10^3	26.23
10	1.65×10^3	16.42

The values are the average of two independently performed experiments.

Paramecium sp. RB1 consumed all three sizes of the microparticles tested. The grazing rates were high for 2 and 5 µm but decreased by 50% for 10 µm particles. The clearance rates decreased with an increase in particle size.

To verify the uptake of microparticles, cells of *Paramecium* sp. RB1 were examined microscopically.

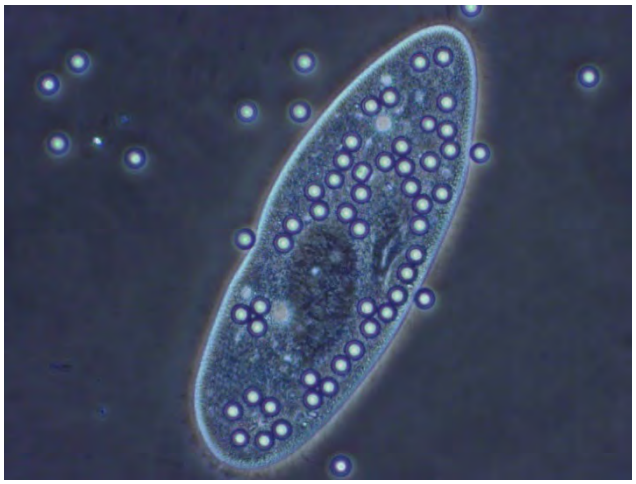

Figure 2.26. Uptake of 10 µm diameter polystyrene microbeads by *Paramecium* sp. RB1 after 24 hours incubation in Chalkley's medium at 25 ± 2ºC in the dark.

Microbeads of diameter 10 µm were ingested and detected within the cells of *Paramecium* sp. RB1.

Influence of temperature on growth and grazing of *Paramecium* sp. RB1

The influence of temperature on growth and grazing of *Paramecium* sp. RB1 was determined by observing the growth of the ciliate with *E. coli* ATCC 8739 at three different temperatures.

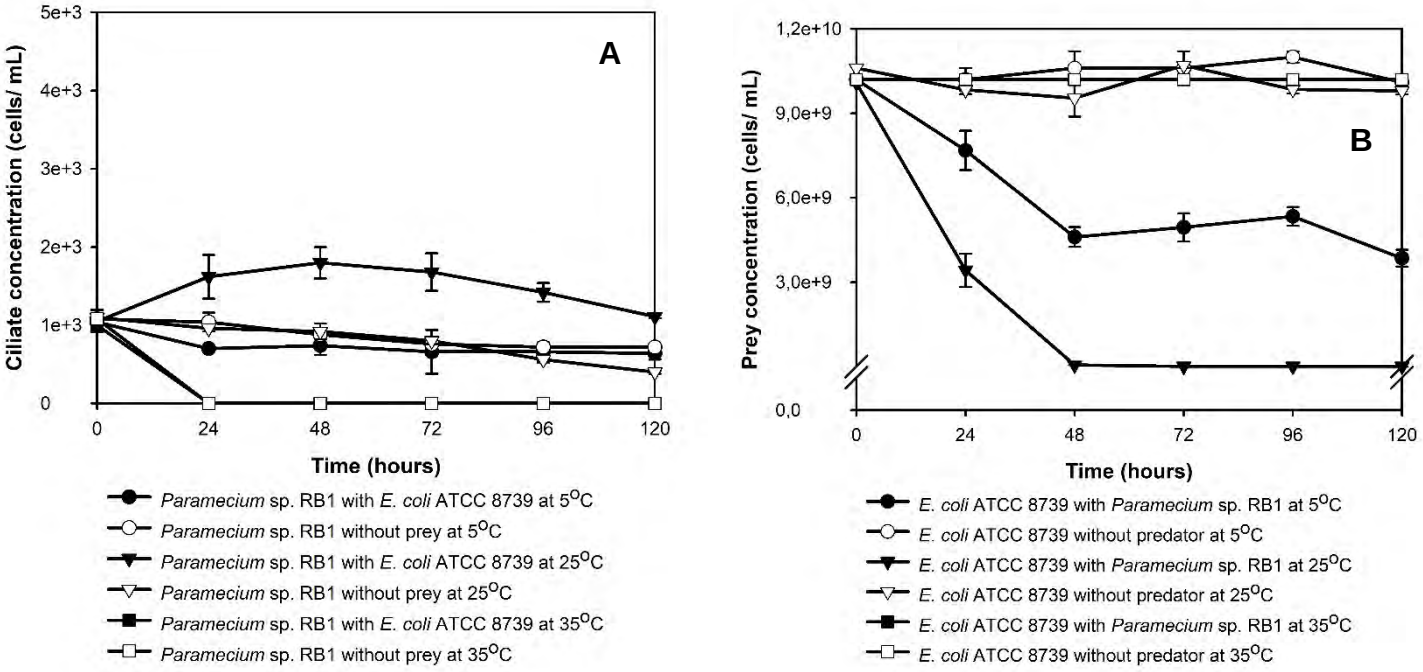

Figure 2.27. Growth of *Paramecium* sp. RB1 with *E. coli* ATCC 8739 (A) and the change of *E. coli* ATCC 8739 in the presence and absence of *Paramecium* sp. RB1 (B) at the temperature of 5, 25, and 35ºC in Chalkley's medium in the dark. All data are means of two independently performed experiments. Error bars represent the difference between means.

Growth of *Paramecium* sp. RB1 was only observed at 25ºC, while 35ºC was lethal to this ciliate. No growth was observed at 5ºC, though at this temperature, a reduction of *E. coli* ATCC 8739 concentrations was observed but was lower than at 25ºC. At 35ºC, no reduction in *E. coli* ATCC 8739 concentrations was observed.

The experimental data generated was used to establish the growth and grazing kinetics of *Paramecium* sp. RB1 feeding on *E. coli* ATCC 8739 at different temperatures.

Table 2.11. Growth and grazing rates of *Paramecium* sp. RB1 feeding on *E. coli* ATCC 8739 at different temperatures in Chalkley's medium in the dark.

Temperature (ºC)	Specific growth rate (hr^{-1})	Generation time (hr)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
5	0	0	1.33×10^4	11.83
25	0.0112	63.73	$1.99 \times 10^5 – 1.18 \times 10^5$	19.81
35	-	-	0	0

The values are the mean of two independently performed experiments. (-) indicated that ciliate cells were not detected after 24 hours of incubation. Grazing rates were corrected for the increased number of individual cells number during exponential growth.

Growth of *Paramecium* sp. RB1 was only observed at 25ºC. Though the growth of *Paramecium* sp. RB1 was not observed at 5ºC, *E. coli* ATCC 8739 was consumed by the ciliate. Grazing and clearance rates of *Paramecium* sp. RB1 were higher at 25ºC than at 5ºC. The temperature of 35ºC was lethal to *Paramecium* sp. RB1, cells of this ciliate were not observed after 24 hours of incubation at this temperature, and consequently, no grazing took place.

2.3.3.3. Growth and grazing studies of *Tetrahymena* sp. RB2

The predator-prey interaction of *Tetrahymena* sp. RB2 was studied with various microorganisms to determine the growth and grazing characteristics and prey selectivity of this ciliate and the nutritional quality of various microbial prey.

The response of *Tetrahymena* sp. RB2 to different prey concentrations

The influence of prey concentrations on the growth and grazing of *Tetrahymena* sp. RB2 was studied with *E. coli* ATCC 8739 at different concentrations.

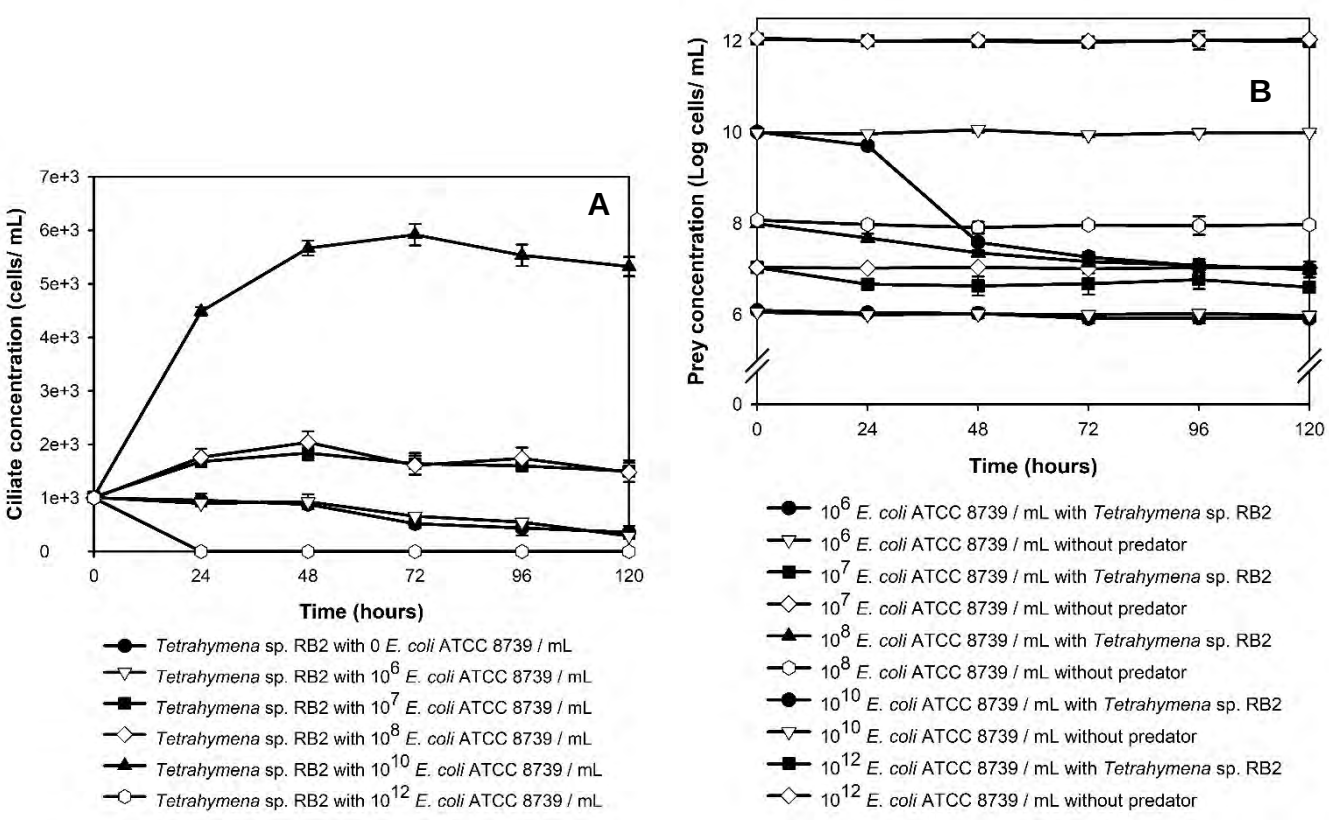

Figure 2.28. Growth of *Tetrahymena* sp. RB2 with *E. coli* ATCC 8739 (A) and the change of *E. coli* ATCC 8739 in the presence and absence of *Tetrahymena* sp. RB2 (B) at different prey concentrations in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between the means.

The growth of *Tetrahymena* sp. RB2 was observed in the presence of *E. coli* ATCC 8739 between cell numbers of 10^7 to 10^{10} bacteria x mL^{-1}, with the highest growth observed at 10^{10} bacteria x mL^{-1}. The bacterial concentration of 10^6 bacteria x mL^{-1} was insufficient to cause an increase in ciliate cell numbers, while 10^{12} bacteria x mL^{-1} caused lysis of ciliate cells. In the presence of ciliates, prey concentrations from 10^7 to 10^{10} bacteria x mL^{-1} were reduced by more than 90%, whereas prey concentrations remained unchanged in the absence of ciliates and at concentrations of 10^6 and 10^{12} bacteria x mL^{-1}.

The experimental data generated during growth experiments were used to establish the growth and grazing characteristics of *Tetrahymena* sp. RB2 at varying concentrations.

Table 2.12. Growth and grazing kinetics of *Tetrahymena* sp. RB2 feeding on *E. coli* ATCC 8739 at different concentrations in Chalkley's medium at 25 ± 2ºC in the dark

Bacterial x mL^{-1}	Specific growth rate (hr^{-1})	Generation time (hr)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
10^6	0	0	0	0
10^7	0.0122	58.39	$1.33 \times 10^2 – 7.35 \times 10^1$	12.41
10^8	0.0148	46.26	$1.59 \times 10^3 – 7.82 \times 10^2$	16.06
10^{10}	0.0351	20.54	$2.08 \times 10^5 – 4.08 \times 10^4$	20.25
10^{12}	-	-	0	0

Values are the average of two independently performed experiments. (-) indicate that ciliate cells were not detected after 24 hours incubation. Grazing rates were corrected for the increased number of individual cells number during exponential growth.

Growth and grazing rates of *Tetrahymena* sp. RB2 were detected at bacterial concentrations from 10^7 to 10^{10} cells x mL^{-1} and increased with an increase in prey concentrations. The highest growth and grazing rates were detected at 10^{10} bacteria x mL^{-1}. Grazing was not detected at concentrations of 10^6 bacteria x mL^{-1}, and the highest concentrations tested, 10^{12} bacteria x mL^{-1}, caused lysis of the ciliate cells.

In addition to the change of total cell counts of *E. coli* ATCC 8739 in the presence of *Tetrahymena* sp. RB2, the impact of *Tetrahymena* sp. RB2 grazing on the viability of *E. coli* ATCC 8739 at different concentrations was determined with plate count methods.

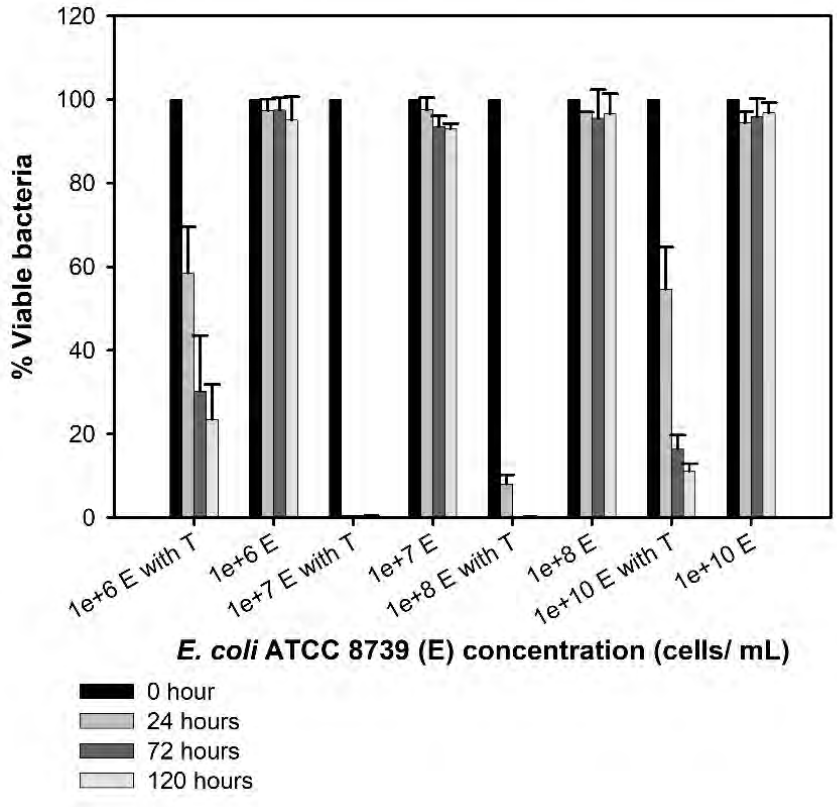

Figure 2.29. The change of viable counts (% CFU x mL^{-1}) of *E. coli* ATCC 8739 at different concentrations in the presence of *Tetrahymena* sp. RB2 (10^3 cells x mL^{-1}) (T) and in the absence of predator in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between the means.

Grazing of *Tetrahymena* sp. RB2 resulted in more than 90% reduction of viable *E. coli* ATCC 8739 from the concentration of 10^7 to 10^{10} bacteria x mL^{-1} and about 80% reduction of viable *E. coli* ATCC 8739 at 10^6 bacteria x mL^{-1} after 5 days incubation. Reduction of viable bacteria was solely due to grazing of *Tetrahymena* sp. RB2 as no considerable reduction was observed in the absence of the isolated ciliate *Tetrahymena* sp. RB2.

Growth and grazing of *Tetrahymena* sp. RB2 on various bacterial species

The ability of the *Tetrahymena* sp. RB2 to consume and grow on various bacterial prey was studied to determine the nutritional quality of the bacterial prey as well as growth and grazing characteristics and feeding selectivity of the isolate *Tetrahymena* sp. RB2 on selected microbial prey.

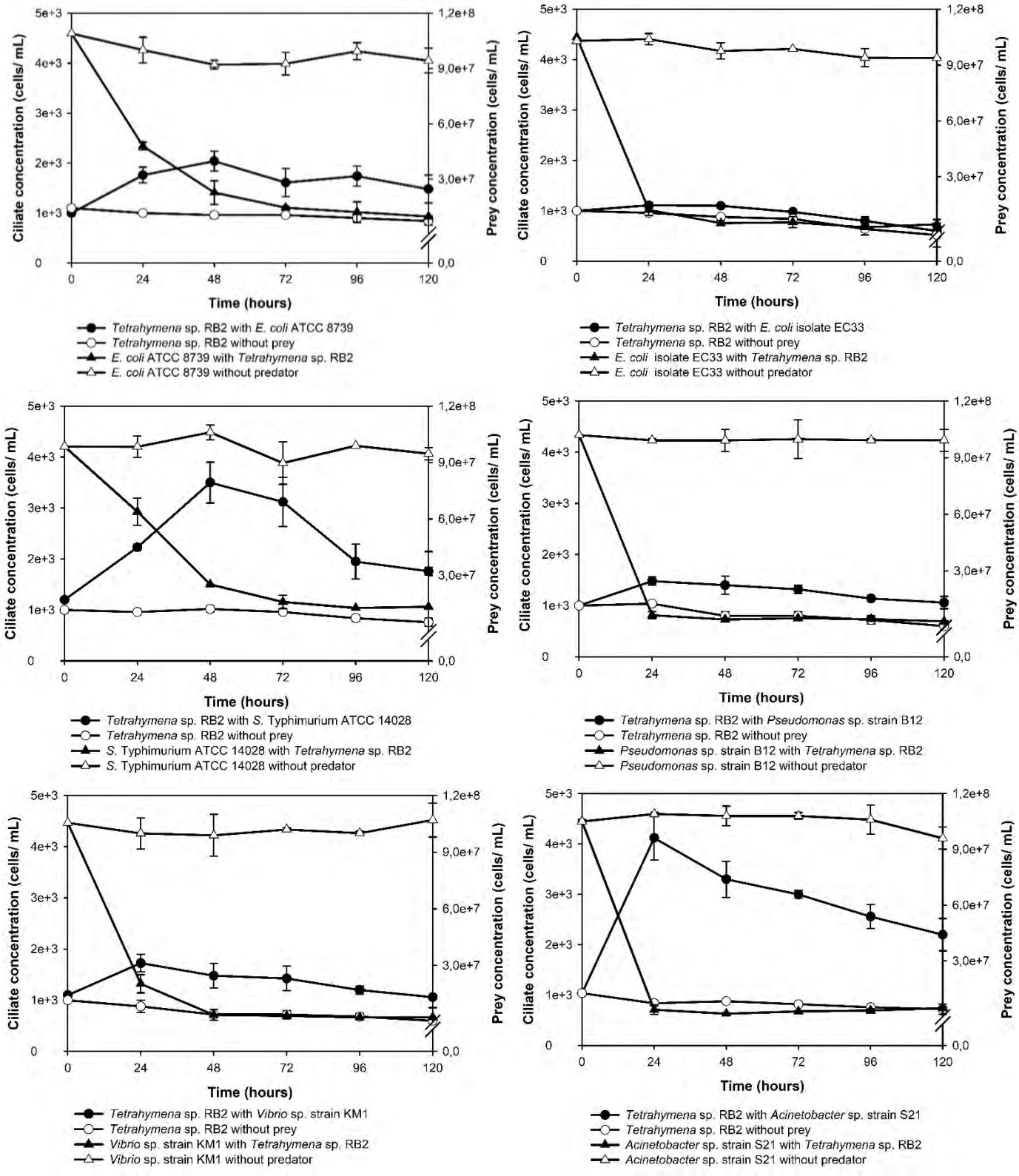

Figure 2.30. Growth of *Tetrahymena* sp. RB2 with various Gram-negative bacteria (10^8 bacteria x mL^{-1}) in Chalkley's medium at 25 ± 2ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between means.

The growth of *Tetrahymena* sp. RB2 correlated with the decrease of bacterial prey concentrations, while no growth was observed in the absence of bacterial prey. *Tetrahymena* sp. RB2 did not show selective feeding on any of the six selected Gram-negative bacterial, resulting in a 90% reduction of bacterial cell numbers. Nevertheless, the growth varied with each bacterial strain, with the highest growth detected with the non-motile coccus-shaped *Acinetobacter* sp. strain S21. Although *Tetrahymena* sp. RB2 consumed cells of antibiotic-resistant *E. coli* isolate EC33 with more than 90% reduction; this bacterial strain did not cause considerable growth of the ciliate.

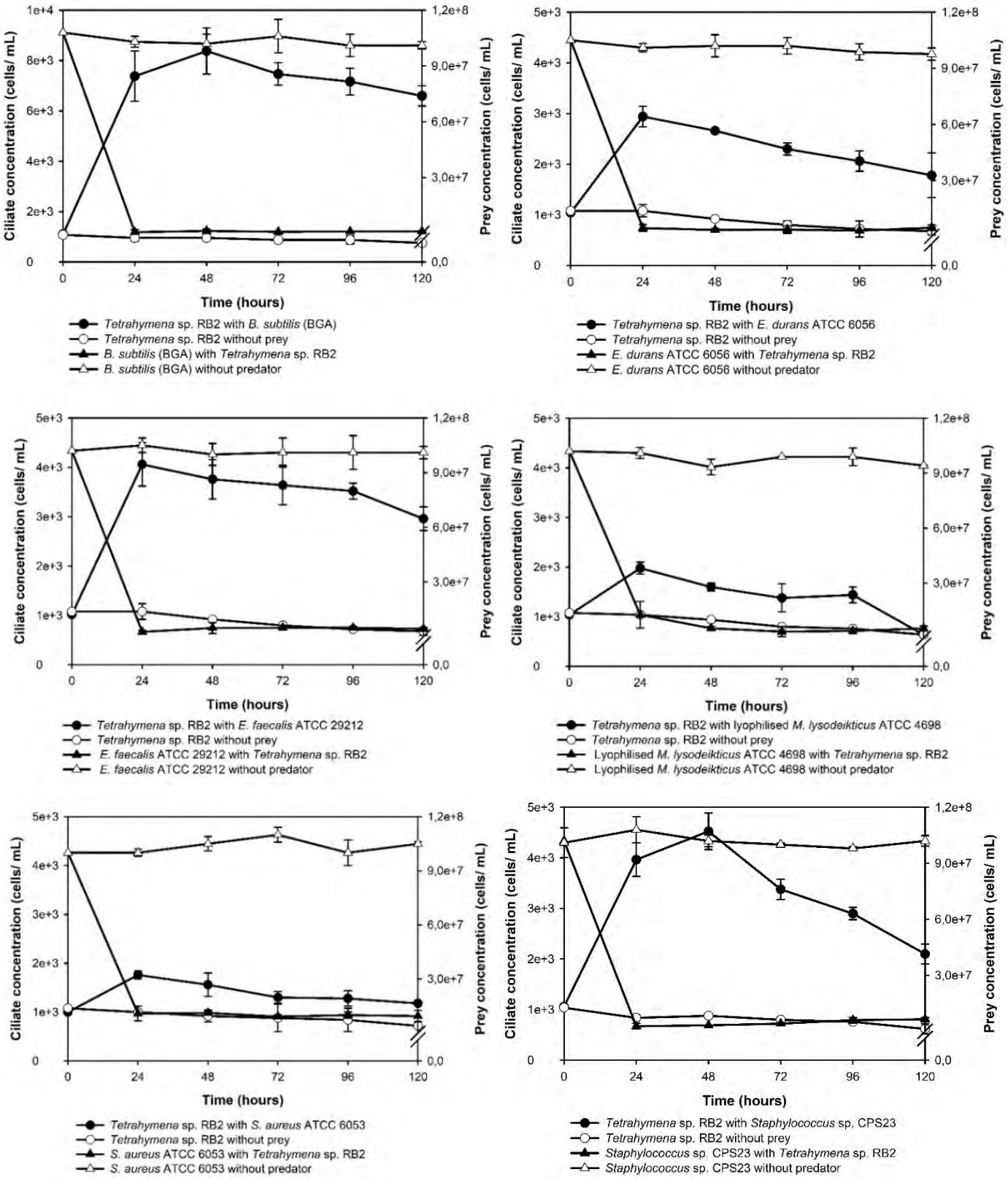

Figure 2.31. Growth of *Tetrahymena* sp. RB2 with various Gram-positive bacteria (10^8 bacteria x mL^{-1}) in Chalkley's medium at 25 ± 2ºC in the dark. All data are the average of two independently performed experiments. Error bars represent the difference between means.

Growth of *Tetrahymena* sp. RB2 was solely due to the consumption of Gram-positive bacterial species used as prey, and ciliate numbers increased with the decrease in prey concentrations. However, in the absence of bacterial prey, growth was not observed. This ciliate did not show selective feeding, as all the Gram-positive bacteria selected were consumed and reduced to below 10^7 bacteria x mL^{-1}. However, the growth of *Tetrahymena* sp. RB2 varied with each bacterial species, indicating different nutritional values. Grazing by this ciliate resulted in a 90% reduction of prey cell numbers, and the highest growth was observed with the spore-forming rod-shaped *Bacillus subtilis* BGA.

Growth of *Tetrahymena* sp. RB2 on various eukaryote species

The ability of *Tetrahymena* sp. RB2 to ingest other microscopic eukaryotic organisms was studied using algal and fungal species as prey organisms.

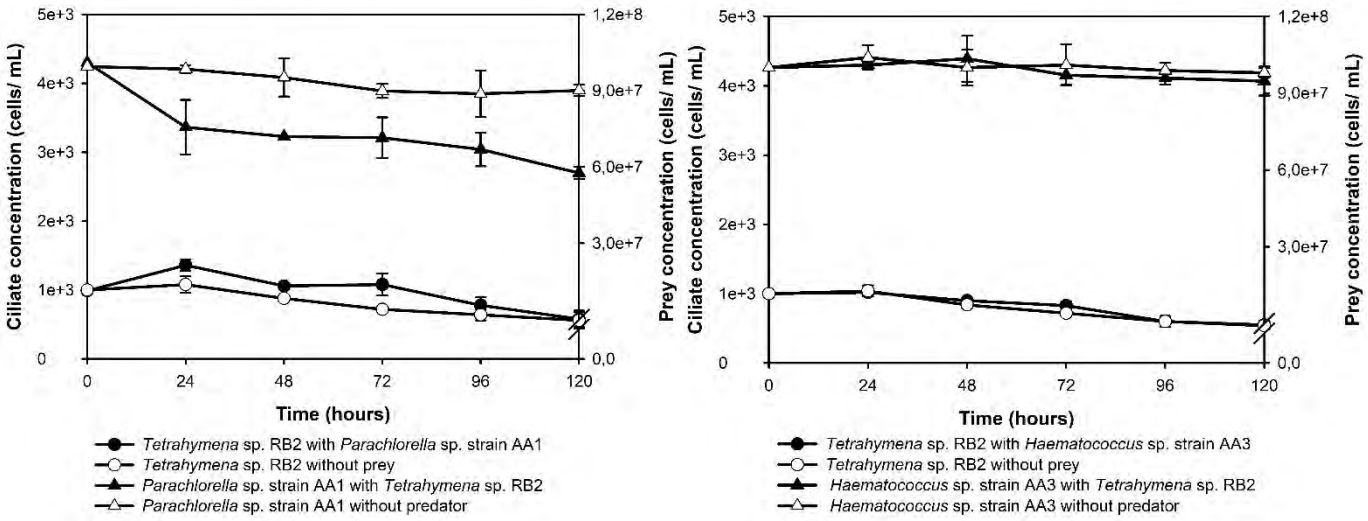

Figure 2.32. Growth of *Tetrahymena* sp. RB2 with two algal prey (10^8 cells x mL^{-1}), *Parachlorella* sp. strain AA1 and *Haematococcus* sp. strain AA3 in Chalkley's medium at 25 ± 2ºC in the dark. All data are the average of two independently performed experiments. Error bars represent the difference between means.

Only minute growth of *Tetrahymena* sp. RB2 was observed with *Parachlorella* sp. strain AA1 as prey with about 30% of algal cells consumed. *Haematococcus* sp. strain AA3 cells were not ingested by the ciliate as concentrations of algal cells remained unchanged throughout the incubation period, and growth of the ciliate was not observed.

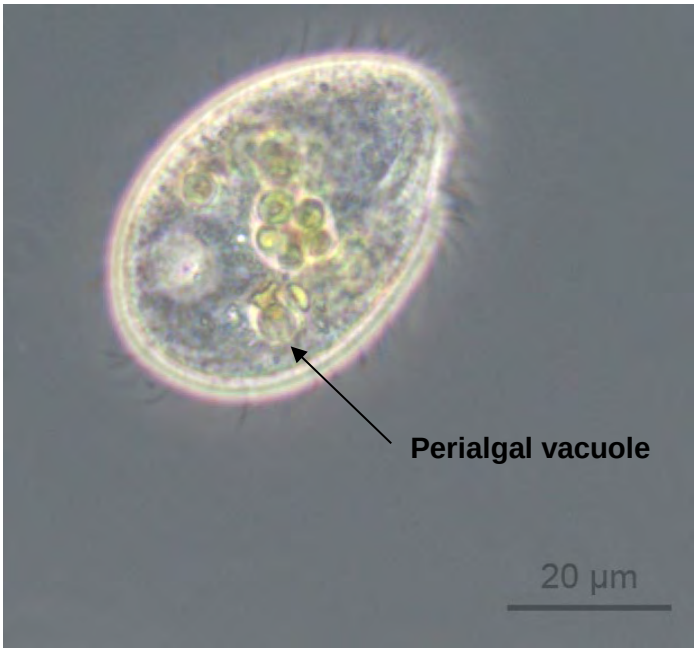

Figure 2.33. A live cell of *Tetrahymena* sp. RB2 with *Parachlorella* sp. strain AA1 cells after 24 hours incubation in Chalkley's medium at 25 ± 2ºC in the dark.

Tetrahymena sp. RB2 ingested cells of *Parachlorella* sp. strain AA1. The algal cells were detected within food vacuoles of the ciliate. The space between the algal cell and the membrane surrounding the cell is indicative of a perialgal vacuole.

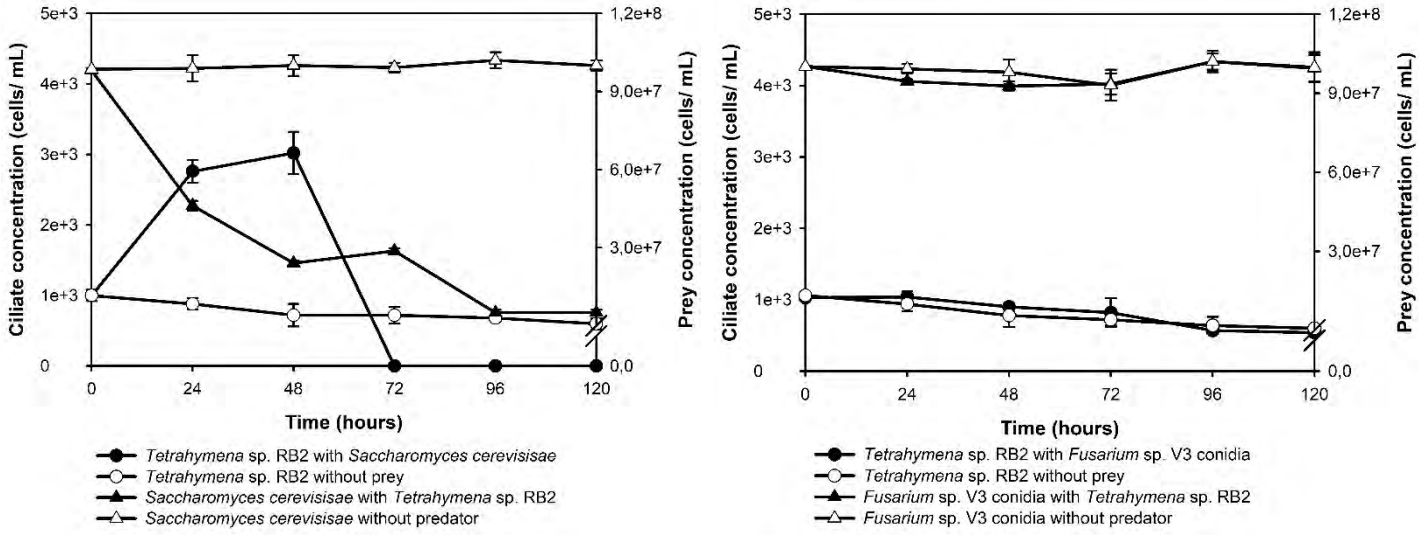

Figure 2.34. Growth of *Tetrahymena* sp. RB2 with two fungal prey (10^8 cells x mL^{-1}), *Saccharomyces cerevisiae* and *Fusarium* sp. V3 conidia in Chalkley's medium incubated at 25 ± 2ºC in the dark. All data are the average of two independently performed experiments. Error bars represent the difference between means.

Growth of *Tetrahymena* sp. RB2 proceeded with a decrease in *Saccharomyces cerevisiae* cell numbers, while no growth was observed with conidia of *Fusarium* sp. V3. Once yeast cells were consumed to below 3 x 10^7 cells x mL^{-1}, the population of *Tetrahymena* sp. RB2 declines and after 72 hours and *Tetrahymena* sp. RB2 cells were not detected in the culture. The reduction of yeast cell concentrations was solely due to the grazing of *Tetrahymena* sp. RB2, resulting in about 80% reduction of yeast cell numbers.

From the growth experiments, the data generated was used to determine the growth and grazing kinetics of *Tetrahymena* sp. RB2 feeding on various microbial organisms.

Table 2.13. Growth and grazing kinetics of *Tetrahymena* sp. RB2 on various microbial prey in Chalkley's medium at 25 ± 2ºC in the dark

Prey	Specific growth rate (hr^{-1})	Generation time (hrs)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
Gram-negative bacteria (10^8 cells x mL^{-1}):				
E. coli ATCC 8739	0.0148	48.26	$1.59 \times 10^3 – 7.82 \times 10^2$	16.06
E. coli isolate EC33	0.0043	175.76	$4.10 \times 10^3 – 3.71 \times 10^3$	37.03
Pseudomonas sp. strain B12	0.0163	43.08	$3.98 \times 10^3 – 2.69 \times 10^3$	39.01
S. Typhimurium ATCC 14028	0.0247	28.34	$1.51 \times 10^3 – 4.45 \times 10^2$	13.32
Vibrio sp. strain KM1	0.0187	38.04	$3.23 \times 10^3 – 2.07 \times 10^3$	30.56
Acinetobacter sp. strain S21	0.0575	12.27	$3.88 \times 10^3 – 9.86 \times 10^2$	38.61
Gram-positive bacteria (10^8 cells x mL^{-1}):				
Staphylococcus sp. CPS23	0.0332	21.10	$2.14 \times 10^3 – 5.06 \times 10^2$	18.73
S. aureus ATCC 6538	0.0236	29.48	$3.73 \times 10^3 – 2.11 \times 10^3$	37.20
E. faecalis ATCC 29212	0.0575	12.13	$5.00 \times 10^3 – 1.26 \times 10^3$	40.00
E. durans ATCC 6056	0.0433	16.12	$4.42 \times 10^3 – 1.57 \times 10^3$	38.46
Lyophilised *Micrococcus lysodeikticus* ATCC 4698	0.0268	26.09	$4.22 \times 10^3 – 2.22 \times 10^3$	35.75
B. subtilis BGA	0.0423	16.52	$2.42 \times 10^5 – 3.17 \times 10^4$	18.77
Algae (10^8 cells x mL^{-1}):				
Haematococcus sp. strain AA3	-	-	0	0
Parachlorella sp. strain AA1	0.0132	53.57	$1.07 \times 10^3 – 7.71 \times 10^2$	10.43
Fungi (10^8 cells x mL^{-1}):				
Saccharomyces cerevisiae	0.0229	30.88	$1.55 \times 10^3 – 5.10 \times 10^2$	16.31
Fusarium sp. V3 conidia	-	-	0	0

All data are the mean values of two independently performed experiments. (-) indicate that growth was not observed. Grazing rates were corrected for the increased number of individual cells number during exponential growth.

Tetrahymena sp. RB2 did not show selective feeding on any of the bacterial species tested but showed selective feeding on eukaryotes microorganisms. Growth and grazing rates of *Tetrahymena* sp. RB2 varied with each species, and the growth rates on eukaryotes ingested were within the same range as those for bacterial species. This ciliate had high growth rates with most Gram-positive bacteria. The highest growth was observed with both Gram-positive *E. faecalis* ATCC 29212 and Gram-negative *Acinetobacter* sp. strain S21.

The response of *Tetrahymena* sp. RB2 to prey availability fluctuations

Aquatic environments are characterized by fluctuation in prey availability, as with other ciliates, *Tetrahymena* sp. RB2 cell numbers increased when prey concentration was sufficient and declined once prey concentration was below the threshold. Therefore, the response of *Tetrahymena* sp. RB2 to change in prey availability and its ability to recover from starvation was studied with *E. coli* ATCC 8739.

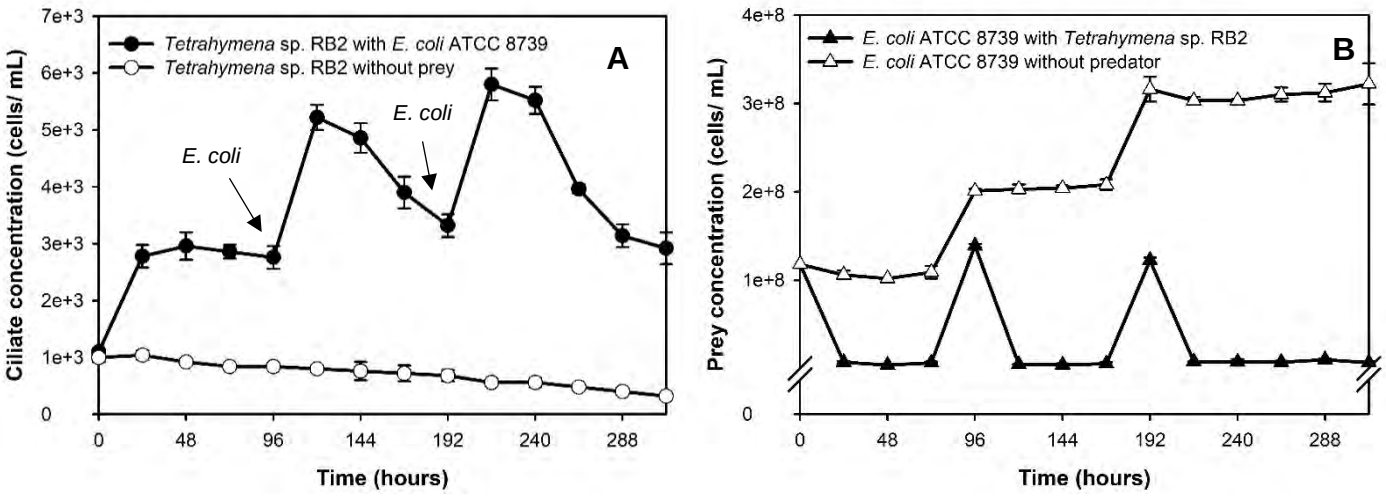

Figure 2.35. Growth of *Tetrahymena* sp. RB2 grazing on *E. coli* ATCC 8739 (A) and change of *E. coli* ATCC 8739 in the presence and absence of *Tetrahymena* sp. RB2 (B) after addition of fresh fodder of *E. coli* ATCC 8739 (10^8 bacteria x mL^{-1}) at 0, 96, and 192 hours in Chalkley's medium at 25 ± 2ºC in the dark. All data are the average of two independently performed experiments. Error bars represent the difference between means.

Tetrahymena sp. RB2 grew again with each addition of *E. coli* ATCC 8739. Once the prey concentration was consumed to below 10^7 bacteria x mL^{-1}, the ciliate population declined. Thus, *Tetrahymena* sp. RB2 can recover after limited periods of starvation and multiply again when prey becomes available at levels sufficient to support growth.

Influence of particle size on the grazing of *Tetrahymena* sp. RB2

Prey or particle size is known to play a significant role in determining the predator-prey relationships. To demonstrate the effect of particle size on the grazing rates, the *Tetrahymena* sp. RB2 isolate was fed with latex microparticles of various sizes.

Table 2.14. Grazing rates of *Tetrahymena* sp. RB2 on polystyrene microbeads of different sizes in Chalkley's medium at 25 ± 2ºC in the dark

Microparticle size (µm diameter)	Grazing rate (particle x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
2	2.88×10^3	28.74
5	2.56×10^3	23.96
10	0	0

The values are the mean of two independently performed experiments.

Tetrahymena sp. RB2 was only able to ingest 2 and 5 µm microbeads particles indicating that these particles were within the preferred size range that can be ingested by this ciliate. Grazing and clearance rates were highest for 2 µm diameter microbeads, which decreased slightly for 5 µm diameter microbeads.

To verify the uptake of microparticles, the cells of *Tetrahymena* sp. RB2 were examined microscopically.

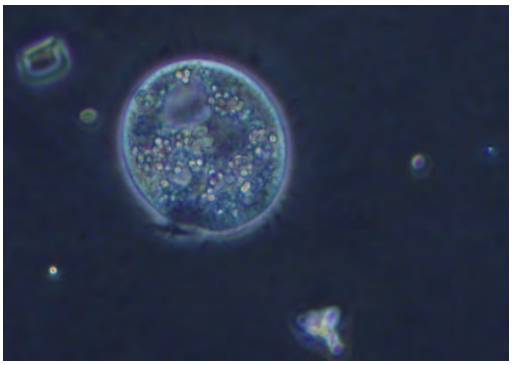

Figure 2.36. A live cell of *Tetrahymena* sp. RB2 with 5 µm microplastic particle present within the ciliate cell after ingestion and 24 hours feeding in Chalkley's medium at 25 ± 2ºC in the dark.

When examined microscopically, the microbeads of 5 µm diameter were ingested within the cell and packaged in food vacuoles of *Tetrahymena* sp. RB2.

Influence of temperature on growth and grazing of *Tetrahymena* sp. RB2

The influence of temperature on *Tetrahymena* sp. RB2 was determined by monitoring the growth and grazing of ciliate feeding on *E. coli* ATCC 8739 concentration at three different temperatures.

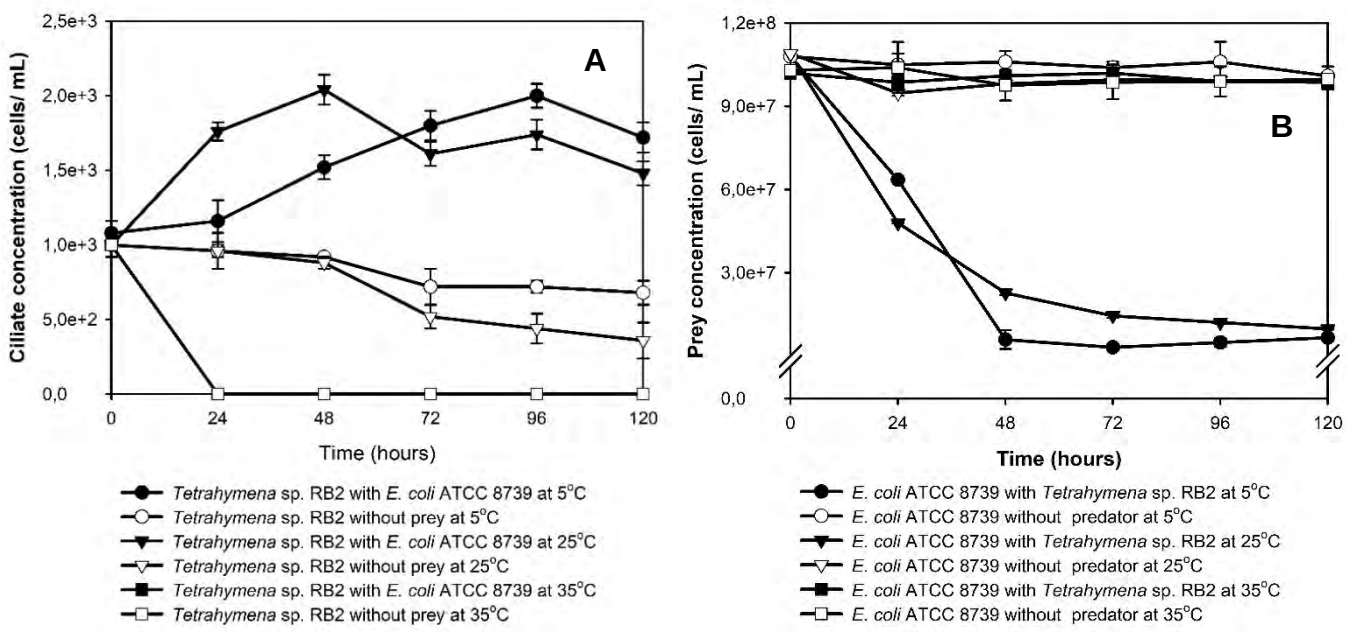

Figure 2.37. Growth of *Tetrahymena* sp. RB2 with *E. coli* ATCC 8739 (A) and the change of *E. coli* ATCC 8739 concentrations in the presence and absence of *Tetrahymena* sp. RB2 (B) in Chalkley's medium at temperatures of 5, 25, and 35ºC in the dark. All data are means of two independently performed experiments. Error bars represent the difference between the means.

Growth of *Tetrahymena* sp. RB2 was observed at the temperatures of 5ºC and 25ºC. However, as expected, growth at 5ºC was slower than at 25ºC with a lag phase of 24 hours, and thereafter the exponential growth for 72 hours was observed. While at 25ºC, no lag phase was observed. The temperature of 35ºC was lethal to *Tetrahymena* sp. RB2, cells of this ciliate were not detected after 24 hours incubation. Though growth was much slower at 5ºC than at 25ºC, the reduction of *E. coli* ATCC 8739 was similar at both temperatures.

The experimental data generated from the growth of *Tetrahymena* sp. RB2 with *E. coli* ATCC 8739 was used to establish the growth and grazing kinetics of the ciliate at different temperatures.

Table 2.15. Growth and grazing kinetics of *Tetrahymena* sp. RB2 with *E. coli* ATCC 8739 at different temperatures in Chalkley's medium in the dark

Temperature (ºC)	Specific growth rate (hr^{-1})	Doubling time (hr)	Grazing rate (bacteria x $pred^{-1}$ x hr^{-1})	Clearance rate (nL x $pred^{-1}$ x hr^{-1})
5	0.0061	108.56	$1.00 \times 10^3 – 9.94 \times 10^2$	9.22
25	0.0148	48.26	$1.59 \times 10^3 – 7.82 \times 10^2$	16.06
35	-	-	0	0

The values are the average of two independently performed experiments. (-) indicate that ciliate cells were not present in cultures after 24 hours incubation. Grazing rates were corrected for the increased number of individual cells number during exponential growth.

Growth and grazing of *Tetrahymena* sp. RB2 was detected at both 5 and 25ºC. Though grazing rates of *Tetrahymena* sp. RB2 at 25ºC were only slightly higher than at 5ºC, growth rates at 25ºC were two-fold higher than growth rates at 5ºC.

2.3.4. Oxygen uptake rates of the isolated ciliates

The respiration of the three isolated ciliates, which is assumed to represent the metabolic rates, was studied with an oxygen electrode. This metabolic activity widely depends on the physiological state of the cell; therefore, the oxygen uptake was determined for starving cells (endogenous respiration) and actively feeding cells (exogenous respiration).

Table 2.16. Respiration rates of isolated ciliates of starved and actively feeding ciliate cells with heat-killed bacterial prey at 25ºC in Chalkley's medium

Predator/ prey	Respiration rates nL O_2 x ciliate^{-1} x hr^{-1}	
	Respiration of starving ciliates	Respiration with prey cells present
***Coleps* sp. RB3**		
E. coli ATCC 8739	9.39×10^{-3} (1.60×10^{-4})	1.35×10^{-2} (7.00×10^{-4})
S. Typhimurium ATCC 14028	9.67×10^{-3} (5.50×10^{-4})	1.13×10^{-2} (7.00×10^{-4})
Staphylococcus sp. CPS23	1.09×10^{-2} (9.00×10^{-4})	1.29×10^{-2} (1.40×10^{-4})
***Paramecium* sp. RB1**		
E. coli ATCC 8739	2.38×10^{-2} (6.00×10^{-4})	3.54×10^{-2} (6.20×10^{-3})
S. Typhimurium ATCC 14028	3.23×10^{-2} (6.00×10^{-3})	3.77×10^{-2} (3.00×10^{-3})
Staphylococcus sp. CPS23	3.09×10^{-2} (5.40×10^{-3})	3.56×10^{-2} (3.20×10^{-3})
***Tetrahymena* sp. RB2**		
E. coli ATCC 8739	3.31×10^{-3} (8.70×10^{-4})	4.28×10^{-3} (2.29×10^{-3})
S. Typhimurium ATCC 14028	3.53×10^{-3} (3.33×10^{-3})	4.34×10^{-3} (2.52×10^{-3})
Staphylococcus sp. CPS23	2.85×10^{-3} (4.20×10^{-4})	2.97×10^{-3} (5.90×10^{-4})

The values presented are the means of two independently performed experiments. Values in brackets represent the difference between means.

The respiration rates of starving ciliate cells (endogenous respiration) were lower than that of actively feeding cells. Upon the addition of heat-killed bacterial cells to a suspension containing a starved culture of the isolated ciliates, the respiration rates increased by 18 to 44% for *Coleps* sp. RB3, 15 to 49% for *Paramecium* sp. RB1 while respiration of *Tetrahymena* sp. RB2 increased by 4 to 29%.

2.3.5. Uptake of food particles and formation of food vacuoles by the isolated ciliates

Phagotrophy of ciliates has generally been studied with particles that can be traced from the point of ingestion to egestion. Filter feeding, a mechanism employed by bacterivorous ciliates to take up food particles, involves the use of the oral cavity where food particles are collected in the cytostomes via cilia membranelles and packaged in food vacuoles where digestion occurs. Therefore, a short-term experiment designed to monitor the uptake of fluorescently labelled particles and the formation of food vacuoles was carried out to determine the kinetics of food uptake and food vacuole formation.

2.3.5.1. Uptake of food particles by *Coleps* sp. RB3

Phagotrophy of *Coleps* sp. RB3 was studied using FLB *E. coli* ATCC 8739 to determine the uptake of food particles and the formation of food vacuoles.

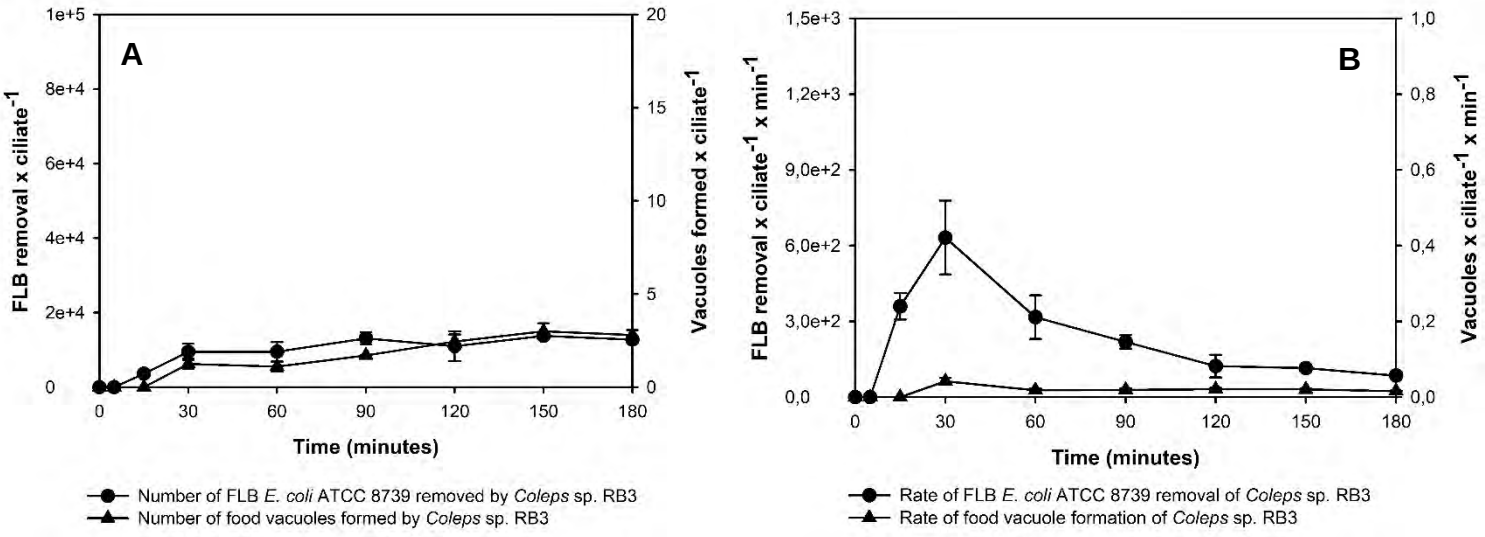

Figure 2.38. The number of bacterial cells removed and the number of food vacuoles formed (A), and the rate of prey cell removal and food vacuole formation (B) by *Coleps* sp. RB3 feeding on fluorescently labelled *E. coli* ATCC 8739 in Chalkley's medium at 25 ± 2ºC in the dark. Error bars represent the SEM for at least 20 ciliate cells (n=3).

The presence of FLB *E. coli* ATCC 8739 induced uptake and formation of food vacuoles in *Coleps* sp. RB3. Labelled bacterial cells were consumed and packaged in food vacuoles within the cells of *Coleps* sp. RB3. Vacuoles containing ingested FLB *E. coli* ATCC 8739 were only detected in ciliate cells after 15 minutes of incubation. There was an initial linear increase in the rates of FLB uptake, which decreased after the maximum rates were reached. The rates of uptake and vacuole formation reached their peak at 1.32 x 10^3 particles x ciliate^{-1} x minute^{-1} and 0.010 vacuoles x ciliate^{-1} x minute^{-1} after 30 minutes.

To verify uptake of FLB *E. coli* ATCC 8739, the cells of *Coleps* sp. RB3 were examined microscopically using Confocal Laser Scanning Microscopy (CLSM).

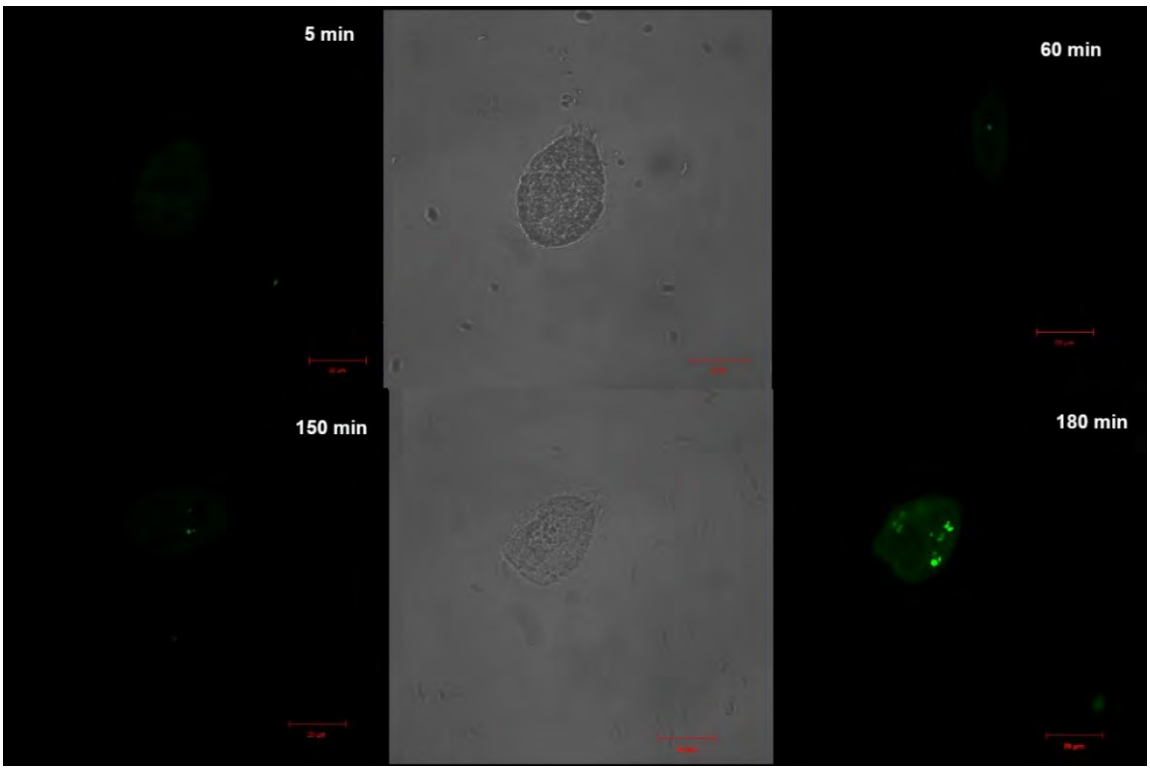

Figure 2.39. CLSM micrographs of *Coleps* sp. RB3 showing the formation of food vacuoles containing fluorescently labelled *E. coli* ATCC 8739 over time in Chalkley's medium at 25 ± 2ºC in the dark.

FLB *E. coli* ATCC 8739 cells were ingested and packaged in food vacuoles within the cell of *Coleps* sp. RB3. Food vacuoles were only detected after 30 minutes of feeding on labelled prey cells, with an average of 3 food vacuoles formed per cell of *Coleps* sp. RB3 after 3 hours of incubation.

Microparticles have been employed in various studies of protist feeding. They can be easily traced as they are not digested to evaluate the food vacuole cycle within the ciliate. Therefore, fluorescently labelled microbeads were fed to the cells of *Coleps* sp. RB3 to study the uptake and food vacuole formation.

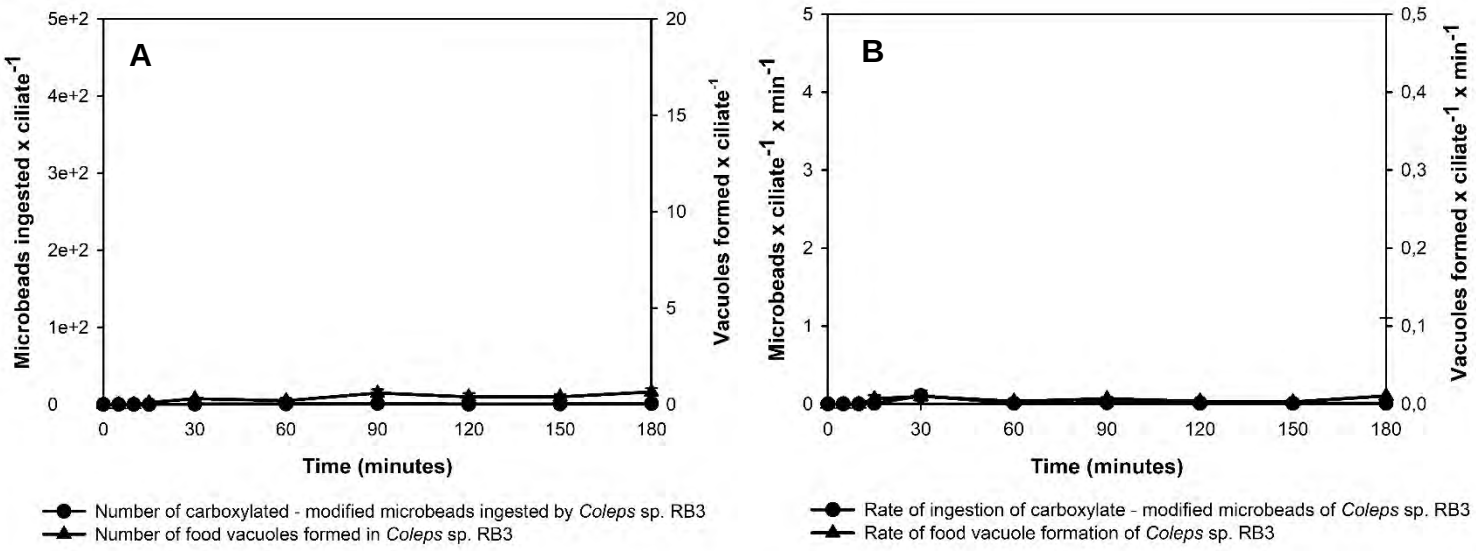

Figure 2.40. The number of ingested 2 µm microbeads and the number of food vacuoles formed (A) and the rate of microbeads uptake and vacuole formation (B) by *Coleps* sp. RB3 feeding on 2 µm red fluorescently labelled carboxylate-modified microbeads in Chalkley's medium at 25 ± 2ºC in the dark. Error bars represent the SEM for at least 20 ciliate cells (n=3).

Although the presence of microbeads induced particle uptake and food vacuole formation, an average of 1.25 microbeads per individual ciliates were detected within cells of *Coleps* sp. RB3 after 180 minutes of feeding. A lag time of 15 minutes was observed before food vacuoles containing 2 µm microbeads were detected

CLSM analysis was not successful in capturing an image of *Coleps* sp. RB3 with ingested 2 µm fluorescently labelled microbeads.

2.3.5.2. Uptake of food particles by *Paramecium* sp. RB1

Phagotrophy of *Paramecium* sp. RB1 was studied with FLB *E. coli* ATCC 8739 to determine the rate of uptake of food particles and food vacuole formation.

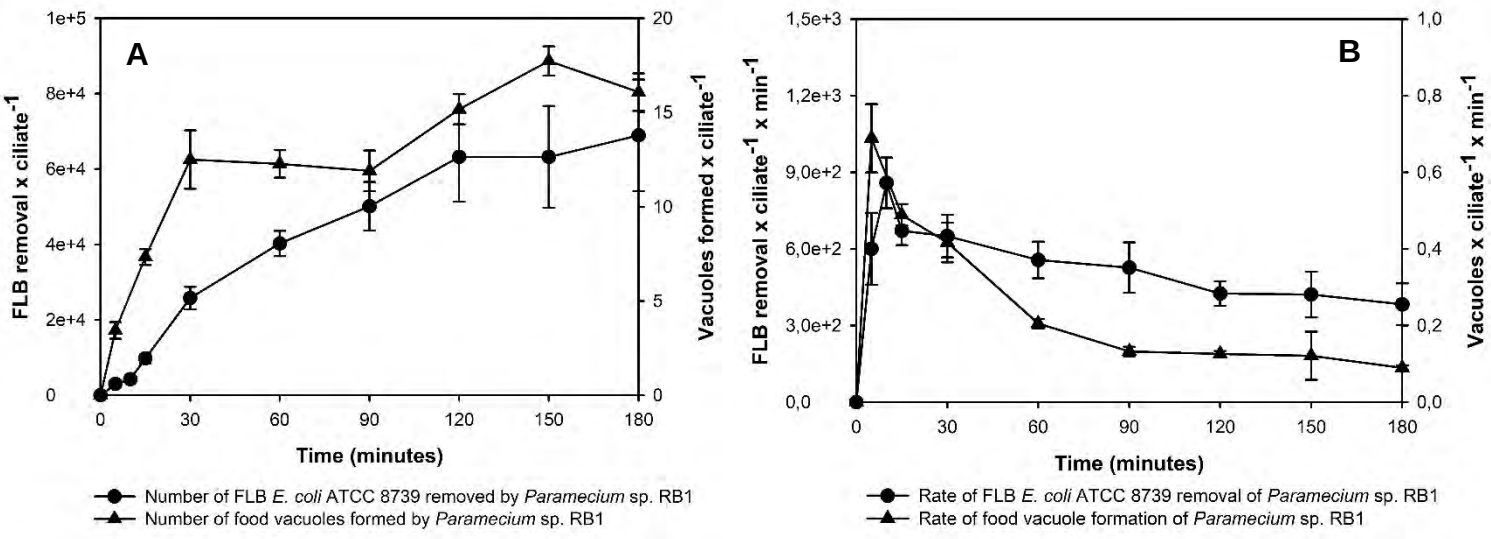

Figure 2.41. The number of bacterial cells removed and the number of food vacuoles formed (A) and the rate of prey cell removal and food vacuole formation (B) by *Paramecium* sp. RB1 feeding on fluorescently labelled *E. coli* ATCC 8739 in Chalkley's medium at 25 ± 2ºC in the dark. Error bars represent the SEM for at least 20 ciliate cells (n=3).

Paramecium sp. RB1 readily ingested FLB *E. coli* ATCC 8739 as vacuoles containing FLB particles were detected after 5 minutes of feeding. Increase in the number of food vacuoles containing FLB *E. coli* ATCC 8739 in *Paramecium* sp. RB1 proceeded with an increase in the number of FLB cells removed in the cultures. A linear increase in the rate of particle uptake and food vacuole formation was observed, which then decreased after the maximum rates were reached. The maximum rate of removal, 1.41 x 10^3 bacteria x ciliate^{-1} x minute^{-1}, was reached after 10 minutes of feeding, while that of vacuole formation (0.69 vacuoles x ciliate^{-1} x minute^{-1}) was reached after 5 minutes.

To verify the uptake of FLB *E. coli* ATCC 8739 by *Paramecium* sp. RB1, the cells were analyzed by Confocal Laser Scanning Microscopy (CLSM).

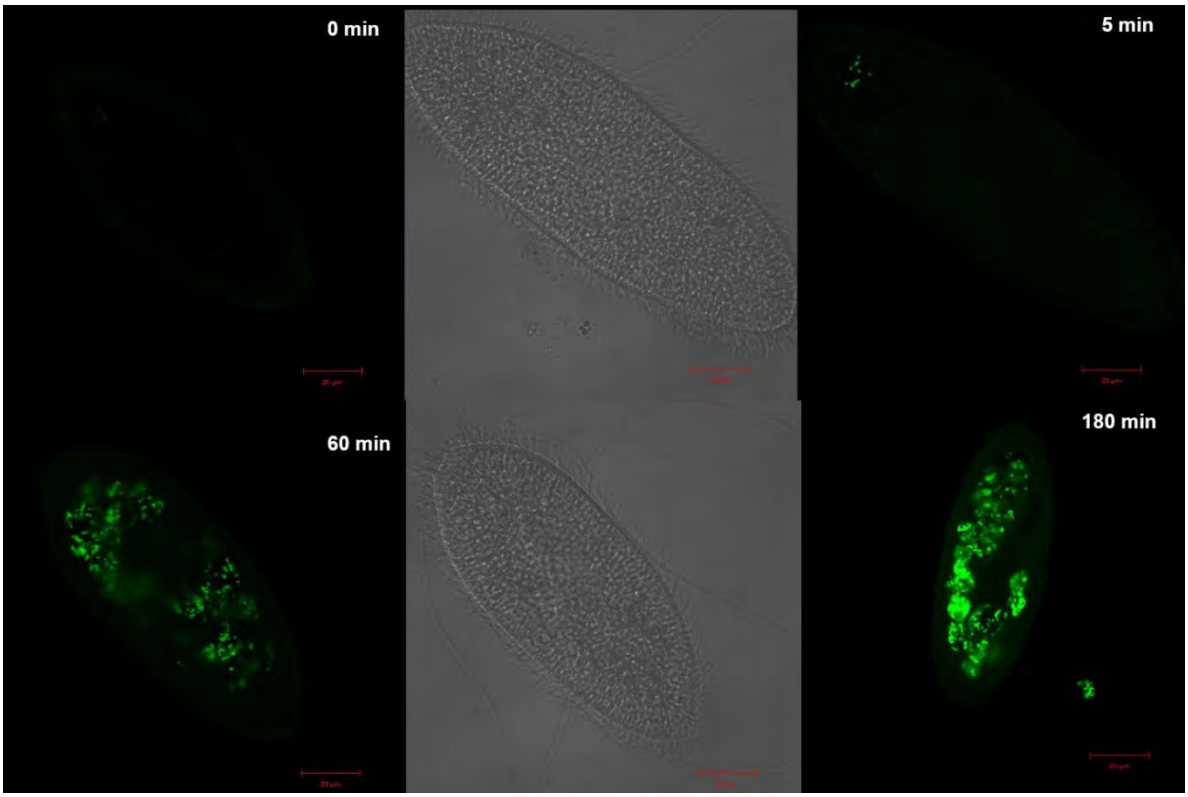

Figure 2.42. CLSM micrographs of *Paramecium* sp. RB1 showing the formation of food vacuoles containing fluorescently labelled *E. coli* ATCC 8739 over time in Chalkley's medium at 25 ± 2ºC in the dark.

The presence of FLB *E.coli* ATCC 8739 induced uptake and formation of food vacuoles within the cells of *Paramecium* sp. RB1. The vacuoles containing FLB *E. coli* ATCC 8739 were detected within 5 minutes of feeding on labelled prey. After 3 hours of incubation, an average of 17 vacuoles containing labelled *E. coli* ATCC 8739 were formed within the cells of isolate *Paramecium* sp. RB1.

In addition, artificial food particles were fed to *Paramecium* sp. RB1 to determine the rate of ingestion and food vacuole formation on non-biological particles.

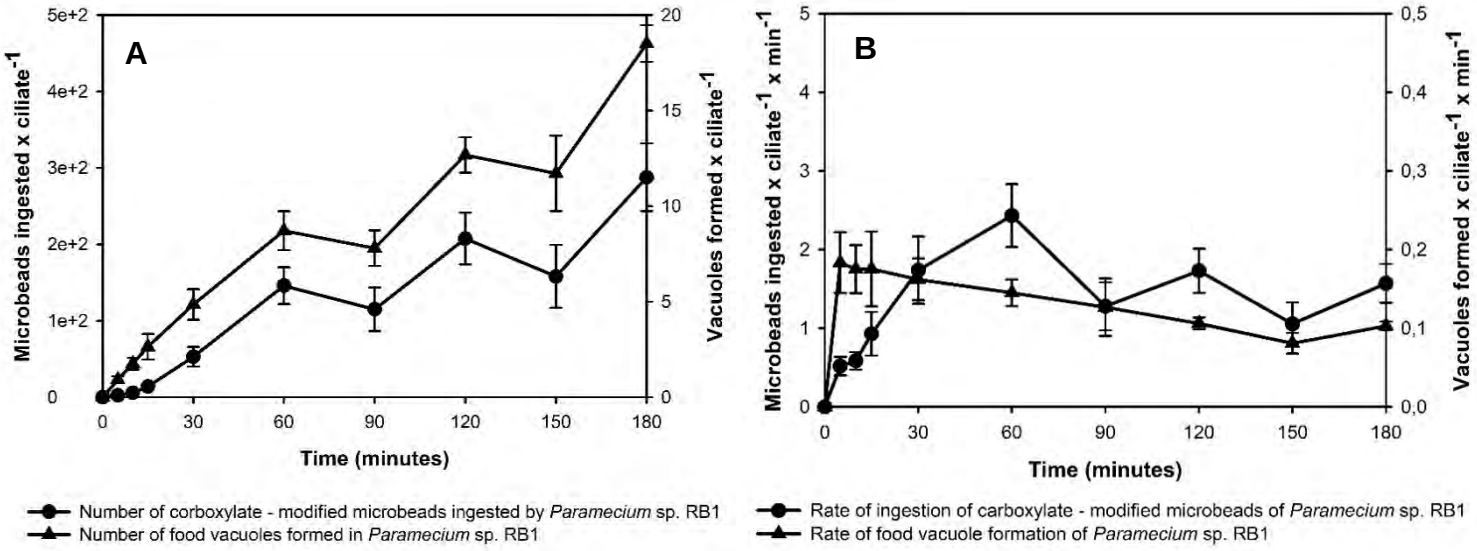

Figure 2.43. The number of ingested 2 µm microbeads and the number of food vacuoles formed (A) and the rate of microbeads uptake and food vacuole formation (B) by *Paramecium* sp. RB1 feeding on 2 µm red fluorescently labelled carboxylate-modified microbeads in Chalkley's medium at 25 ± 2ºC in the dark. Error bars represent the SEM for at least 20 ciliate cells (n=3).

Similar to the FLB *E. coli* ATCC 8739, the presence of microbeads induced uptake and formation of vacuoles in cells of *Paramecium* sp. RB1 and the number of the vacuoles formed increased with the increase in particles ingested by this ciliate. While the rate of food vacuole formation reached its peak at 0.18 vacuoles x ciliate^{-1} x minute^{-1} after 5 minutes of feeding and subsequently declining to 0.1 vacuoles x ciliate^{-1} x minute^{-1}, the maximum ingestion rate of 2.38 particles x ciliate^{-1} x minute^{-1} was reached after 60 minutes.

The uptake of red fluorescently labelled carboxylate-modified microbeads by *Paramecium* sp. RB1 was verified by microscopic analysis using Confocal Laser Scanning Microscopy (CLSM).

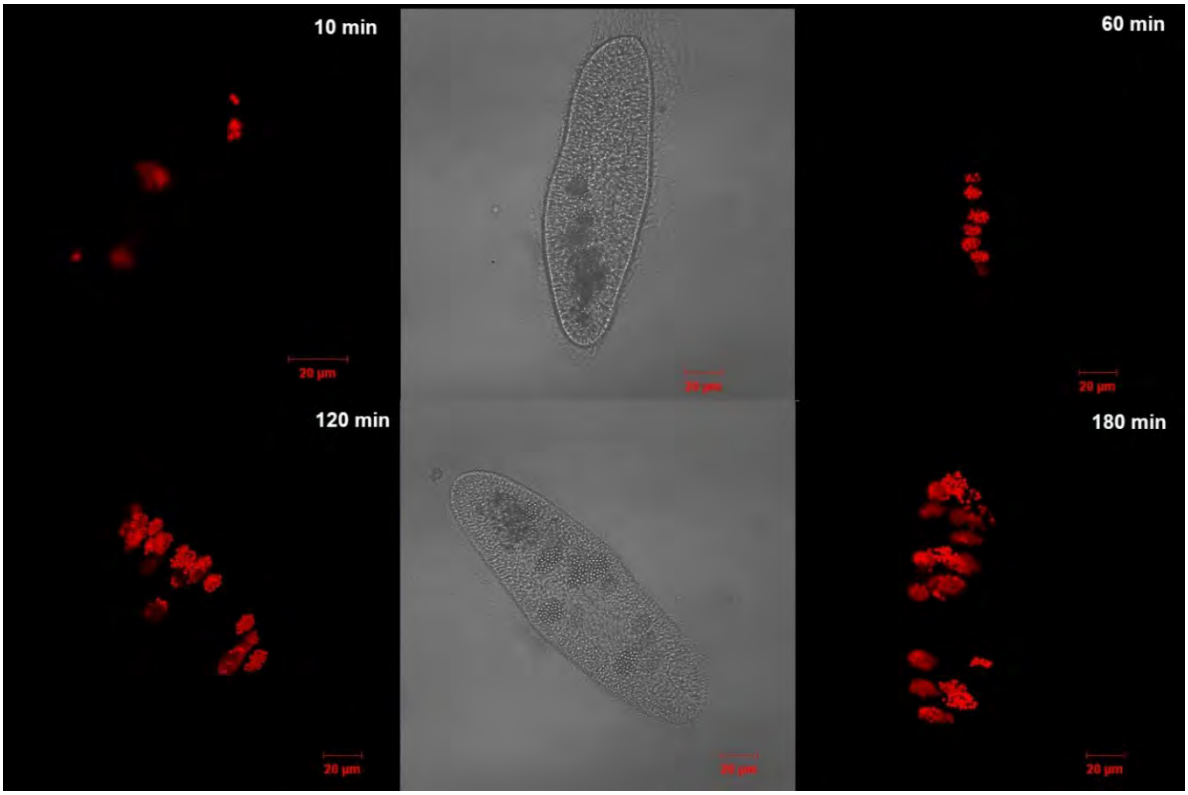

Figure 2.44. CLSM micrographs of *Paramecium* sp. RB1 showing formation on food vacuoles containing 2 µm red fluorescently labelled carboxylate-modified microbeads over a period of 180 minutes of feeding in Chalkley's medium at 25 ± 2ºC in the dark.

As shown in Figure 2.43., *Paramecium* sp. RB1 ingested 2 µm red fluorescently labelled carboxylate-modified microbeads and packaged these in food vacuoles. The average number of food vacuoles formed at the end of 3 hours incubation was 19 vacuoles x ciliate^{-1}.

2.3.5.3 Uptake of food particles by *Tetrahymena* sp. RB2

The uptake and food vacuole formation in *Tetrahymena* sp. RB2 was monitored using FLB *E. coli* ATCC 8739 to determine the phagotrophy kinetics of *Tetrahymena* sp. RB2.

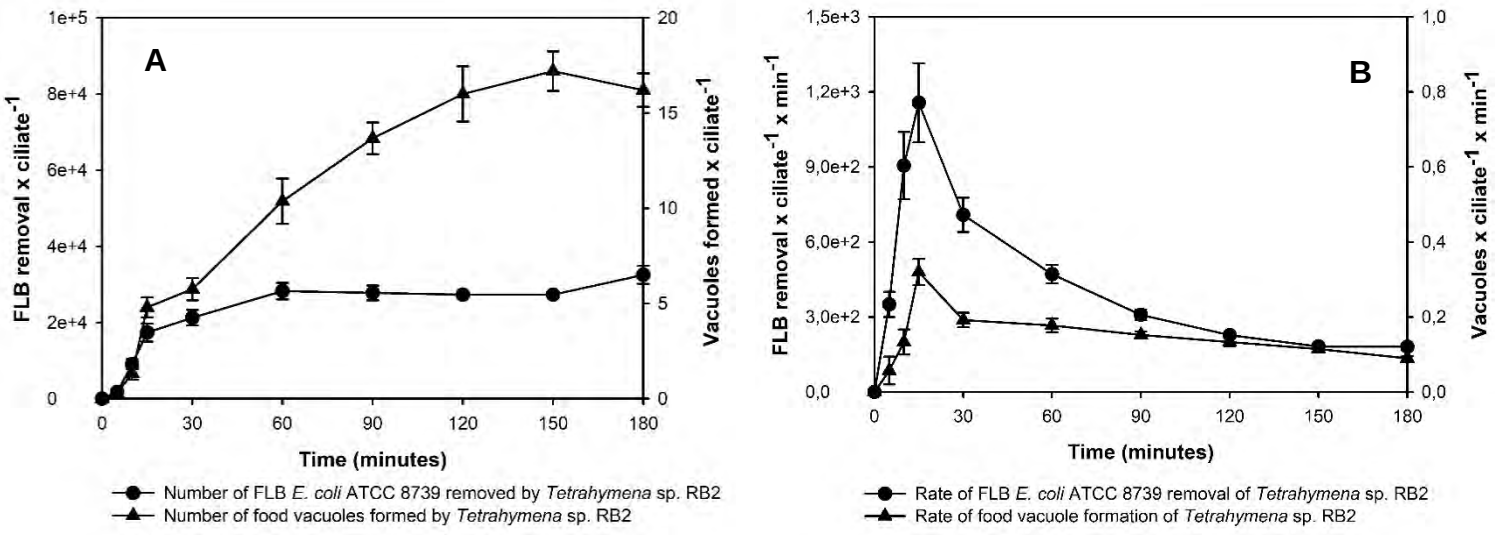

Figure 2.45. The number of bacterial cells removed and the number of food vacuoles formed (A) and the rate of prey removal and food vacuole formation (B) by *Tetrahymena* sp. RB2 feeding on fluorescently labelled *E. coli* ATCC 8739 in Chalkley's medium at 25 ± 2ºC in the dark. Error bars represent the SEM for at least 20 ciliate cells (n=3).

The presence of FLB *E. coli* ATCC 8739 induced the formation of food vacuoles in cells of *Tetrahymena* sp. RB2 and increased over time along with the increase in the number of particles reduced by the ciliate. The maximum rate of removal was 1.16 x 10^3 cells x ciliate^{-1} x minute$^{-1,}$ and the rate of food vacuole formation, 0.32 vacuoles x ciliate^{-1} x minute^{-1} were both reached after 15 minutes of feeding on FLB *E. coli* ATCC 8739.

The uptake of FLB *E. coli* ATCC 8739 by *Tetrahymena* sp. RB2 was confirmed by Confocal Laser Scanning Microscopy (CLSM).

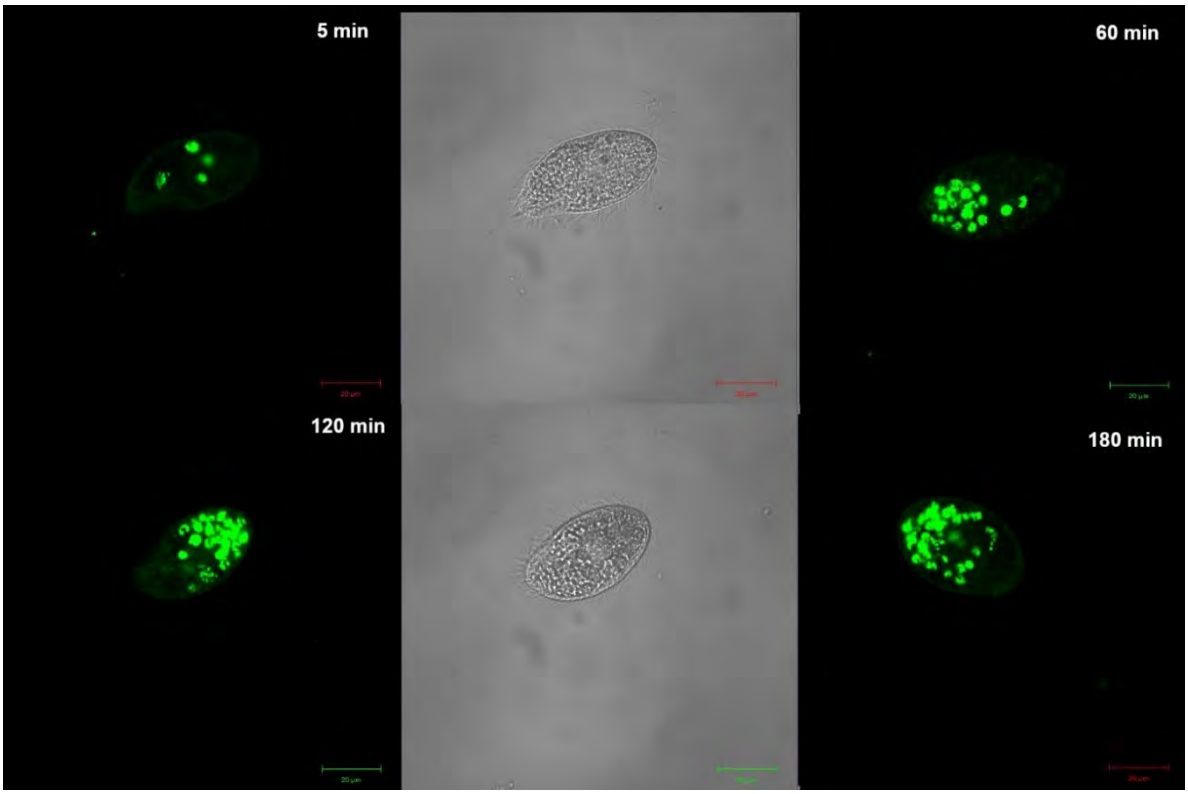

Figure 2.46. CLSM micrographs of *Tetrahymena* sp. RB2 showing formation on food vacuoles containing fluorescently labelled *E. coli* ATCC 8739 over a period of 180 minutes of feeding in Chalkley's medium at 25 ± 2ºC in the dark.

The presence of FLB *E. coli* ATCC 8739 induced uptake and formation of food vacuoles in *Tetrahymena* sp. RB2. The food vacuoles containing FLB cells were detected within 5 minutes of feeding. An individual cell of *Tetrahymena* sp. RB2 had an average of 16 food vacuoles present after 3 hours of feeding on FLB *E. coli* ATCC 8739.

In addition, 2 µm microparticles were added to the culture of *Tetrahymena* sp. RB2 to determine the uptake kinetics of this ciliate on non-nutritious particles.

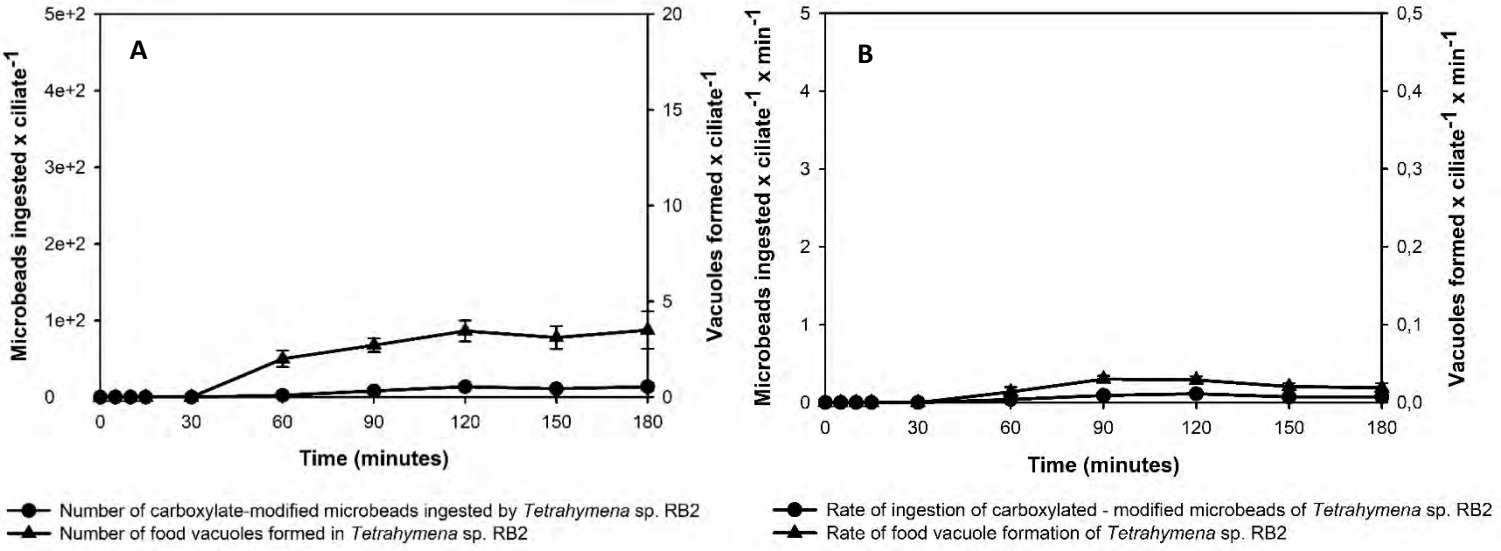

Figure 2.47. The number of ingested 2 µm microbeads and the number of food vacuoles formed (A) and the rate of microbeads uptake and vacuole formation (B) by *Tetrahymena* sp. RB2 feeding on 2 µm red fluorescently labelled carboxylate-modified microbeads in Chalkley's medium at 25 ± 2ºC in the dark. Error bars represent the SEM for at least 20 ciliate cells (n=3).

As with FLB *E. coli* ATCC 8739, *Tetrahymena* sp. RB2 was able to ingest fluorescently labelled red carboxylated microbeads, which induced the formation of food vacuoles. Vacuoles containing the red particles were, however, only detected after 60 minutes of feeding and increased with an increase in the number of particles ingested. The maximum rate of ingestion, which was 0.174 particles x ciliate^{-1} x minute^{-1}, was reached after 180 minutes of feeding, while the maximum rate of vacuole formation, 0.075 vacuoles x ciliate^{-1} x minute^{-1}, was reached after 90 minutes of feeding on these microbeads.

The uptake of red fluorescently labelled carboxylate-modified microbeads by *Tetrahymena* sp. RB2 was verified by Confocal Laser Scanning Microscopic (CLSM) analysis.

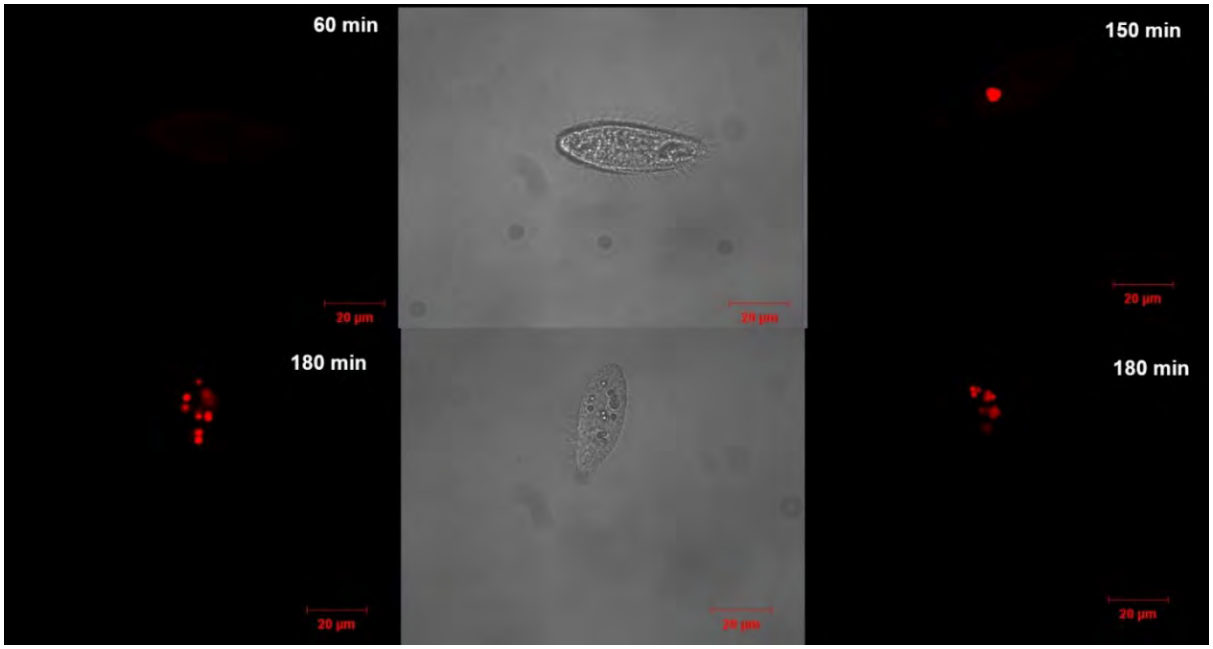

Figure 2.48. CLSM micrographs of *Tetrahymena* sp. RB2 showing the formation of food vacuoles over time while feeding on 2 µm red fluorescently labelled carboxylate-modified microbeads in Chalkley's medium at 25 ± 2ºC in the dark.

Food vacuoles containing red fluorescently labelled microbeads were detected inside the cells of *Tetrahymena* sp. RB2 after 30 minutes of feeding. An average of 4 vacuoles x ciliate^{-1} were formed by this ciliate while feeding on red fluorescently labelled microbeads.

2.4. Discussion

Identification and characterization of isolated ciliates

Predator-prey interaction involving protists and bacteria has been extensively studied in freshwater environments. However, studies have focused mostly on flagellates as important consumers of bacteria. Because of their ubiquitous nature and feeding strategy, ciliates are an important group of bacterivorous protists that plays a major role in the regulation of bacteria in various aquatic environments (Pernthaler, 2005; Corno *et al*., 2008; Hisatugo *et al.,* 2014; Weisse 2017). As such studies are scarce for South Africa, three ciliates were successfully isolated from the freshwater samples collected from a local stream in Pietermaritzburg, KwaZulu-Natal, South Africa. These ciliates were selected and identified based on the typical phenotypic characteristics of species belonging to the phylum *Ciliophora*, i.e., ciliature and swimming pattern. Based on the light microscopy and SEM analysis, all three isolates were holotrich ciliates and were presumptively identified to belong to the genus of *Coleps* (Figure 2.1. and 2.2.), *Paramecium* (Figure 2.3. and 2.4.), and *Tetrahymena* (Figure 2.5. and 2.6.), as they resembled the typical morphology of species within these genera.

Ciliates were traditionally and are still classified taxonomically using their distinct morphological characteristics via microscopic analysis (Finlay, 2004; Dopheide *et al*., 2009; Foissner *et al.,* 2007). Ciliates are a diverse clade of eukaryotic organisms that are morphologically complex and distinct. Their functional cellular features allowed for classification to the genus level (Gao *et al*., 2016; Abraham *et al*., 2019; Trogant *et al.,* 2020). This, however, does not provide information on their genetic relatedness and can be insufficient in identifying species that exhibit almost similar structural features. Therefore, molecular techniques have become increasingly important over the last three decades and are now integrated with morphological characterization for the identification and taxonomic classification of protists (Weisse, 2014; Warren *et al.,* 2017). To confirm genus identity, molecular analysis was employed for two of the three isolated ciliates and determine their genetic relatedness with the species that have been previously identified and published. The small ribosomal unit has been proven to be the best marker for genus and species identification in eukaryotes as it comprises of both conserved and variable regions (Regensbogenova *et al.,* 2004; Zhang *et al*., 2015). The primers used for the amplification of the 18S rRNA gene extracted from

ciliates were designed to amplify almost a full length of the small ribosomal unit gene, which ranges from 1500 to 1900 base pairs in ciliates. PSSU-342f and Medlin B primers, and EukF and EukR primers, which amplify the 18S rRNA gene fragment are protozoa and eukarya specific that are complementary to the conserved region, thus provide an accurate taxonomical classification (Medlin *et al*., 1988; Karnati *et al*., 2003; Regensbogenova *et al.,* 2004). Isolate RB1 and RB2 shared more than 99% sequence similarity with species of the class *Oligohymenophorea*. For taxonomic assignments of eukaryotes, a sequence similarity level of more than 97% is indicative of the genus assignment (Caron *et al*., 2009; Wu *et al*., 2015; Gimmler *et al*., 2016). Therefore, isolate RB1 belongs to the genus *Paramecium* with 100% similarity to *Paramecium multimicronucleatum,* and isolate RB2 belongs to the genus *Tetrahymena* with 100% similarity to *Tetrahymena setosa* (Table 2.2. and 2.3.). The phylogenetic tree, which indicates the genetic relatedness of organisms, revealed that the isolated ciliates cluster with the ciliates of the class *Oligohymenophorea*. While RB1 was grouped with selected ciliates of genus *Paramecium* representing the "*aurelia*" group (Figure 2.8.), RB2 was grouped with species of the genus *Tetrahymena* representing *Tetrahymena* "*borealis*" group (Figure 2.9.).

Species of the genus *Coleps* are classified as *Prostomatea* within the order *Prorodontida*. They are characterized by typical morphological characteristics, i.e., are a barrel-shaped cell with armour-like calcified plates, an oral cavity at the anterior end, and prominent spikes at the posterior end of the cell (Noland, 1925; Corliss, 1979; Foissner *et al*., 2008; Chen *et al*., 2012; Lu *et al*., 2016). They are morphologically similar, with more than 20 species assigned to the genus *Coleps*; however, they have a distinct feature that can be described into either of the 5 morphospecies (Foissner *et al*., 2008; Pröschold *et al.,* 2021).

Species of the genus *Paramecium* are classified as *Oligohymenophorea* within *Peniculida*. They are typically characterized by a distinct morphological characteristic that has only been described for this ciliate, i.e., the position of the nucleus between two contractile vacuoles surrounded by six water canals located at both ends of the cell (Fokin and Chivilev, 2000; Bhamare *et al*., 2012). Despite being the well-studied ciliate within the peniculine ciliates, the taxonomy and phylogeny of *Paramecium* is yet to be resolved, and some species are still regarded as invalid. Based on the morphological characteristics, only 18 morphospecies are considered valid presently

and are grouped into two distinct clades of *Paramecium*, the “bursaria” and “aurelia” groups. Species of the subgroup “*aurelia*”, including *Paramecium multimicronucleatum*, are morphologically and biologically similar with a typical cigar-shaped cell and are confined to freshwater environments (Sonneborn, 1975; Strüder-Kypke *et al.,* 2000; Krenek *et al.,* 2015).

Ciliates of the genus *Tetrahymena* are classified as *Oligohymenophorea* within *Hymenostomatida;* they have a distinct oral morphology that is typical for species of this genus (Elliot, 1959). Only 44 species of *Tetrahymena* are recognised presently, and most are considered as free-living bacterivores (Doerder, 2018). Although molecular analysis has provided distinction of species of genus *Tetrahymena* into two major clades, the “*borealis*” group being larger and diverse than the “*australis”* group, their morphological characteristics are similar (Chantangsi and Lynn, 2008).

All three ciliates are regularly detected in various freshwater environments and have been detected in wastewater treatment plants, even in three of the aeration tanks of wastewater treatment plants in the province of Eastern Cape, South Africa (Sibewu *et al*., 2008).

Influence of prey concentration on the growth and grazing of isolated ciliates

Ciliates are mainly heterotrophs that obtain their energy by consumption of food particles (Sherr *et al*., 1986; Montagnes *et al*., 2008; Gong *et al*., 2016). For the three isolated ciliates, heterotrophy was demonstrated as their growth was solely due to the consumption of bacterial prey or other live microscopic eukaryotes. Ciliate numbers increased with a decrease in digestible prey concentrations and declined without prey. Studies have shown that the growth of ciliates is concentration-dependent and that the ingestion rate is a function of prey concentration (Fenchel, 1980a; Jonsson, 1986; Šimek *et al*., 2000; Weisse *et al*., 2002). It is therefore not surprising that the growth of the isolated ciliates increased with an increase in bacterial concentration from 10^7 to 10^{10} bacteria x mL^{-1} for *Coleps* sp. RB3 (Figure 2.10.) and *Tetrahymena* sp. RB2 (Figure 2.28.), and from 10^9 to 10^{10} bacteria x mL^{-1} for *Paramecium* sp. RB1 (Figure 2.18.). The results demonstrate that there is a critical concentration of bacteria required to support ciliate growth; for *Coleps* sp. RB3 and *Tetrahymena* sp. RB2 that concentration was 10^7 bacteria x mL^{-1}, while 10^9 bacteria x mL^{-1} was the threshold

concentration for *Paramecium* sp. RB1. The threshold concentration that has been previously reported for *Tetrahymena* sp. was 1.8 x 10^7 bacteria x mL^{-1} (Seto & Tazaki, 1971; Berk *et al*., 1976). Although the growth of *Paramecium* sp. RB1 was not observed at concentrations of 10^8 bacteria x mL^{-1}, grazing rates were detected, albeit very low; thus, the prey concentration was only sufficient to sustain the population of this ciliate (Table 2.8.).

Ingestion and food vacuole formation is induced by the presence of food particles, and their rate is determined by food concentrations. When prey concentration is below the threshold level, the contact probability between the prey and the predator is reduced; thus, the grazing rate is limited by food particles (Zubkov and Sleigh, 1996). At high concentrations, the rate at which the ciliate cell forms food vacuoles and digest ingested food particles becomes a limiting factor. Filter feeders shift and process a constant volume of water per unit time; therefore, the ingestion rates increase with an increase in prey concentration until maximum growth rate has been reached (Laybourn and Stewart, 1975; Fenchel, 1980a). Similar to growth rates, the grazing rates of all three isolated ciliates increased with an increase in prey concentration with maximum grazing rates at 10^{10} bacteria x mL^{-1} (Table 2.4., 2.8., and 2.12.). Prey concentration above 10^{10} bacteria x mL^{-1} caused cell lysis within 30 minutes of the addition of prey biomass due to prey clogging the oral groove and probably reduced oxygen flow due to the densely concentrated cultures.

In addition to reducing the total bacterial numbers, the grazing activities of the three isolated ciliated reduced viability of *E. coli* ATCC 8739. The highest impact of ciliate grazing on the viability of bacteria, 90% reduction, was observed from 10^7 to 10^{10} bacteria per mL. Even at a concentration below the threshold (10^7 bacteria x mL), viable *E. coli* ATCC 8739 was reduced by 80% by *Tetrahymena* sp. RB2, 60% by *Paramecium* sp. RB1, while *Coleps* sp. RB3 achieved a 90% reduction of viable bacteria (Figure 2.11., 2.19., and 2.29.). This indicates that grazing by ciliates is important in controlling and reducing bacteria in aquatic environments, particularly in environments with sufficient prey numbers to support growth and grazing.

Fenchel (1980b) reported that a prey concentration of 10^6 bacteria x mL^{-1} is sufficient to allow the growth of ciliates and that bacterivory of ciliates is considered important when bacterial abundance is above 5 x 10^6 per mL in freshwater environments. For

the three isolated ciliates, bacterivory can therefore only occur in environments where bacterial density is above 10^7 per mL. In oligotrophic aqueous natural environments, ciliates do not encounter bacteria at high density except in polluted water, sludge, and sediments rich in organic matter where bacterial densities can reach and even exceed 10^8 bacteria x mL^{-1}. The influence of prey concentration on the growth and feeding of ciliates, even at high bacterial concentrations, is useful for determining their feeding potential and ecological impact in such natural environments and man-made systems such as wastewater treatment plants.

Growth and grazing of the isolated ciliates on various bacteria

Bacteria are the predominant food source of bacterivorous ciliates in aquatic environments. Studies have demonstrated that different species of bacteria may be of different nutritional value to ciliates; moreover, different ciliate species may have different responses to the same bacterial species (Curds and Vandyke, 1966; Barna and Weis, 1973; Taylor and Berger, 1976; Matz and Kjelleberg, 2005; Posch *et al.,* 2015). The isolated ciliates exhibited different growth patterns and growth rates with different species of bacteria; thus, growth responses of the isolated ciliates were species-specific (Figure 2.12., 2.13., 2.20., 2.21., 2.30. and 2.31.).

The growth of the isolated ciliates followed a typical growth curve. A lag phase that is influenced by the type of bacteria on which the ciliate is grazing; thus, it was observed when grazing on some bacteria, and the length varied with the type of prey. The lag time before population increase was observed with *Coleps* sp. RB3 when grazing on *Pseudomonas* sp. strain B12 and *Vibrio* sp. strain KM1 (Figure 2.12.) and *Paramecium* sp. RB1 with *B. subtilis* BGA, which lasted for 24 hours (Figure 2.21.). Given enough prey, ciliates have the potential to reach their maximum population density, decreasing prey concentrations to below their required growth threshold, after which the ciliate population starts to decline. The growth of ciliates has been generally studied at bacterial concentrations that naturally occur in aquatic environments. Madoni *et al.* (1990) reported doubling times of *Coleps hirtus* ranging from 34 – 44 hours when feeding on bacteria at a concentration of 2 – 5 x 10^5 cells x mL^{-1}. Maximum generation times of 7.62 hours (at 30ºC, pH 9) and 8.35 hours (at 27ºC, pH 7) were reported for two strains of *P. caudatum*, strain PC1, and strain PC2 at concentrations of 10^6

bacteria per mL (Alves *et al.*, 2016). Under optimal conditions, *T. thermophila* can grow rapidly with a doubling time of less than 2 hours (Cassidy-Hanley, 2012). While growing in an axenic medium at 30ºC, *T. thermophila* had a generation time of only 1.4 hours (Kiy and Tiedtke, 1992). Generation times established for *Coleps* sp. RB3 ranged from 22 – 62 hours for Gram-negative bacteria and much slower growth with Gram-positive bacteria with recorded generation times of 45 – 111 hours when grazing at concentrations of 10^{10} bacteria x mL^{-1} at 25ºC (Table 2.5.). Growth of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 with selected bacteria was slower than previously reported, with generation times established for *Paramecium* sp. RB1 ranging from 21 – 105 hours at 10^{10} bacteria x mL^{-1} (Table 2.9.) and *Tetrahymena* sp. RB2 ranged from 12 – 176 hours at 10^{8} bacteria x mL^{-1} (Table 2.13.) at 25ºC.

Once prey concentration is below the threshold concentration, the ciliate population starts to die off due to starvation. Habte and Alexander (1978) and Watson *et al.* (1981) observed that *T. pyriformis* cease feeding before prey density is completely depleted. While some ciliates can endure starvation for days, others die off within 1 or 2 days without sufficient food available. This has an impact on the microbial food web as ciliate may not be available as prey for larger protists or zooplankton for the transfer of energy and carbon matter (Montagnes *et al.,* 1996). Only *Coleps* sp. RB3 died off once the prey concentration of some bacterial species had reached the critical threshold, while *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 endured for longer with limited prey concentrations, and their cells were detected at the end of 5 days incubation period.

Ciliates are described as voracious bacterial grazers, which have been reported to produce grazing rates of more than 10^{3} bacteria x $ciliate^{-1}$ x $hour^{-1}$ and clearance rates ranging from 12 to 156 nL x $ciliate^{-1}$ x $hour^{-1}$ (Sherr *et al.,* 1988; Kisand and Zingel, 2000; Pauli *et al.,* 2001; Zingel *et al*., 2007). Grazing rates of freshwater ciliates for bacteria were reported to range from 3 – 5 x 10^{5} bacteria per ciliate x hour, with most values between 3 x 10^{2} and 5 x 10^{3} bacteria per ciliate x hour (Bott and Kaplan, 1990; Eisenmann *et al*., 1998). Grazing and clearance rates for the three isolated ciliates were within the range that has been previously reported for ciliates. When grazing at prey concentrations of 10^{10} bacteria x mL^{-1}, *Coleps* sp. RB3, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 had grazing rates ranging from 10^{4} to 10^{5} bacteria x $ciliate^{-1}$ x $hour^{-1}$, and their clearance rates ranged from 5 to 33 nL x $ciliate^{-1}$ x $hour^{-1}$ for *Coleps*

sp. RB3, 13 to 40 nL x ciliate^{-1} x hour^{-1} for *Tetrahymena* sp. RB2 and 5 to 34 nL x ciliate^{-1} x hour^{-1} for *Paramecium* sp. RB1 (Table 2.5., 2.9., and 2.13.).

Fenchel (1980b) demonstrated that holotrich ciliates can clear from 3 x 10^3 to 10^4 times their own cell size per hour. A small ciliate like *Tetrahymena pyriformis* has the potential to clear up to 5 x 10^{-5} mL h^{-1} (50 nL x hr^{-1}). In comparison, the larger-sized *Paramecium caudatum* can achieve clearance rates of 2 x 10^{-4} to 10^{-3} mL h^{-1} (200 to 1000 nL x hr^{-1}) depending on the size of the cell. However, the study by Fenchel (1980b) was carried out using latex beads and did not consider factors that may influence grazing on live prey cells. Different species of ciliates feed on different species of bacteria at varying efficiency; some prey organisms are easily ingested due to their morphological characteristics, while some may escape phagocytosis or be rejected by the protists (González *et al*., 1990b; Šimek *et al.,* 1997; Rønn *et al*., 2002; Corno and Jürgens, 2006). Characteristics such as the type of bacteria, cell size, motility, and surface properties are major determinants influencing the grazing efficiency of ciliates (Matz and Kjelleberg, 2005). Ciliates are selective feeders, and their ingestion is mainly influenced by the size of the prey; there is a preferred size range of prey at which ciliate can ingest, and this is a function of the oral morphology of the ciliate (Beaver and Crisman, 1989; Šimek and Chrzanowski, 1992; Jürgens and Šimek, 2000; Jürgens and Matz, 2002; Jürgens and Massana, 2008). Motility and surface properties of prey may enhance or reduce contact probability between prey and predator (Gurijala and Alexander, 1990; Hahn and Höfle, 2001; Matz and Jürgens, 2001; Jürgens and Matz, 2002). The feeding selectivity of ciliates is recognized as an important mechanism that governs the microbial communities (Bautista-Reyes and Macek, 2012). From the data generated in this study, the three isolated ciliates did not show selective feeding on bacterial species tested, as the grazing rates for all bacteria ranged between 10^3 and 10^5 bacteria per individual ciliate per hour (Table 2.5., 2.9., and 2.13.). Thus, the morphology of bacterial prey did not have an apparent influence on the grazing and ingestion of the three isolated ciliates.

Bacterial species were consumed by ciliates at almost similar rates with usually more than 90% reduction of bacterial cell numbers except for *E. faecalis* ATCC 29212 and *Staphylococcus* sp. CPS23 for *Coleps* sp. RB3 (Figure 2.13.) and *E. coli* isolate EC33 for *Paramecium* sp. RB1 (Figure 2.20.). However, there was variation in the growth of the isolated ciliates on various bacterial species tested (Table 2.5., 2.9., and 2.13.).

While consumption of some bacteria resulted in high growth yields, some did not support growth at all, and some even caused the death of the isolated ciliates. The growth rate of the predator is determined by the digestibility and food quality (as determined by the Carbon: Nitrogen: Phosphorus content) of the prey as well as the prey response once ingested within the ciliate (Zubkov and Sleigh, 1996; Gruber *et al.,* 2009; Gerea *et al*., 2013). When prey cells are consumed, they are packaged into food vacuoles where digestion occurs. Acid phosphatase, which breaks down the cell wall of prey, is released once the food vacuole fuses with the lysosome to digest the ingested food particles (Fenchel, 1980b; González *et al.,* 1993). This process is described as the digestion phase of the food vacuole process and is described as orderly and fixed; thus, any ingested food particles in the food vacuoles are processed and digested for the same length of time (Strom and Morello, 1998; Thurman *et al*., 2010). Verity (1991) attributed the difference in the growth of two tintinnid ciliates to poor quality prey and slow digestion. Digestion is affected by the nature of bacterial prey (Sherr *et al*., 1988; Weekers *et al*., 1993); bacteria may be completely digested, partially digested, or undigested and even survive the vacuole passage time (Boenigk *et al*., 2001).

Studies have demonstrated that Gram-positive bacteria are less suitable prey than Gram-negative bacteria due to their different cell wall characteristics (Plante, 2000; Rønn *et al.,* 2002). Gram-positive bacteria have a thick cell wall of 15 to 80 nm thickness with several layers of peptidoglycan, which takes longer to break down in the food vacuoles, whereas Gram-negative bacteria have an outer membrane and a single peptidoglycan layer of about 10 nm thickness (Mai-Prochnow *et al*., 2016). This was demonstrated when a Gram-positive, *Mycobacterium chlorophenolicum,* was unaffected by predation by soil protozoa (Rønn *et al*., 2002). Mycobacterial cell walls have an outer membrane rich in mycolic acid content, which is assumed to serve as a major barrier to the penetration of antimicrobial agents (Denyer and Maillard, 2002). González *et al*. (1990b) reported that natural assemblages of ciliates digested FLB *E. faecalis* at slower rates than FLB *E. coli,* although ingestion rates on these bacteria were similar. Only *Coleps* sp. RB3 showed better growth with Gram-negative bacteria as higher growth rates were observed with most Gram-negative bacteria than for Gram-positive bacteria (Table 2.5.).

Paramecium sp. RB1, conversely, had the highest growth rate with Gram-positive cocci-shaped *Staphylococcus* sp. CPS23 (generation time 21 hours) and poor growth were observed with a Gram-negative rod-shaped *Pseudomonas* sp. strain B12 (generation time 105 hours) (Table 2.9.). This ciliate did not show any apparent preference for Gram-positive or Gram-negative bacterial species, and clearance rates were highest when grazing on both Gram-negative cocci-shaped *Acinetobacter* sp. strain S21 and Gram-positive cocci-shaped *Staphylococcus* sp. CPS23. *Paramecium* species were previously reported to have high growth yields with Gram-negative bacteria. Barna and Weis (1973) demonstrated that Gram-negative bacteria such as *Enterobacter cloacae*, *P. aeruginosa* and *P. fluorescens* supported high growth yields of *Paramecium bursaria* while grazing on Gram-positive *Bacillus laterosporus* and *Bacillus polymyxa* caused poor growth, and *Sarcina flava* and *Micrococcus* sp. even caused lysis of the ciliate. Surprisingly, *Tetrahymena* sp. RB2 showed high growth rates on mostly Gram-positive bacteria (Table 2.13.). *Tetrahymena* sp. RB2 also grew well with the Gram-positive, rod-shaped *B. subtilis* BGA, a highly motile and endospore-forming bacterium. Similar to other ciliates, *Tetrahymena* species were previously reported to produce high growth yields with Gram-negative bacteria (González *et al*., 1990a; Dillon and Parry, 2009). *Tetrahymena pyriformis* was reported to digest Gram-negative *E. coli* K12 and *Pseudomonas aeruginosa* faster than a Gram-positive *Staphylococcus aureus* (Thurman *et al*., 2010).

Although the Gram status may influence the digestibility of the bacterial prey, it is not an absolute factor that determines growth rates as some Gram-positive bacteria were favourable, and some Gram-negative bacteria were unfavourable. Reducing permeability through the cell wall is one of the strategies that is used by bacteria to resist antimicrobial compounds. A study by Martinez *et al.* (2001) demonstrated that *E. coli* O157:H7 remained intact and in a perfect reactive state when cells were treated with *p*-nitrophenyl phosphate at low concentrations, and this bacterial strain shifted its expression of the outer membrane porins reducing permeability by 70%. This mechanism has only been reported on Gram-negative bacteria as a mechanism for bacterial resistance against antibiotics (Hancock, 1997; Denyer and Maillard, 2002). The reduction of the permeability of the bacterial cell wall as a defense mechanism against protist predation is unknown at present, and this will be a subject of interest in future studies of bacterial resistance against digestion within food vacuoles. In

addition, it has been reported that antibiotic-resistant bacteria frequently exhibit metal resistance (Pal *et al.,* 2017; Nguyen *et al.,* 2019). Metals such as Copper (Cu) and Zinc (Zn) are released from the lysosome to kill ingested prey in the phagosome of protists, thus, antibiotic resistance in bacteria could regulate digestion within protist food vacuoles (Hao *et al.,* 2016; Buracco *et al.,* 2018).

Once ingested, bacterial cells may sense the conditions of the food vacuole and produce metabolites that may have a lethal effect on the host cells (Lainhart *et al.,* 2009). Antibiotic resistance *E. coli* isolate EC33 and *Staphylococcus* sp. CPS23 caused a more rapid decline of *Paramecium* sp. RB1 and *Coleps* sp. RB3 cell numbers than in their absence, indicating that predation on these bacterial species had a toxic effect on these ciliate isolates (Figure 2.13. and 2.20.). These two bacterial strains are not known to produce toxic metabolites; however, some strains of *E. coli* have been reported to produce Shiga toxins that cause lysis of *Tetrahymena* sp. (Lainhart *et al.,* 2009).

Not all bacteria were equally suitable for the three isolated ciliates. Therefore, following the criteria to categorize the nutritional quality of bacteria based on the effect on ciliates as described by Curds and Vandyke (1966), the bacteria used in this study were grouped as toxic, unfavourable, and favourable bacteria. Toxic bacteria are described as those that caused a decline in the ciliate cell numbers, unfavourable bacteria as those that slightly support growth but do not result in doubling of cell numbers, and favourable bacteria as those that support growth and cause more than doubling of cell numbers. Only antibiotic-resistant *Staphylococcus* sp. CPS23 was toxic to *Coleps* sp. RB3, Gram-positive bacteria were unfavourable except for the lyophilized *M. lysodeikticus* ATCC 4698*,* while all Gram-negative bacteria were favourable to *Coleps* sp. RB3. Bacteria that had a toxic effect on *Paramecium* sp. RB1 were antibiotic-resistant *E. coli* isolate EC33. The Gram-negative *Pseudomonas* sp. strain B12 and Gram-positive bacteria *B. subtilis* BGA, *E. durans* ATCC 6056, and *S. aureus* ATCC 6538 were unfavourable to *Paramecium* sp. RB1. No bacteria selected had a toxic effect on the cells of *Tetrahymena* sp. RB2, only the Gram-negative antibiotic-resistant *E. coli* isolate EC33 and *Pseudomonas* sp. strain B12 were unfavourable to this ciliate.

Growth and grazing of the isolated ciliates on algae

Ciliates are mainly bacterivorous; however, several studies have suggested that ciliates cannot survive in marine and some freshwater environments on bacteria alone as the bacterial biomass in these environments may not be sufficient to meet the energy demand of ciliates (Fenchel, 1980b; Hadas *et al*., 1998; Rosetta and McManus, 2003). Studies have demonstrated that ciliates are able to feed on other eukaryotes, and in some freshwater environments, their presence is associated with the presence of algal biomass (Berk *et al*., 1991; Dziallas *et al*., 2012). As observed by Goulder (1972), a large population of ciliates was detected when the algae *Scenedesmus* was present in high density in a eutrophic pond; some ciliates such as the bacterivorous *Loxodes magnus* even contained up to 76 algal cells per ciliate cell. Algae have the ability to form a symbiotic relationship with heterotrophic organisms, and this phenomenon is frequently detected with ciliates such as *Paramecium bursaria* in aquatic environments (Dziallas *et al*., 2012).

Although bacterivorous ciliates can ingest algae, growth is generally lower than when feeding on bacteria. This was observed with *Colpoda steinii,* which had doubling times that were 1.5 to 3 times lower when feeding on picocyanobacteria (*Synechococcus* sp.) and picoeukaryotes than on bacteria (Hadas *et al*., 1998). Poor growth of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 was observed with *Parachlorella* sp. strain AA1 (Figure 2.22. and 2.32.). The endosymbiotic relationship between ciliates and algae is well described for *Paramecium bursaria*. This relationship is induced by the ingestion of algal cells packaged in food vacuoles, the ingested cells evade digestion by budding off from the digestive vacuole with the digestive vacuole membrane, which protects the algal cell from lysosome fusion. The enclosed algal cells in the digestive vacuole membrane, the so-called perialgal vacuole, then escape to the cytoplasm and localize beneath the host cell surface (Kodama and Fujishima, 2010). Before establishing endosymbiosis, ingested algae are partially digested in the food vacuoles, thus providing sufficient nutrients for the slight population increase of ciliates. The process of transforming algal cells within the food vacuole to perialgal takes longer (>30 minutes) than the process of digestion, from the formation of digestive vacuole to the release of lysosome enzyme in *Paramecium bursaria,* which takes about 5 minutes (Kodama and Fujishima, 2009; 2010).

When observed under the light microscope, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 contained single algal cells that appeared as perialgal vacuoles (Figure 2.23. and 2.33.). Perialgal vacuoles are distinguished from the digestive vacuole by the space between the food vacuole membrane and the algal cell and are located on the peripheral cytoplasm of ciliates (Meier *et al*., 1984; Kodama and Fujishima, 2016). Thus, the ingested *Parachlorella* cells possibly induced and established endosymbiosis with the isolated *Paramecium* sp. RB1 (Figure 2.23.). Endosymbiosis of *Chlorella* sp. has not been reported for *Tetrahymena*. Based on the possible formation of perialgal vacuoles (Figure 2.33.), the prey *Parachlorella* sp. strain AA1 possibly established a symbiotic relationship with *Tetrahymena* sp. RB2. However, this would require additional studies to evaluate the relationship of algae with *Tetrahymena* sp. RB2. Endosymbiosis with algae has been reported for species of genera *Paramecium* and *Coleps* (Stabell *et al*., 2002; Toonoka and Watanabe, 2007; Kodama and Fujishima, 2010; Dziallas *et al*., 2012). Although they ingest algae, species of the genus *Tetrahymena* were not known to harbour endosymbiotic algae. It is only recently that *Tetrahymena utriculariae* became the first reported *Tetrahymena* forming an endosymbiotic relationship with the algae *Micractinium* sp. (Kapsetaki *et al*., 2016; Pitsch *et al*., 2017).

Consumption of *Haematococcus* sp. strain AA3 did not result in the growth of *Paramecium* sp. RB1 (Figure 2.22.). *Haematococcus* sp. strain AA3 vegetative cells were harvested at the non-motile phase of its life cycle, and this phase is characterized by the formation of a new primary wall that may have been difficult to digest in the food vacuole (Hagen *et al*., 2002; Han *et al*., 2012). Endosymbiosis of ciliates with *Haematococcus* spp. has not been reported. *Coleps* sp. RB3 cell numbers, conversely, declined more in the presence of algal species than in the absence of algal species (Figure 2.14.). Because the grazing of this ciliate did not result in a reduction of algae cell numbers, the prey cell size were possibly outside the preferred size range that can be ingested by this ciliate. Species of the genus *Coleps* commonly form a symbiosis with algae, and green *Coleps* spp. were reported to grow better and survive longer than *Coleps* spp. without endosymbionts (Stabell *et al.,* 2002).

Paramecium species can harbour several hundred symbiotic algae (Kodama and Fujishima, 2016). The individual cells of *Paramecium* sp. RB1 had grazing rates of 420 to 10^3 algae per hour on *Parachlorella* sp. strain AA1 and *Haematococcus* sp.

strain AA3 with clearance rates of 5 – 10.66 nL per hour (Table 2.9.). Despite its small size, *Tetrahymena* sp. RB2 had grazing rates on *Parachlorella* sp. strain AA1 similar to the larger *Paramecium* sp. RB1, ranging from 775 to 10^3 algal per hour (Table 2.13.). When cells of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 were examined microscopically, only a few *Parachlorella* sp. strain AA1 cells were detected within ciliates. As the grazing experiments were carried out under dark conditions, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 would lose the symbiotic algal cells as endosymbiosis of algae with ciliates is light-dependent; under dark conditions, ciliates lose their symbionts, and the number of algae within the host cell declines as the algae cannot photosynthesize and the respiration rates of algal cells are increased (Kodama and Fujishima, 2014). When incubated under light conditions, a strain of the green *Paramecium bursaria* grew and showed no decline for 80 days, while under dark conditions, this ciliate showed only low growth rates (Karakashian, 1963; Kadono *et al*., 2004). Endosymbionts can maintain the host ciliates in the absence of other food prey. Most of the ciliates with algal endosymbionts are common inhabitants of freshwater lakes and ponds, and typically, the algal endosymbiont is a *Chlorophyte*, often *Chlorella* spp. (Stoecker *et al*., 2009).

Interaction of ciliates with algae can present a challenge as some algae species produce toxins that can act against predators. For example, ingestion of lyophilised blue-green algae, *Fischerella epiphytica, Gleotrichia echinulate,* and *Nostoc linckia* were toxic to cells of *P. caudatum* (Ransom *et al.,* 1978). Even without ingestion of algal cells, the microcystins produced by cyanobacteria inhibited the growth of *T. pyriformis* (Ward and Codd, 1999). Nonetheless, the toxicity of algae on ciliates varies with species, as some ciliates were unaffected by the presence of algae. The abundance of *Spirostomum* sp. increased in the presence of toxic cyanobacteria with high concentrations of microcystins in the cells and also with high concentrations of microcystins dissolved in the water, though this ciliate abundance was decreased in the presence of *Aphanizomenon flos-aquae,* which did not produce toxins (Kosiba *et al.,* 2019).

Growth and grazing of isolated ciliates on fungal species

Compared to the extensive literature on ciliate interaction with algae, little is known about ciliate interaction with fungi (Seppey *et al*., 2017). Protists feeding on fungi are common; nevertheless, the studies on protist-fungi interaction have been limited to amoebae. Several species of amoebae have been described to feed on yeast, mycelium, and spores of fungi (Casadevall *et al*., 2019). A bacterivorous *Acanthamoeba castellani* was shown to rapidly kill yeast cultures of *S. cerevisiae*, reducing more than half of the cells after 90 min of feeding, while *Cryptococcus neoformans* caused lysis of its host cell (Steenbergen *et al*., 2001). *Vermamoeba vermiformis* readily ingested conidia of *Aspegillus fumigatus* and *Fusarium oxysporum*, but the uptake did not reduce the viability of conidia; instead promoted filamentation and growth of the fungus (Novohradská *et al*., 2017). Fungal species are common inhabitants in aquatic environments; they are abundant and diverse, with more than 3000 species known to be associated with aquatic ecosystems (Christian *et al*., 2011). They, therefore, encounter constant protozoal predation as they occupy the same niche in aquatic environments. Studies have shown that yeast cells are readily ingested and killed by ciliates (Frager *et al*., 2010). This interaction is widely employed in studying the ingestion and digestion process of ciliates as yeast stained with pH indicator dye can be used to demonstrate the change in pH inside food vacuoles (Mast, 1947). The three isolated ciliates readily ingested *S. cerevisiae* reducing concentrations by up to 70% and producing grazing rates of 10^3 yeasts per hour, which were within the range observed for bacterial species at similar prey concentrations. Even the growth rates of *Tetrahymena* sp. RB2 and *Coleps* sp. RB3 was within the range observed for bacterial species (Table 2.5. and 2.13.). *Paramecium* sp. RB1, however, did not grow with the yeast (Figure 2.24.); this was due to the concentration of yeast not being sufficient to support the growth of the ciliate. No growth was observed when grazing on conidia of *Fusarium* sp. V3 and the population of *Coleps* sp. RB3 decreased more rapidly in the presence of fungal spores than in their absence (Figure 2.15.). Digestion of fungal spores is a specialized mechanism that has only been observed in a few species of amoebae and the ciliate *Grossglockneria acuta,* which feed mainly on yeasts and other fungi (Miller, 1963; Petz *et al*., 1986). Fungi produce spores that have a melanized cell wall that is highly resistant to degradation, and their digestion requires perforation of the cell wall that

causes lysis of conidia (Old, 1978). Filter feeding ciliates such as the three isolates, *Coleps* sp. RB3, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 lacks a feeding tube that would puncture the cell wall of a fungal spore.

Studying the interaction of ciliates with fungi is equally important as bacterial interaction in aquatic environments. The constant grazing pressure has resulted in fungi developing mechanisms to survive protist predation, and the response of fungi to protist predation varies with each species. Even intracellular survival of fungi within protists has been reported for amoebae. However, a pathogenic fungus, *Cryptococcus neoformans,* which caused the death of *Acanthamoeba polyphaga* and *A. castellani*, was ingested and killed by three species of *Paramecium* (*P. multimicronucleatum*, *P. aurelia,* and *P. tetraurelia*) (Steenbergen *et al*., 2001; Frager *et al*., 2010).

The response of isolated ciliates to prey availability fluctuation

One of the important characteristics of aquatic environments is the variability of food availability over time. Such fluctuations indicate that microbial populations may experience intermittent periods of growth and decline determined by food levels (Jonsson, 1986). Therefore, organisms inhabiting these environments must be well adapted to prey density fluctuations and be able to change growth rates in response to prey availability. Ciliate populations can increase, causing a decrease in prey concentration until prey becomes limited. Once the prey concentration becomes limited, the ciliates enter starvation mode to enable long-term survival. Ciliates that do not form survival structures such as cysts undergo physiological changes such as reduction of metabolic rates and enzyme synthesis, a decrease of oxygen uptake, and the reduction of cell size to ensure cellular survival (Jackson and Berger, 1985; Fenchel, 1989). Furthermore, starvation can induce autophagy, where non-functional cytoplasmic components are degraded and utilized as nutrients to maintain basic cell functioning (Kiel, 2010). Once the prey density is sufficient to support growth, starving ciliate cells feed immediately and increase in cell volume. The time it takes for the cell to resume cell division after feeding depends on the length of starvation and varies with the species.

Tetrahymena sp. RB2 seems to be well adapted to the fluctuation of prey density as there was an immediate increase in cell numbers within 24 hours with each addition

of fresh prey suspension, and the ciliate reduced prey concentration to 10^7 bacteria x mL^{-1} (Figure 2.35.). *Coleps* sp. RB3 and *Paramecium* sp. RB1 only recovered and resumed cell division after cell starvation with the first addition of *E. coli* ATCC 8739, reducing prey concentration to about 10^8 bacteria x mL^{-1} (Figure 2.16. and 2.25.). No increase in cell numbers was observed upon the second addition of *E. coli* ATCC 8739 suspension. The *Coleps* sp. RB3 population remained stationary for 24 hours and subsequently declined, while *Paramecium* sp. RB1 continued to decline until cells were not detected in the culture.

Grazing on artificial food and the Influence of particle size

Paramecium sp. RB1 did not show selective feeding on the microbial species tested (Table 2.9.). *Tetrahymena* sp. RB2 did not ingest *Haematococcus* sp. strain AA3 and the spores of *Fusarium* sp. V3 (Table 2.13.), while the growth of *Coleps* sp. RB3 was not observed with any of the algal species (*Parachlorella* sp. strain AA1 and *Haematococcus* sp. strain AA3) and spores of *Fusarium* sp. V3 (Table 2.5.). Ciliates are described as selective feeders that mainly select their prey based on the size of the particle (Hansen *et al*., 1994). Therefore, the particle size limit and its influence on the grazing of the isolated ciliates were determined using polystyrene microparticles of 2, 5, and 10 µm diameter. *Coleps* sp. RB3 and *Tetrahymena* sp. RB2 only ingested 2 and 5 µm microbeads and the grazing rates were highest for 2 µm (Table 2.6. and 2.14.). The large buccal cavity of *Paramecium* sp. RB1 allowed the cell to ingest even 10 µm microbeads (Table 2.10.), while its grazing and clearance rates decreased with an increase in particle size. These results agree with previous studies reporting that ciliates cannot discriminate food particles but mainly select prey based on the size and that the grazing efficiency is high for the preferred size range (Børsheim, 1984; Jonsson, 1986; Jürgens and Símek, 2000). The particle size that can be ingested by ciliates is limited by the oral morphology; thus, small ciliates ($\leq$50 µm) can only ingest small-sized particles while large ciliates ($\geq$50 µm) can ingest a wider range of particle sizes. This explains the inability of *Tetrahymena* sp. RB2 to consume the large cells of *Haematococcus* sp. strain AA3 and spores of *Fusarium* sp. V3 as the cell sizes were above the size limit that can be ingested.

The size of prey that can be ingested by *Coleps* species has been reported to be up to 5 µm; however, their feeding strategies allow them to feed on prey that is larger than their cell size (Klaveness, 1984). *Coleps* spp. exhibit different feeding mechanisms; they are filter feeders that feed on suspended prey and are also raptorial feeders and scavengers that function when preferred prey is not available (Buonanno *et al*., 2014). They possess extrusome toxicysts that are discharged after contact with the prey cell; their toxic compounds enter into the prey cell, causing necrotic death. The substances discharged from the toxicysts by *Coleps* have been demonstrated to be highly toxic to other ciliates such as *Paramecium tetraurelia*, *Spirostomum teres*, *Euplotes aediculatus*, *Oxytricha* sp., and *Urocentrum turbo* (Foissner *et al*., 2008; Buonanno and Ortenzi, 2016; 2018). They then attach to prey and feed on prey organelles by suction (Auer *et al*., 2004). The effect of paralytic substances released from toxicysts depends on the susceptibility of the prey membrane (Rosati *et al*., 2008). The toxicysts of *Coleps* sp. RB3 may have been ineffective against algal cells (the cell size of algae tested was above the size limit); thus, the growth was not observed with these species. In case the toxicyst is ineffective against prey, the substances released may dissolve in the medium and act against its own cell; this, however, cannot occur in nature as the ciliate detaches from the prey after releasing toxicyst to avoid autotoxicity (Buonanno *et al*., 2014). This may have caused the *Coleps* sp. RB3 population to decline more rapidly in the presence of prey than its absence (Figure 2.14.). The spores of *Fusarium* sp. V3 are curved rods in the presence of *Coleps* sp. RB3, their concentration was reduced (Figure 2.15.). It is possible that this ciliate attached to the spore and utilized a suction mechanism for ingestion.

The three isolated ciliates showed high grazing efficiency for particle sizes up to 5 µm, and this is the typical size range of most microorganisms present as prey in freshwater environments (Dolan and Simek,1997). The inability of ciliates to distinguish between nutritive and non-nutritive particles may contribute to the bioaccumulation of microplastic pollutants in higher animals (Sieburth *et al*., 1978). Ciliates are an integral part of the microbial food web in aquatic environments; they are grazed by larger protists, zooplankton, fish larvae (Porter *et al*., 1985; Stabell, 1996; Finlay and Estenban, 1998; Sherr and Sherr, 2002; Zingel *et al.,* 2019). High ingestion rates of zooplankton feeding on ciliates have been reported; for example, a copepod *Eucyclops serrulatus* exhibited ingestion rates ranging from 3 to 39 ciliates x hour^{-1} per

individual copepod when feeding on *P. caudatum* (Reiss and Schmid-Araya, 2011). Microplastics are considered emerging pollutants and are a major concern in aquatic environments, particularly in aquatic food webs (Wright *et al*., 2013; Sun *et al*., 2017). Their concentration increases with a decrease in particle size due to the progressive breakdown of plastic debris. As they are abundant in surface waters and occupy the same size range as plankton (≤5 mm), they are bioavailable for ingestion by microorganisms representing lower trophic levels (Browne *et al*., 2007; Beers *et al*., 2018; Carbery *et al.,* 2018; Everaert *et al.,* 2018). Microplastics have been detected in aquatic organisms such as crustaceans (*Gammarus pulex*), annelids (*Lumbriculus variegatus*), gastropods (*Potamopyrgus antipodarum*), and ostracods (*Notodromas monacha*), and ingestion of microplastic may cause an adverse effect on the animal (Wagner *et al*., 2014; Ogonowski *et al*., 2016). Suspension and filter feeders are more prone to ingestion of microplastics because they feed on suspended particles, and their feeding mechanism does not discriminate particles within the preferred size of ingestion (Scherer *et al*., 2018). Thus, the filter-feeding mechanism allows ciliates to ingest large quantities of microparticles and subsequently a possible trophic transfer to higher organisms upon the ingestion of ciliates. For example, a rotifer, *Brachionus calyciflorus* ingested *T. pyriformis* cells with up to 15 microplastic particles (0.5 µm) per ciliate at a rate of3.3 ciliates x rotifer^{-1} x hour^{-1} (Mohr and Adrian, 2000; Joaquim-Justo *et al.,* 2004).

Effect of temperature on growth and grazing of ciliate

The temperature had an effect on the physiological functions (i.e., growth and grazing rates) of the three isolated ciliates. As expected, the growth of these ciliates increased with an increase in temperature between 5 and 25ºC. While *Coleps* sp. RB3 growth was observed at all three temperatures tested, 35ºC was lethal to the cells of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 (Figure 2.17., 2.27. and 2.37.). Growth of bacterivorous ciliates has been reported to occur over a temperature range of 5 to 30ºC; at low temperatures, the cell division is slow, and it increases with an increase in temperature until the optimum temperature, after which growth rates decline. Outside the physiological range, some ciliates form survival structures that can withstand high temperatures while others are killed (Rose and Caron, 2007; Verni

and Rosati, 2011). A *Tetrahymena pyriformis* that can withstand temperatures as high as 35ºC has been reported; however, it only survived for 9 hours at this temperature (Slater, 1954). The maximum growth rate for *Tetrahymena pyriformis* was reported to occur at 27.5ºC, with a temperature below 20ºC and a temperature of 30ºC, delaying cell division (Thormar, 1962). Cell division of *Paramecium* sp. was observed between 16ºC and 28ºC (Phelps, 1946). The growth of *Coleps* species has only been studied at temperatures between 9ºC and 21ºC, and their cells have been isolated at temperatures from 6.2ºC to 28.5ºC (Noland, 1925; Weisse and Rammer, 2006). This study demonstrates that *Coleps* ciliates can grow and survive at higher temperatures ($\geq$30ºC) than previously reported.

Although the growth of *Paramecium* species has not been reported at temperatures above 30ºC, vacuole formation in *P. caudatum* and *P. aurelia* was reported to increase with an increase in temperature from 4 to 40ºC (Lee, 1942; Duncan *et al*., 2011). Temperature influences the efficiency of food uptake by increasing the rate of food vacuole formation. Thus, grazing and clearance rates of all three isolated ciliates increased with an increase in temperatures from 5ºC to 25ºC for *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2, and 5ºC to 35ºC for *Coleps* sp. RB3 (Table 2.7., 2.11. and 2.15.). An increase in uptake rates does not essentially cause an increase in growth rates as more prey particles are ingested than needed to sustain growth (Laybourn and Stewart, 1975). The rate at which food vacuoles are formed is higher than the rate at which they are processed, thus increasing grazing efficiency (James and Read, 1957; Weisse and Rammer, 2006). This was observed with *Coleps* sp. RB3, as there was no increase in growth rates from 25ºC to 35ºC. Its grazing and clearance rates, however, increased by a factor of more than 2, with a rapid reduction of bacterial cell numbers within 24 hours at 35ºC (Table 2.7.).

In South Africa, the water temperature in rivers, streams, lakes, and ponds can seasonally range from 7.5 to 35ºC (Dallas, 2008). The temperature in freshwater environments can change by up to 10ºC, especially in small water bodies that take up air temperature; therefore, microorganisms inhabiting such environments may experience substantial variation in temperatures. Temperature fluctuations exceeding 1ºC can have a negative or a positive impact on the growth rates, cell size, and productivity of ciliates (Montagnes and Weisse, 2000; Laakso *et al*., 2003). With global warming expected to cause increasing temperatures in freshwater environments, it

might alter the food web and the productivity in aquatic ecosystems by eliminating microorganisms that cannot withstand higher temperatures (Montagnes *et al*., 2000). Some ciliates can form survival structures to survive extreme conditions and proliferate when conditions are favourable. Those that do not possess such mechanisms have to adapt to variable environmental conditions. A study by Duncan *et al*. (2011) revealed that with frequent exposure to variable temperatures, *Paramecium caudatum* adapted to an increase in temperatures.

Respiration rates of isolated ciliates

The species from the genera *Coleps*, *Paramecium*, and *Tetrahymena* are aerobic organisms that require oxygen and obtain energy through aerobic respiration (Fenchel, 2014). Accordingly, oxygen consumption was observed, and the respiration rates of the isolated ciliates were estimated using the Clark-type oxygen electrode (Table 2.16.). Although studies of oxygen uptake by ciliates are limited, studies have reported respiration rates for species of the same genera as the three isolated ciliates. Respiration rates of *Coleps hirtus* were reported to be 0.35 nL O_2 x $cell^{-1}$ x hr^{-1} at 25ºC, 1.24 to 4.44 nL O_2 x $cell^{-1}$ x hr^{-1} for growing cells of *Paramecium caudatum*, and 7.07 x 10^{-2} for starved cells, and 0.063 nL O_2 x $cell^{-1}$ x hr^{-1} for growing cells of *Tetrahymena pyriformis* (Fenchel and Finlay, 1983). Respiration rates of protists have been reported, they are described to be highly variable, and they largely depend on the body size, physiological state of the cell, and environmental conditions (Johnson *et al.,* 2009; Ikeda, 2017). For this study, the respiration rates at 25ºC were lower than those previously reported. Measured respiration rates of the three isolated ciliates ranged between 2.85 x 10^{-3} to 3.23 x 10^{-2} nL x hour per individual ciliate for starved cells and increased to 2.97 x 10^{-3} to 3.77 x 10^{-2} nL x hour per individual ciliate upon addition of bacterial prey. Oxygen uptake is related to the body size of organisms (Zeuthen, 1953; Finlay and Esteban, 2009). As expected, the larger-sized ciliate, *Paramecium* sp. RB1 had higher respiration rates per cell than smaller-sized ciliate *Coleps* sp. RB3 and *Tetrahymena* sp. RB2 (Table 2.16.).

The respiration of unicellular organisms is the function of the cell's physiological state (Crawford *et al.,* 1994). When food is limited, a decrease in oxygen uptake by the cell is induced, prolonged starvation results in a decrease in macromolecule synthesis and

a decrease in the number of mitochondria due to autophagy to ensure survival for long periods. The onset of starvation mode varies with species. *Tetrahymena* sp. respiration rates were reported to decrease by 30% during starvation (Fenchel, 2005). The addition of food induces an increase in oxygen uptake. As observed with the three isolated, upon addition of bacterial prey to starving cells, respiration increased by 4 – 49% (Table 2.16.) During lengthy incubation, cells become starved, resulting in the decrease in oxygen uptake, which increased upon the addition of heat-killed bacterial prey. Respiration rate studies are essential as they allow an evaluation of the role of individual species in the ecosystems with varying oxygen concentrations and the response of aerobic ciliates to such changes.

Uptake of food particles and food vacuole formation

The presence of FLB *E. coli* ATCC 8739 induced particle uptake and food vacuole formation in all three isolated ciliates, vacuoles containing labelled bacteria were detected within ciliate cells (Figure 2.38., 2.41., and 2.45.). Ciliates take up food particles in suspension by a highly specialized mechanism called filter feeding. Particles in suspension are swept by a highly organized oral ciliature and collected into a membrane-bound receptacle, finally moved into a receiving vacuole located at the base of the cytostome. Once the receiving vacuole is filled with food particles, it is separated from the oral region to form a sealed food vacuole, and a new receiving vacuole begins to form immediately (Allen, 1974; Fenchel, 1980a; Sherman *et al.,* 1981; Albright *et al*., 1987; Guerrier *et al*., 2017). When feeding rapidly, ciliates can produce large amounts of the required plasma membrane at the cytostome. For example, *Tetrahymena* can form a food vacuole membrane that covers 50% of its average cell volume when growing in a chemically defined medium (Rasmussen, 1976). The process of phagocytosis has been studied using fluorescently labelled bacteria or microparticles that are traced from the point of ingestion, formation of food vacuole to egestion (Sherr *et al*., 1987; Weisse, 2002). Therefore, FLB *E. coli* ATCC 8739 and fluorescently labelled microbeads were used to determine the rate of ingestion and food vacuole formation of the isolated ciliates. *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 readily ingested bacterial cells as food vacuoles containing FLB *E. coli* ATCC 8739 were detected after 5 minutes of feeding; their grazing resulted in

an average of 16 food vacuoles per individual ciliate after 180 minutes of incubation. The process of phagocytosis is well described for species of *Paramecium* and *Tetrahymena,* and it has been demonstrated that these ciliates are capable of rapid production of the food vacuole membrane at appropriate food concentrations (Allen, 1974; Rasmussen, 1976).

The rate of food vacuole formation is influenced by temperature; it was observed that the number of food vacuoles formed in *Tetrahymena thermophila* increased from 1.2 at 22ºC to 2.8 at 30ºC (James and Read, 1957; Sherr *et al*., 1988; Luan *et al*., 2012). The slow rate of food vacuole formed containing FLB *E. coli* ATCC 8739 in *Coleps* sp. RB3 was possibly due to the temperature at which the uptake studies were carried out (i.e., 25ºC), FLB *E. coli* ATCC 8739 were only detected in the cells of *Coleps* sp. RB3 after 10 minutes of incubation, and this ciliate only produced an average of 3 food vacuoles per ciliate after 180 minutes (Figure 2.38.). When growing at 35ºC, grazing rates of *Coleps* sp. RB3 were about 2.5X higher than grazing rates at 25ºC (Table 2.7.), indicating that this ciliate formed more food vacuoles at 35ºC, increasing the rate of ingestion of food particles when feeding on live *E. coli* ATCC 8739.

The rate of ingestion and food vacuole formation can be used as an index for the process of ciliate phagocytosis (Kloetzel, 1974). The sealed food vacuole with ingested particles is then transported to fuse with the lysosome, where acidification of the food vacuole occurs. Acidification transforms the food vacuole into a digestive vacuole, the content is degraded, and nutrients are absorbed (Fok *et al*., 1988; Hausmann, 2002). This process is described as the digestive phase and has been reported to be orderly and fixed. The waste material in the digestive vacuole is transported to be ejected out of the cell through the cytoproct; this phase is described as a non-digestive or defecation phase and is variable (Berger and Pollock, 1981; Fok *et al*., 1984; Thurman *et al*., 2010). Sherr *et al*. (1988) observed that when *Tetrahymena* was feeding on FLB, there was a linear increase in the uptake rate to the maximum, after which the FLB number per cell remained constant and the rate of uptake was equal to the rate of egestion. The time from the beginning of the uptake and the point of levelling was used to estimate the digestive and defecation phases of *Tetrahymena*. This was based on the fact that the rate of uptake is initially high and slows down at the start of digestion, and the levelling out of the rate is due to the equilibrium between ingestion and egestion being established (Berger and Pollock, 1981). Similar to previous

studies, a linear increase of ingestion and food vacuole formation in the isolated ciliates was observed until maximum rates were reached, which then decreased slightly and plateaued until the end of the incubation. The maximum rates of ingestion and food vacuole formation by the isolated ciliates were reached rapidly, the maximal rates of *Paramecium* sp. RB1 were reached after 5 minutes (Figure 2.41.)., *Coleps* sp. RB3 were reached after 10 minutes (Figure 2.38.), while *Tetrahymena* sp. RB2 reached maximum rates after 15 minutes (Figure 2.45.).

The formation of food vacuoles is a function of the uptake rate; equally so, the uptake rate can be limited by the rate of membrane material formed to enable vacuole formation. When prey concentration is sufficient, uptake rates can exceed rates of food vacuole formation, and the excess particles are pumped out of the cytostome with water (Verity, 1991). The maximum rates of food vacuole formation suggest that the digestion of FLB *E. coli* ATCC 8739 in *Tetrahymena* sp. RB2 was started after 15 minutes, for *Paramecium* sp. RB1 after 5 minutes while that of *Coleps* sp. RB3 was initiated after 30 minutes.

The short-term observation of the uptake of fluorescently labelled particles by ciliates allows for the processing time of food vacuoles to be determined. The time intervals of each process involved in the phagocytosis are categorized into four stages; stage 1 is the formation of a new vacuole, which can last for 5 minutes. Mast (1947) stated that the time required to form a new food vacuole in the presence of sufficient food particles is 1 to 2 minutes. Hence, vacuoles containing FLB particles were detected after just 5 minutes in *Tetrahymena* sp. RB2 and *Paramecium* sp. RB1 (Figure 2.41. and 2.45.). The transformation of the food vacuole to a fully functioning digestive vacuole by the release of acid phosphatase is described as stage 2, and it typically lasts for 1.5 minutes. Stage 3 is where the digestive vacuole stays for the longest period, followed by egestion, which is described as stage 4 (Mast,1947; Müller and Röhlich, l961). Acidification in *Paramecium* has been reported to occur 5 minutes after the addition of prey organisms. The digestion phase in ciliates is fixed and varies with ciliate species; it can last for a short period ($\leq$30 minutes) or can be longer and last for more than 1 hour. The digestive phase of *Tetrahymena pyriformis* was reported to lasts for 25 minutes, that of *Paramecium caudatum* was reported to last for 21 minutes, while a vacuole process time in *Halteria grandinella* lasted for 1 hour (Thurman *et al*., 2010).

For every new food vacuole formed, there is a food vacuole that is egested, resulting in a constant number of vacuoles present within the cell after the ciliate has reached its maximal uptake (Dolan and Šimek, 1997). A new vacuole formed undergoes a series of maturation steps before being egested from the ciliate. The time it takes for the maturation of the vacuole formed is described as a vacuole passage time, and this has been studied using fluorescently labelled microparticles. Since they are not digestible, they can be easily traced from vacuole formation to egestion, and any loss of food vacuole containing microparticles indicates the end of a complete vacuole passage cycle (Thurman *et al*., 2010). The vacuole process time has mainly been studied in *Paramecium* and *Tetrahymena*, and the total time from food vacuole formation to egestion has been observed to last approximately 60 to 90 minutes (Müller and Röhlich, l961). Similar to FLB *E. coli* ATCC 8739, labelled microbeads induce uptake and food vacuole formation, and the ingested particles were internalized within the cells of ciliates RB1, RB2, and RB3 packaged in membrane-bound food vacuoles (Figure 2.40., 2.43., and 2.47.). Only *Paramecium* sp. RB1 readily ingested 2 µm microbeads, with an average of 18 vacuoles per ciliate detected after 180 minutes of feeding (Figure 2.43.). *Coleps* sp. RB3 and *Tetrahymena* sp. RB1 had a lag time of 60 and 15 minutes before the microbeads were detected within the cell, and less than 4 vacuoles per cell were formed after 180 minutes (Figure 2.40. and 2.47.).

The use of microparticles in uptake studies can be problematic as microbeads have no nutritional value, and predators are reported to discriminate between nutritive and non-nutritive particles (Gruber *et al.,* 2009). Some species of ciliates have been reported to regulate the uptake of non-nutritive particles by ingesting particles at a slower rate, or the particles are only retained for a short time within the cell (Mast, 1947; Müller *et al.,* 1965; Sherr *et al*., 1988). This was observed in *Paramecium trichum*, which egested the vacuole containing carmine particles 15 minutes after ingestion (Bragg, 1936). Lower uptake of microbeads in a short-term experiment, with an FLB/ microspheres (0.5 µm diameter) uptake ratio of 10:1 was reported for an oligotrich ciliate (*Spirotrichea*). Some ciliates have the ability to sense chemical cues from their preferred prey and respond rapidly to the point source of the organic matter and chemotatically congregate at the location within minutes (Verity, 1988; Fenchel and Blackburn, 1999; Roberts *et al.,* 2011). The function of chemosensory in feeding

has been previously reported in *Tetrahymena* species (Sherr *et al.,* 1987; Dopheide *et al.,* 2011). Furthermore, additional recognition of ingested particles can occur in the food vacuoles for digestion (Dürichen *et al.,* 2016). Ricketts (1971) demonstrated that acidification did not occur in food vacuoles containing non-digestible particles in *Tetrahymena pyriformis*. Thus, ingestion of microbeads is possibly more coincidental. This may have played a role in the slow ingestion of carboxylate-modified polystyrene microbeads.

Both the long-term and short-term experiments are essential as they demonstrate the capability of the isolated ciliates to graze efficiently on suspended bacteria. It confirms that ciliates should be able to control bacterial numbers, thus establishing the functional role of ciliates in diverse freshwater environments. Short-term experiments are essential for providing specific ingestion rates of certain ciliates, while long-term experiments provide community grazing rates and should thus be integrated into the study of protist bacterivory.

The uptake kinetics established from short-term experiments can result in an over- or underestimation of the grazing potential of ciliates (González, 1999; Weisse *et al.,* 2021). The grazing rates determined from long-term experiments were much lower (ranged between 26 - 33 bacteria x ciliate^{-1} x min^{-1} for all three isolated ciliates) than the uptake rates from short-term experiments (ranged between 250 - 600 bacteria x ciliate^{-1} x min^{-1} for all three isolated ciliates). Feeding rates of protists have been shown to decrease when the cell becomes gradually food-satiated. Therefore, the maximum consumption potential of ciliates can be missed with the long-term experiments (Jürgens and Šimek, 2000).

Furthermore, live prey may be consumed and digested differently from the heat-killed FLB bacteria (Weisse, 2002; Gruber *et al.,* 2009). FLB *E. coli* are non-viable heat-killed cells with different physical and biochemical cellular characteristics (heat treatments alter surface chemistry and associated surface ligands of the cell wall) and are chemically modified during the staining process (Katsui *et al.,* 1981; Dolan and Šimek, 1997). Surface modification on food particles can influence uptake rates (Dürichen *et al.,* 2016). Thus, responses of ciliate predation to non-viable prey differ from the live prey, which influences the rate of uptake and digestion. Some ciliates have been reported to discriminate between heat-killed FLB in favour of a live cell, and

this may result in an underestimation of grazing rates (Stabell, 1996; Gruber *et al.,* 2009). For example, Ferrier-Pages and Rassoulzadegan (1994) observed poor growth of the flagellate *Pseudobodo* sp. and ciliate *Strombidium sulcatum* when feeding on heat-killed bacterial prey than with live bacteria. Thus, there can be problems obtaining reliable counts of ingested FLB that represent the grazing rates occurring in natural environments.

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CHAPTER 3

Influence of a lytic bacteriophage on the bacterivory of two isolated ciliates

3.1. Introduction

Natural aquatic environments are complex systems where one species interacts with multiple other species. Lytic viruses are ubiquitous in freshwater, together with bacterivorous protists; they are the most prominent top-down regulators of bacterial populations (Alonso *et al.,* 2000; Zhang *et al*., 2014; Feichtmayer *et al.,* 2017). Interaction between bacteria and viruses is important, not only in the regulation of bacterial populations but also important for carbon flow in the microbial loop (Miki and Yamamura, 2005). Whereas protists are considered generalist grazers that mainly select their prey based on size, bacteriophages are highly species-specific, with most only infecting a single bacterial species. Nonetheless, they each account for comparable proportions of bacterial loss and are key factors in the regeneration of nutrients (Bratbak *et al.,* 1994; Fuhrman and Noble, 1995).

The impact of bacterivorous protists and bacteriophages on bacterial communities has been established. Unlike bacterivorous protists that consume multiple prey particles at one time to reproduce, bacteriophages infect one cell at a time to produce multiple progenies (Örmälä-Odegrip *et al.,* 2015; Dion *et al.,* 2020). The profound differences between grazing and viral lysis present differences in the ecological and biogeochemical effects; while bacterivorous protists reduce the proportion of active bacteria and bacterial net production, lytic viruses may stimulate bacterial net production by returning carbon from lysed bacterial cells, which is used by bacteria for carbon and energy synthesis (Strom, 2000; Evan *et al.,* 2003).

Previous studies have examined the evolution of bacteria against viruses and protist predation. Such studies demonstrated that the presence of more than one predator in the environment might affect (negatively or positively) the impact of another predator on the prey population (Deng *et al*., 2013; Johnke *et al.,* 2017). Studies have reported that the disappearance of viral particles and decay in viral infection in aquatic environments is accelerated by the presence of protists (Suttle and Chen, 1992; Clarke, 1998). Grazing on viruses has mostly been reported for flagellates. However,

studying the interaction of lytic bacteriophages and bacterivorous protists when feeding on bacteria is essential to understand as this predator-prey might influence population dynamics and the microbial food web in aquatic ecosystems.

Little is known on the interaction between bacterial viruses and bacterivorous ciliates and on the effect of this interaction on bacteria (Weinbauer *et al.,* 2003). Örmälä-Odegrip *et al.* (2015) demonstrated that the presence of *Tetrahymena thermophila* and *Acanthamoeba castellanii* lowered the susceptibility of *Serratia marcescens* to a lytic bacteriophage. A study by Friman and Bucklin (2014) demonstrated that the presence of a bacteriophage reduced *Pseudomonas aeruginosa* anti-predatory defense (biofilm formation) against *Tetrahymena thermophila*. These bacteriophage-protist-bacteria interaction studies also demonstrated that grazing and viral lysis vary in different environments.

Therefore, the aim of this study was to evaluate the interaction of a bacteriophage with isolated ciliates feeding on *E. coli* bacterium.

3.2. Materials and methods

As the culture of *Coleps* sp. RB3 was lost during the pandemic campus lockdown; henceforth, experiments were conducted with the two remaining isolates, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2.

The study organisms

The two bacterivorous ciliates (*Paramecium* sp. RB1 and *Tetrahymena* sp. RB2) were isolated from freshwater samples from the Blackborough stream (KwaZulu-Natal, South Africa), as described in chapter 2. The isolates were maintained in Chalkley's medium at room temperature (25 $\pm$ 2ºC) in the dark.

E. coli ATCC 25922 was obtained from the American Type Culture Collection and was routinely grown in Nutrient broth at 37°C and 100 rpm.

The obligatory lytic bacteriophage NPM infecting *E. coli* ATCC 25922 was kindly provided by Ms. Nontando Ntuli in the Microbiology department at UKZN. This phage was isolated from water samples from the Darvil wastewater treatment plant in KwaZulu-Natal, South Africa, using *E. coli* ATCC 25922 as the host. It has a T4 like morphology with a distinct head and tail and is a member of the family *Myoviridae* (personal communication with Ms. N. Ntuli)

Preparation of cultures

The experiments were carried out in duplicates in 5 mL Chalkley's medium at 25 $\pm$ 2ºC in the dark. Prior to initiating the experiment, the organisms were cultured separately and prepared as follows:

Bacterial culture – *E. coli* ATCC 25922 was grown overnight in Nutrient broth incubated in a rotary shaker at 37°C and 100 rpm. The cells were harvested by centrifugation at 10 000 *g* for 10 minutes and washed twice with 0.85% NaCl. The cells were then resuspended in 5 mL Chalkley's medium to a final concentration of 10^{10} bacteria x mL^{-1}.

Ciliate cultures – *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 were harvested by centrifugation at 500 *g* for 5 min and washed twice with fresh Chalkley's medium. Cells were resuspended in 5 mL Chalkley's medium to a final concentration of 10^3 ciliates x mL^{-1}.

Bacteriophage NPM – 100 µL stock culture of bacteriophage was mixed with 100 µL of overnight *E. coli* ATCC 25922 in 500 µL 0.85% NaCl. The suspension was mixed with molten 0.6% soft-agar and overlayed onto Nutrient agar plates. Plates were then incubated at 37°C for 24 hours. To obtain bacteriophage lysate, a single plaque was pipetted from the prepared plates into 500 µL 0.85% NaCl. The suspension was added to a 10 mL culture of *E. coli* ATCC 25922 incubated for 60 minutes at 37°C at 100 rpm. The culture was incubated for an additional 2 hours in the rotary shaker. Debris was removed from the lysate by centrifugation for 10 min at 10 000 *g* at 5°C and then filtered with 0.2 µm sterile Syringe Filters (Acrodisc® Sterile Syringe Filters). The bacteriophage lysate was added to 5 mL Chalkley's medium to a final concentration of 10^8 PFU x mL^{-1}.

The experiments were initiated by the addition of appropriate volumes of each microorganism from a stock culture in Chalkley's medium to a final volume of 5 mL. Flasks were incubated at 25ºC statically in the dark. Seven different communities were established:

1. Bacteriophage
2. Bacteriophage + *E.coli* ATCC 25922
3. Bacteriophage + *E.coli* ATCC 25922 + one ciliate
4. Bacteriophage + one ciliate
5. One ciliate + *E.coli* ATCC 25922
6. One ciliate
7. *E. coli* ATCC 25922

The change in concentrations for each microorganism was monitored every 24 hours for 5 days as follows:

Bacteriophage (PFU x mL^{-1}) – Phage NPM concentrations were monitored by counting plaque-forming unit (PFU) using the double-layer agar method. 100 µL was aliquoted and serially diluted in 0.85% NaCl to achieve the desired final concentrations. Plates were incubated at 37ºC for 24 hours.

Ciliates (ciliate x mL^{-1}) – *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 were monitored by direct cell counts microscopically. 50 µL of the culture was aliquoted, and cells were immobilized with 0.1% formaldehyde.

***E. coli* ATCC 25299 (Bacteria x mL^{-1})** – Bacterial cell numbers were monitored microscopically by direct cell counts using a Neuber improved type Counting chamber (0.02 mm depth, 0.0025 mm^2 area). In addition to direct cell counts, a change in viable bacteria was determined by viable plate counts on nutrient agar and expressed as colony-forming unit per mL (CFU x mL^{-1}).

3.3. Results

Lytic viruses occupy the same niche as bacterivorous protists in aquatic environments and prey on similar organisms. Studies have shown that the presence of protists influences virus infecting bacteria. Therefore, this study was carried out to evaluate the potential influence of a lytic bacteriophage on ciliate grazing.

3.3.1. Interaction of *Paramecium* sp. RB1 and bacteriophage NPM

The growth and interaction of *Paramecium* sp. RB1 and phage NPM was studied in the presence and absence of *E. coli* ATCC 25922 as prey.

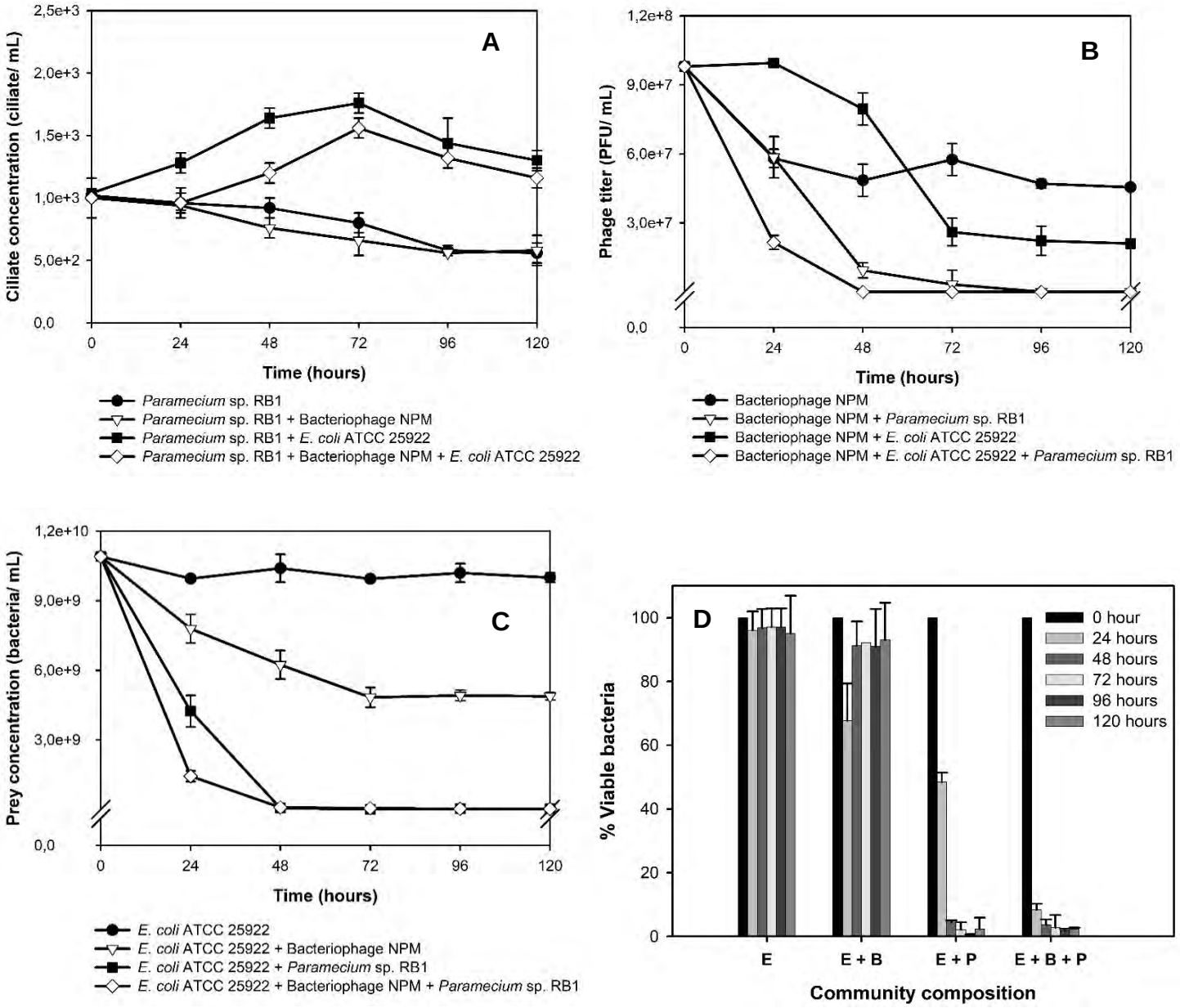

Figure 3.1. Interaction of *Paramecium* sp. RB1, bacteriophage NPM and *E. coli* ATCC 25922. A – Growth of *Paramecium* sp. RB1, B – Change in bacteriophage NPM titer, C – change in *E. coli* ATCC 25922 concentration, D – Viable counts of *E. coli* ATCC 25922 in monoculture (ciliate/ bacteriophage/ bacterium), co-cultures of ciliate + bacteriophage, ciliate + bacteria, bacteriophage + bacteria, and ciliate (P) + bacteriophage (B) + bacteria (E) in Chalkley's medium at 25 ± 2ºC in the dark.

The presence of bacteriophage NPM in the co-culture with the bacterial prey slightly reduced the growth of *Paramecium* sp. RB1, the lag phase, which was observed in the presence of bacteriophage, was not observed in the absence of bacteriophage while grazing on *E. coli* ATCC 25922. The decline of bacteriophage in the presence of *E. coli* ATCC 25922 was observed after 24 hours, while in the absence of a bacterial host, the decline was observed within 24 hours. The bacteriophage titer declined in the presence of *Paramecium* sp. RB1, though in the presence of bacteria, the decline of phage titer was apparently more rapid. Both phage NPM and *Paramecium* sp. RB1 reduced bacterial cell numbers; the bacteriophage achieved 40% reduction, while the ciliate achieved more than 90% reduction of bacterial cell concentrations. In the presence of both the ciliate and the bacteriophage, bacterial cell concentrations were reduced by more than 90%. Phage NPM achieved an initial reduction of 40% of viable *E. coli* ATCC 25922, subsequently increasing after 24 hours incubation. While a mono-culture of *Paramecium* sp. RB1 and a co-culture of *Paramecium* sp. RB1 and phage NPM achieved more than 90% reduction of viable bacteria.

The growth and grazing kinetics of *Paramecium* sp. RB1 when feeding on *E. coli* ATCC 25922 in the presence and absence of bacteriophage NPM was determined from the data generated.

Table 3.1. Growth and grazing kinetics of *Paramecium* sp. RB1 in the absence and presence of lytic bacteriophage in Chalkley's medium at 25 ± 2ºC in the dark

Co-culture composition of *Paramecium* sp. RB1 with	Specific growth rate (hr^{-1})	Generation time (hr)	Grazing rate (bacteria x $ciliate^{-1}$ x hr^{-1})	Clearance rate (nL x $ciliate^{-1}$ x hr^{-1})
E. coli ATCC 25922	0.00808	86.59	$1.46 \times 10^5 – 8.60 \times 10^4$	13.35
E. coli ATCC 25922 + phage NPM	0.00731	95.57	$1.51 \times 10^5 – 8.55 \times 10^4$	13.82

The values presented are the average of two independently performed experiments. Grazing rates were corrected for the increase in ciliate numbers during growth experiments.

The presence of the bacteriophage affected the growth of *Paramecium* sp. RB1 when grazing on *E. coli* ATCC 25922; the growth rate of the ciliate was slightly reduced in

the presence of the bacteriophage, and the generation time was about 9 hours longer. However, the grazing and clearance rates were similar in the absence and presence of the bacteriophage.

3.3.2. Interaction of *Tetrahymena* sp. RB2 and bacteriophage NPM

As for *Paramecium* sp. RB1, the growth, and interaction of *Tetrahymena* sp. RB2 with lytic bacteriophage NPM was studied in the presence and absence of *E. coli* ATCC 25922.

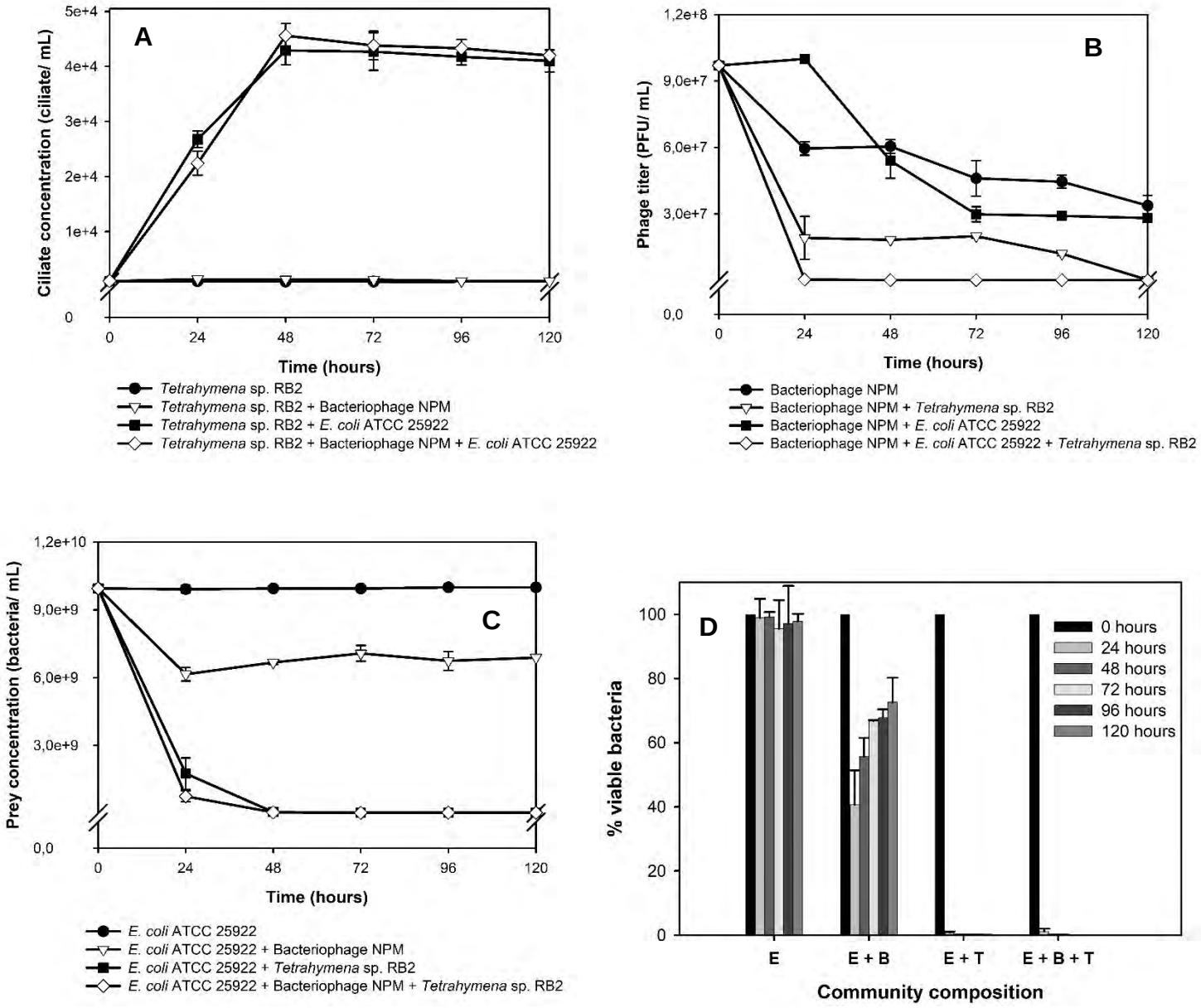

Figure 3.2. Interaction of *Tetrahymena* sp. RB2, bacteriophage NPM and *E. coli* ATCC 25922. A – Growth of *Tetrahymena* sp. RB2, B – Change in phage NPM titer, C – Change in *E. coli* ATCC 25922 concentration, D – Viable counts of *E. coli* ATCC 25922 in monoculture (ciliate/ bacteriophage/ bacterium), co-cultures of ciliate + bacteriophage, ciliate + bacteria, bacteriophage + bacteria, and ciliate (T) + bacteriophage (B) + bacteria (E) in Chalkley's medium at 25 ± 2ºC in the dark.

The presence of lytic bacteriophage NPM did not have any apparent effect on the growth of *Tetrahymena* sp. RB2. There was no difference observed in the growth of the ciliate when feeding on *E. coli* ATCC 25922 in the presence and absence of the

lytic bacteriophage. Conversely, *Tetrahymena* sp. RB2 had an effect on the bacteriophage. In the presence of *E. coli* ATCC 25922, Bacteriophage decline was observed after 24 hours of incubation, while in the absence of a bacterial host, a decline was within 24 hours. In the presence of the ciliate, there was a more rapid decline of the phage titer than in the bacteriophage mono-culture. The presence of *Tetrahymena* sp. RB2 reduced phage particle concentrations even without bacterial cells present. The bacteriophage reduced the total bacterial count by 40% in Bacteriophage + *E. coli* ATCC 25922 co-culture for 24 hours feeding which subsequently increased thereafter. However, no difference was observed in the reduction in bacterial concentrations between the mono-culture of *Tetrahymena* sp. RB2 and co-culture of *Tetrahymena* sp. RB2 and bacteriophage. *Tetrahymena* sp. RB2 achieved a reduction of more than 99% of viable bacteria after 48 hours, while the bacteriophage achieved only 60% after 24 hours. In co-culture with *Tetrahymena* sp. RB2 and bacteriophage NPM, viable *E. coli* ATCC 25922 was reduced by more than 90%.

Growth and grazing kinetics of *Tetrahymena* sp. RB2 when feeding on *E. coli* ATCC 25922 in the presence and absence of phage NPM were determined from the data generated during growth experiments.

Table 3.2. Growth and grazing kinetics of *Tetrahymena* sp. RB2 in the absence and presence of lytic bacteriophage in Chalkley's medium at 25 ± 2ºC in the dark

Co-culture composition of *Tetrahymena* sp. RB2 with	Specific growth rate (hr^{-1})	Generation time (hr)	Grazing rate (bacteria x $ciliate^{-1}$ x hr^{-1})	Clearance rate (nL x $ciliate^{-1}$ x hr^{-1})
E. coli ATCC 25922	0.0788	9.32	$2.06 \times 10^5 – 4.72 \times 10^3$	20.64
E. coli ATCC 25922 + phage NPM	0.0792	8.87	$2.07 \times 10^5 – 4.54 \times 10^3$	20.75

The values presented are the average of two independently performed experiments. Grazing rates were corrected for the increase in ciliate numbers during growth experiments.

The presence of the bacteriophage only had a slight effect on the growth and grazing rates of *Tetrahymena* sp. RB2 when feeding on *E. coli* ATCC 25922.

3.4. Discussion

The growth of both ciliates was observed in the presence and absence of lytic bacteriophage NPM when feeding on *E. coli* ATCC 25922. This study demonstrates that the two bacterial predators, which have different strategies to utilize bacteria, have a different effect on bacterial cell numbers. The grazing activity of the ciliates, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 had the strongest effect on bacteria as it achieved more than a 90% reduction of the total bacterial cell numbers (Figure 3.1C and 3.2C). Conversely, infection by the bacteriophage caused an initial rapid decrease of up to 40% of bacterial concentrations (Figure 3.1C and 3.2C). Zhang *et al.* (2014) observed the temporary decrease of bacterial biomass due to bacteriophage infection, the increase in bacterial biomass was attributed to the utilization of cell content released from lysed bacterial cells by non-infected cells. The nucleic acids and proteins released can be assimilated by bacteria and serve as a source of Phosphorus and Nitrogen, which are essential for bacterial growth (Brüssow, 2007; Pinchuk *et al.,* 2008).

Natural environments are characterized by the varying availability of nutrients over time. Bacteria adapt to low nutrients or starvation by reducing the abundance and activity of the protein biosynthesis apparatus (e.g., ribosome), thus changing the pattern of transcription or reducing transcription activity (Watson *et al.,* 1998; Bergkessel *et al.,* 2016; Lempp *et al.,* 2020). As the lytic phages require viable cells for replication, the change in transcription activity may result in the infected cell becoming less suitable for the development of bacteriophage progeny virions (Golec *et al.,* 2011). A temperate phage can enter the lysogenic life cycle, where its genome is maintained in the host cell until bacterial growth resumes. A decline in bacteriophage titer was observed after 24 hours of incubation with the host bacterial cells, *E. coli* ATCC 25922 (Figure 3.1B and 3.2B). Experiments were carried out in a defined mineral salts (Chalkley's medium), which does not support bacterial growth. Non-growing *E. coli* cells are less metabolically active and have a repressed DNA replication. Thus, infection activity and further replication of bacteriophages is reduced (Łoś *et al.,* 2007; Bergkessel *et al.,* 2016).

The co-incubation of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2, each with a lytic bacteriophage, resulted in the decline of the phage NPM titer. Removal of

bacteriophage particles by ciliates has been reported for *Tetrahymena* and *Euplotes,* and these two ciliates even inactivated the virus (Clarke, 1998; Hennemuth *et al.,* 2008; McMinn *et al.,* 2020). The results obtained demonstrate that even a large ciliate like *Paramecium* sp. RB1 can remove bacteriophage particles from a co-culture (Figure 3.1B). Moreover, in the absence of bacterial prey, the decrease in phage NPM titer in co-culture with *Paramecium* sp. RB1 demonstrates that the uptake of viral particles by ciliates is more than coincidental ingestion and a by-product of the ingestion of bacteria. Interactions of *Paramecium* spp. with viruses have been reported with picornaviruses. Karalyan *et al.* (2012) observed that *Paramecium caudatum* actively removed and inactivated picornaviruses and that the number of ciliate cells increased more in the co-incubation medium with picornaviruses particles and *Paramecium caudatum* than in medium without viral particles. The size of bacteriophages typically ranges from 24 to 200 nm (the size of the isolated phage NPM is 150 nm) and that of picornaviruses from 22 to 30 nm; thus, ciliates can ingest nanosized particles, smaller than commonly assumed. A study by Fenchel (1980) already found that some species of ciliates, including *Paramecium* and *Tetrahymena*, retained particles ranging from 300 nm to 30 µm.

The bacteriophage enters the ciliate cell as for bacterial prey, by phagocytosis of the viral particles. Pinheiro *et al.* (2007) observed that the time course for the removal of phage corresponded with the formation of food vacuoles. Ingested viruses can be digested and inactivated within food vacuoles. Nonetheless, phages resistant to such digestion have been reported. Hennemuth *et al.* (2008) demonstrated that the T4 polypeptide was degraded in food vacuoles of *Tetrahymena* sp. A similar study by Akunyili *et al.* (2008) reported a ΦX174 phage highly resistant to digestion by *T. thermophila*, *T. silvana*, and *T. pyriformis*; the phage particles were ingested and packaged in food vacuoles but were recovered in the faecal pellets of the ciliates. Consumption of phages, however, did not provide any nutritional benefits to either *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 as growth was not observed in the co-culture with the phage (Figure 3.1A and 3.2A). Viruses are non-motile, occur in great numbers in aquatic environments and wastewater (10^6 – 10^9 particles per mL), and are easy to access by filter feeders. Studies have estimated that viruses can be a source of nutrients for protists if present at concentrations greater than bacteria (Gonzàlez and Suttle, 1993; Bratbak *et al.,* 1994; Sime-Ngando, 2014). The nutrient

quality of viruses was estimated for flagellates; to obtain an equal amount of carbon as bacteria, they would require 1000 times more viruses than bacteria (Deng *et al.,* 2014). As ciliates are typically larger than flagellates and have different feeding strategies, they may require 50 times greater concentrations of viruses than nanoflagellates (Ichinotsuka *et al.,* 2006).

The grazing activity of ciliates can result in the reduction of bacteriophage abundance. The presence of *E. coli* ATCC 25922 in the co-culture with ciliates accelerated the decline of the phage NPM titer and resulted in the higher removal of phage particles (Figure 3.1B and 3.2B). One of the key steps in the infection of bacteria is the attachment of bacteriophage onto proteins or lipopolysaccharide of the outer membrane in the case of susceptible Gram-negative bacterium. Bacteriophage adsorption initiates the infection process, and studies have shown that a single bacterium can adsorb multiple phage particles (Ellis and Delbrück, 1938; Silva, 2016). With each bacterial cell ingested, multiple phage particles adsorbed on the bacterial surface are simultaneously ingested by ciliates, thus causing a higher removal of viral particles in the presence of susceptible bacteria. Because ciliates and phages consume the same type of prey, an antagonistic interaction is to be expected, i.e., viral infection may reduce the number of bacterial cells available for ciliates consumption; likewise, grazing by ciliates reduces bacterial cells available for infection for phage progeny production (Weinbauer *et al.,* 2007; Deng *et al.,* 2014; Feichtmayer *et al.,* 2017). The presence of the phage and the infection of bacterial prey did not have an effect on the grazing and clearance rates of the isolated ciliates (Table 3.1. and 3.2.). It can, therefore, be assumed that the isolated ciliates consumed phage-infected bacteria at the same rates as non-infected bacteria. Nonetheless, the presence of phage slightly reduced the growth rates of *Paramecium* sp. RB1 (Table 3.1.). It can also be assumed that the consumed viruses did not provide additional nutrients to either *Paramecium* sp. RB1 or *Tetrahymena* sp. RB2. Grazing on phage-infected bacterial cells may have some effect on the digestibility or nutritional quality of bacteria. This could occur if bacterial physiology or behaviour changed drastically after a viral infection, although no evidence for this is available (Miki and Yamamura, 2005). If not digested within food vacuoles, susceptible bacteria may be infected by the ingested lytic virus within food vacuoles. Aijaz and Koudelka (2017) reported a 6-fold increase of the lysogen formation in food vacuoles of *Tetrahymena* when compared

with control without predator. Thus, confinement within the phagosome of the ciliate facilitated the transfer of phages between ingested bacteria. The consequence of this interaction is the potential of bacteria to acquire virulent traits, transforming them into human pathogens.

By removing both viruses and bacteria, ciliates play a crucial and complex role in aquatic ecosystems. The inactivation of viruses and bacteria by ciliates is crucial in maintaining water quality. Viruses are generally more abundant (e.g., one to three orders of magnitude) than bacteria in aquatic ecosystems (Wommack and Colwell, 2000; Jacquet *et al.,* 2010). Therefore, studying the uptake of viruses together with bacteria at more environmentally relevant concentrations and proportions would be of great interest in future studies to better understand the implications of multiple species interactions in natural and man-made environments such as wastewater treatment plants.

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CHAPTER 4

Survival of bacteria after ingestion by isolated ciliates

4.1. Introduction

Protists grazing on bacteria is one of the essential ecological processes in the aquatic ecosystems that control bacterial biomass (Sherr and Sherr, 2002; Gong *et al.,* 2016). The constant grazing pressure from predators has made it necessary for bacteria to develop strategies to resist protist predation. Bacteria have therefore developed various mechanisms enabling them to resist protist predation by either resisting ingestion or resisting digestion (Matz *et al.,* 2002; Anderson *et al.,* 2011). The predation resistance of bacteria thus influences the efficiency of protist bacterivory as this depends on the success of sequential steps of ingestion and digestion (First *et al.,* 2012). Consequences of these are bacteria that can survive and persist in aqueous environments and present a challenge to eradicate (Brandl *et al.,* 2005).

The potential role of protists as reservoirs for pathogenic bacteria is now receiving increased interest as this has major implications for human public health (Brown and Barker, 1999; Barker *et al.,* 1999; Vaerewijck *et al.,* 2014). Bacteria that can resist digestion once ingested can survive and even multiply within the cells of protists. Furthermore, intracellular survival within the host enhances resistance against antimicrobial treatments such as disinfection, use of antibiotics, and heat inactivation (King *et al.,* 1988; Denoncourt *et al.,* 2014). In addition, the presence of different bacteria in food vacuoles might accelerate horizontal gene transfer leading to antibiotic resistance transfer (Schlimme *et al.,* 1997). Since the first study that demonstrated intracellular survival of pathogenic *Legionella* spp. in *Acanthamoeba*, many protist species have been reported to host various pathogenic bacteria (Rowbotham, 1980).

Ciliates have been demonstrated to effectively act as reservoirs for bacteria (Greub and Raoult, 2004; Erken *et al.,* 2013; Lin *et al.,* 2016). They are frequently colonized by microorganisms that can be highly infectious or non-infectious (Görtz, 2001; First *et al.,* 2012). Ciliates are considered as voracious grazers that account for a major reduction of bacterial biomass in aquatic environments (Beaver and Crisman, 1989; Zingel *et al.,* 2007). They also dominate during sludge treatments in wastewater

treatment plants, thus are exposed to a great number of potentially pathogenic bacteria and are regularly released with treated wastewater effluent into receiving water bodies (Martin-Cereceda *et al.,* 1996; Madoni, 2011). Ciliates are regularly isolated even from drinking water systems and fresh produce (Gourabathini *et al.,* 2008; Lambrecht *et al.,* 2015). Therefore, more research is required to understand the interaction of ciliates with various bacteria and how such a predator-prey relationship may impact the survival and epidemiology of bacteria, particularly pathogenic bacteria.

The aim of this study was to determine the proportion of bacterial survival within isolated ciliates after ingestion and to examine how the disinfection with chlorine affects bacteria ingested by isolated ciliates.

4.2. Materials and methods

4.2.1. Preparation of co-cultures of isolated ciliates and bacteria

Preparation of predator organisms

Ciliate cultures in Chalkley's medium were harvested and washed twice with the same medium by centrifugation at 500 *g* for 5 minutes. Washed ciliate cells were suspended in fresh Chalkley's medium and left overnight to starve and empty their food vacuoles. The ciliate cultures were then treated with 100 µg x mL^{-1} Ampicillin and 100 µg x mL^{-1} Gentamicin for 2 hours to inactivate any remaining viable bacteria, after which the ciliate cells were washed thrice and resuspended in 5 mL fresh Chalkley's medium.

Preparation of bacterial prey

Overnight cultures of bacteria grown in Nutrient broth were harvested by centrifugation at 10 000 *g* for 10 minutes and washed thrice with 0.85% NaCl. The washed cells were then resuspended in fresh Chalkley's medium containing isolated ciliates (10^3 ciliates x mL^{-1}) and used to establish co-cultures of bacteria and ciliates.

All feeding experiments with the three ciliate isolates were performed in 5 mL Chalkley's medium with various bacterial prey organisms listed in Table 2.1. (Chapter 2). All experiments were performed in duplicate at 25ºC ± 2ºC in the dark under static conditions. Two sets of controls were employed; control 1 was a mono-axenic culture of the ciliate predator without the bacterial prey, while control 2 was bacterial prey in Chalkley's medium without the predator to account for non-predatory changes.

To establish a co-culture, ciliate cells in 5 mL Chalkley's medium at a concentration of 10^3 cells x mL^{-1} were fed with a bacterial culture at a concentration of 10^8 bacteria x mL^{-1} for *Tetrahymena* sp. RB2 and 10^{10} bacteria x mL^{-1} for *Coleps* sp. RB3 and *Paramecium* sp. RB1. Ciliates were allowed to feed on bacteria for 24 hours.

4.2.2. Evaluation of morphological changes of bacterial prey in the co-culture with ciliates

The possible change of bacterial cell morphology was monitored over 5 days in response to ciliate grazing. 100 µL samples of cultures were examined microscopically to observe any changes in the morphology of bacteria such as cell size, filamentation, or formation of aggregates in the presence and absence of the three isolated ciliates.

4.2.3. Quantification of viable bacteria ingested within the food vacuoles of the isolated ciliates

The viability of bacteria in the food vacuoles of ciliates after 24 hours of incubation was carried out with two of the isolated ciliates, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2.

After 24-hour incubation, uningested bacterial cells were removed from the co-culture by filtration using Whatman Cyclopore membrane filters of a pore size of 8 µm and a diameter of 47 mm using gravity flow. A single cell of a ciliate was drawn out under a light microscope and transferred to 100 µL of sterile 0.85% NaCl in a sterile Eppendorf tube and then placed in an ice bath to lyse the cells. The solutions (100 µL) containing lysed ciliate cells in NaCl were inoculated onto the Nutrient agar by spread plating in replicates. The presence of viable bacterial cells within food vacuoles was determined by establishing Colony Forming Units (CFU x ciliate^{-1}). To verify the identity of ingested bacteria, an appropriate selective or differential medium that encourages the growth of selected bacteria was inoculated as above from replicate samples by the spread plate method. To verify that bacterial prey was present in the culture, culture filtrates were inoculated onto Nutrient agar and a selective or differential medium listed in Table 4.1. The plates were then incubated at 37ºC for 48 hours; the colonies resembling the expected morphology of the matching controls were counted and streaked onto a fresh selective or differential medium to confirm the identity of selected bacterial prey.

4.2.4. Viability of bacteria in the egested faecal pellets of isolated ciliates

The metabolic activity of the selected Gram-negative bacteria present in the faecal pellets released from the ciliate cells was determined using the DNA gyrase inhibitor, Nalidixic acid, which allows all metabolic processes in bacteria to proceed except cell division, producing elongated cells (Kogure *et al.,* 1979). The starved ciliates were allowed to feed on bacteria for 24 hours, 50 µL of the co-culture was added to 50 µL of SOC medium containing 10 µg x mL^{-1} Nalidixic acid. The flasks were incubated at 37ºC and observed every 30 minutes for 4 hours to monitor for the presence of bacterial cell elongation taking place in faecal pellets released by ciliates. Bacterial cells were judged metabolically active if the size of their cells exceeds twice the length of the average cell and jetting out of the pellet (Kogure *et al.,* 1979).

SOC medium was prepared by aseptically combining sterile SOB medium (consisting of 0.5% yeast extract, 2% tryptone, 2.5 mM KCl, and 10 mM NaCl at pH 7) with sterile 10 mM $MgSO_4$, 10 mM $MgCl_2$, and 20 mM glucose.

4.2.5. Chlorine treatment of isolated ciliates feeding on bacteria

The response of isolated ciliates to chlorine solutions

The chlorine treatments were carried out using Sodium hypochlorite solution with 5% free chlorine (Associate Chemical Enterprise Pty Ltd – ACE, South Africa). Axenic cultures of the three isolated ciliates were treated with different chlorine concentrations to quantitatively assess their susceptibility to free chlorine. Ciliate cells at a concentration of 10^3 cells x mL^{-1} were treated with chlorine solutions containing 0, 1, 2, 2.5, 5, and 10 mg free chlorine per litre. The response of the isolated ciliates after exposure to chlorine solutions was examined microscopically every 5 minutes for 1 hour. The effect of chlorine on ciliates was judged based on the observation of ciliatory beating, swimming speed, and death due to cell lysis.

Viability of bacteria in ciliate cells after chlorine disinfection

Co-culture chlorine disinfection treatment was carried out after 24 hours of feeding on bacterial prey. Uningested bacteria were removed from the culture by filtration following the method described in section 4.2.3. Isolated ciliate cells were transferred to 100 µL of chlorine solutions (0, 1, 2, 2.5, and 5 mg free chlorine per litre) and exposed for 30 minutes. At the end of contact time, non-lysed ciliate cells were drawn out from chlorine solutions under a light microscope, washed once, and transferred to fresh 100 µL sterile 0.85% NaCl in Eppendorf tubes and placed in ice to lyse the cells. To quantify viable bacteria present inside food vacuoles, replicate solutions (100 µL) containing lysed cells in saline solution were inoculated onto nutrient agar and a selective or differential medium by spread plating in replicates.

To evaluate the effectiveness of chlorine inactivation on bacteria, chlorine disinfection was carried out with uningested filtered bacterial cells from co-cultures and bacterial cells from mono-cultures of bacteria. Bacterial cells were exposed to different chlorine concentrations for 30 minutes (0, 1, 2, 2.5, and 5 mg free chlorine per litre). After contact time, bacterial cells were harvested by centrifugation at 10 000 *g* for 10 minutes, washed twice, resuspended in 100 µL 0.85% NaCl, and then inoculated by spread plating on nutrient agar and specific or differential agar in replicates. The plates were incubated at 37ºC, and the colonies formed enumerated after 48 hours.

Viability of bacteria in faecal pellets egested from ciliates after chlorine disinfection

Metabolic activities of selected Gram-negative bacteria in faecal pellets expelled from the isolated ciliates after treatment with chlorine solutions were determined using the DNA gyrase inhibitor, Nalidixic acid. The 24-hour co-cultures were centrifuged at 500 *g* for 5 minutes and left standing for 10 minutes to separate sedimented faecal pellets from planktonic ciliate cells. The collected faecal pellets were exposed to chlorine solutions at different concentrations (0, 1, 2, 2.5, and 5 mg free chlorine per litre) for 30 minutes. The treated faecal pellets were washed twice with 0.85% NaCl by centrifugation for 5 minutes and then transferred to the SOC medium for the cell elongation assay (section 4.2.4.). The pellets were examined microscopically for cell

elongation every 30 minutes for 4 hours. Bacterial cells were judged metabolically active if elongated cells were jetting out of the pellets (Smith *et al.,* 2012).

Selective and/ or differential media listed in Table 4.1. were used to verify the identity of bacteria recovered from the cells of ciliates.

Table 4.1. Selective or differential media for selected bacterial prey

Bacteria	**Media**
E. coli ATCC 8739	Eosin Methylene Blue Agar
E. coli isolate EC33	Eosin Methylene Blue Agar
S. Typhimurium ATCC 14028	Xylose Lysine Deoxycholate Agar
Pseudomonas sp. strain B12	MacConkey Agar
Vibrio sp. strain KM1	MacConkey Agar
Acinetobacter sp. strain S21	MacConkey Agar
Staphylococcus sp. CPS23	Baird Parker Agar
S. aureus ATCC 6053	Baird Parker Agar
E. faecalis ATCC 29212	Bile Esculin Agar
E. durans ATCC 6056	Bile Esculin Agar

All selective/ differential media used were obtained from Merck (Pty) Ltd, South Africa.

4.3. Results

4.3.1. Morphological response of tested bacteria to grazing by isolated ciliates

In response to protist grazing pressure, some bacteria can shift their population phenotype traits to resist capture by a predator. Therefore, possible changes in bacterial cell morphology were examined microscopically to determine whether selected bacterial prey can express defensive traits such as filamentation or formation of microcolonies as a mechanism to resist ingestion by the three isolated ciliates.

Table 4.2. Cell morphology of bacterial prey in the presence and absence of isolated ciliates over a period of 5 days incubation in Chalkley's medium at 25 ± 2ºC in the dark

Prey	Bacterial cell morphology changes			
	No ciliate	*Coleps* sp. RB3	*Paramecium* sp. RB1	*Tetrahymena* sp. RB2
Gram-negative				
E. coli ATCC 8739	-	-	-	-
E. coli isolate EC33	-	-	-	-
S. Typhimurium ATCC 14028	-	-	-	-
Pseudomonas sp. strain B12	-	-	-	-
Vibrio sp. strain KM1	-	-	-	-
Acinetobacter sp. strain S21	-	-	-	-
Gram-positive				
Staphylococcus sp. CPS23	-	-	-	-
S. aureus ATCC 6053	-	-	-	-
E. faecalis ATCC 29212	-	-	-	-
E. durans ATCC 6056	-	-	-	-

(-) – no change in bacterial cell morphology was observed; cells remained freely suspended and showed no filamentation or aggregation.

When isolated ciliates were feeding on bacteria, no formation of defensive morphotypes such as an increase in cell size, filamentation, and aggregation was observed over 5 days of incubation. In the presence of the isolated ciliates, bacterial cell numbers were reduced by ≥90%, and uningested bacterial cells remained freely suspended or planktonic throughout incubation.

4.3.2. Intracellular survival of ingested bacteria within isolated ciliates

Ingested food particles in ciliates are packaged in food vacuoles and transported to fuse with lysosomes, forming degradative phagolysosomes where digestion of prey occurs. Bacteria have evolved mechanisms to evade digestion once ingested within the predator cell. Therefore, experiments were carried out to determine the intracellular survival capabilities of bacteria within *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2.

4.3.2.1. Viability of ingested bacteria within food vacuoles of *Paramecium* sp. RB1

The ability of bacteria to resist digestion and survive within the cells of *Paramecium* sp. RB1 was determined; the numbers of viable bacteria recovered from cells of the ciliate are presented in Table 4.3.

Table 4.3. Viable bacteria ingested and packaged in food vacuoles of *Paramecium* sp. RB1 and uningested bacteria from the 24-hour co-culture in Chalkley's medium at 25 ± 2ºC in the dark

	Packaged bacteria within ciliate (CFU x ciliate^{-1})		Residual uningested bacteria (Log CFU x mL^{-1})		Planktonic bacteria without ciliate present (Log CFU x mL^{-1})	
Bacterial prey	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar
Gram-negative						
E. coli ATCC 8739	0 (0)	0 (0)	4.73	3.30	9.40	8.47
E. coli isolate EC33	4.50 (1 – 11)	3.00 (1 – 4)	4.51	3.99	9.57	8.10
S. Typhimurium ATCC 14028	0 (0)	0 (0)	3.78	2.41	9.06	8.96
Pseudomonas sp. strain B12	0 (0)	0 (0)	4.33	3.02	8.98	7.82
Vibrio sp. strain KM1	32.50 (18 – 51)	10.00 (1 – 16)	3.00	2.70	8.93	8.00
Acinetobacter sp. strain S21	0 (0)	0 (0)	3.22	2.62	9.18	8.34
Gram-positive						
Staphylococcus sp. CPS23	194.00 (98 – 266)	155.00 (48 – 195)	4.49	4.48	9.31	8.70
S. aureus ATCC 6053	6.00 (3 – 10)	2.00 (1 – 5)	4.88	4.50	8.54	8.11
E. faecalis ATCC 29212	15.75 (4 – 24)	13.50 (1 – 17)	3.84	3.59	8.95	7.90
E. durans ATCC 6056	18.50 (3 – 49)	13.00 (1 – 21)	4.95	4.79	9.29	8.43

The values (CFU x ciliate^{-1}) presented are the median of 20 randomly selected ciliate cells analyzed from two independently performed experiments. The values in brackets represent the range of colony counts per each ciliate cell selected. Log CFU x mL^{-1} is the average of two independently performed experiments.

After 24 hours of feeding, viable bacteria were reduced in co-cultures with *Paramecium* sp. RB1. Ingested bacteria packaged in food vacuoles were recovered as viable bacteria for all Gram-positive bacteria from within cells of *Paramecium* sp. RB1. Only for the Gram-negative antibiotic-resistant *E. coli* isolate EC33 and *Vibrio* sp. strain KM1 were viable cells recovered from the cells of *Paramecium* sp. RB1 after ingestion. The highest numbers of viable bacteria were recovered from *Paramecium* sp. RB1 cells for the Gram-positive antibiotic-resistant *Staphylococcus* sp. CPS23. Treatment with the antibiotics Gentamicin and Ampicillin, and starvation of *Paramecium* sp. RB1 overnight, prior to initiating experiments, was effective in removing viable bacteria from cells of *Paramecium* sp. RB1 as no viable bacteria were detected when analysing starved mono-cultures of this ciliate.

4.3.2.2. Viability of ingested bacteria within food vacuoles of *Tetrahymena* sp. RB2

The ability of bacteria to resist digestion and survive within the food vacuoles of *Tetrahymena* sp. RB2 was determined, the numbers of viable bacteria recovered from cells of ciliate are presented in Table 4.4.

Table 4.4. Viable bacteria ingested and packaged in food vacuoles of *Tetrahymena* sp. RB2 and uningested bacteria from the 24-hour co-culture in Chalkley's medium at 25 ± 2ºC in the dark

Bacterial prey	Package bacteria within ciliate (CFU x ciliate^{-1})		Residual uningested bacteria (Log CFU x mL^{-1})		Planktonic bacteria without ciliate present (Log CFU x mL^{-1})	
	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar
Gram-negative						
E. coli ATCC 8739	2.77 (1 – 5.8)	0.87 (0 – 1.1)	4.26	4.03	7.23	5.91
E. coli isolate EC33	3.15 (0 – 6)	1.00 (1 – 2)	4.81	4.71	7.20	5.22
S. Typhimurium ATCC 14028	0 (0)	0 (0)	4.77	3.81	6.96	6.14
Pseudomonas sp. strain B12	0 (0)	0 (0)	3.11	2.95	6.92	4.38
Vibrio sp. strain KM1	0 (0)	0 (0)	3.04	2.73	6.15	5.75
Acinetobacter sp. strain S21	0 (0)	0 (0)	3.48	2.30	6.68	4.91
Gram-positive						
Staphylococcus sp. CPS23	0.79 (0 – 4)	0.40 (0 – 3)	3.34	2.30	6.38	4.78
S. aureus ATCC 6053	0 (0 – 1)	0 (0 – 1)	3.70	2.90	6.93	5.11
E. faecalis ATCC 29212	0.84 (0 – 2)	0.79 (0 – 1)	3.86	3.01	7.27	5.58
E. durans ATCC 6056	6.84 (2 – 9)	3.00 (2 – 6)	3.02	2.80	6.91	4.80

The values (CFU x ciliate^{-1}) presented are the median of 20 randomly selected ciliate cells analysed from two independently performed experiments. The values in brackets represent the range of colony counts per each ciliate selected. Log CFU x mL^{-1} is the average of two independently performed experiments.

After 24 hours grazing of *Tetrahymena* sp. RB2, viable bacteria were reduced in co-cultures with the ciliate. Some of the ingested bacterial cells were recovered as viable bacteria from within the cells of *Tetrahymena* sp. RB2; of the Gram-negative bacteria tested, only *E. coli* ATCC 8739 and the antibiotic-resistant *E. coli* isolate EC33 were recovered as viable bacteria, whereas all Gram-positive bacteria used in this study were recovered from the food vacuoles of *Tetrahymena* sp. RB2. In the monoculture control of *Tetrahymena* sp. RB2, no viable bacteria were recovered, indicating that starvation overnight and treatment with Gentamicin and Ampicillin effectively removed bacteria from ciliate cells.

4.3.3. Viability of bacteria in vesicles/faecal pellets released from the isolated ciliates

Ciliates produce and expel vesicles/ faecal pellets as part of the digestive process. These vesicles contain digested or undigested food material released from food vacuoles after the digestive cycle. To determine whether bacteria survived the digestion process in the two isolated ciliates, the viability of bacteria was evaluated in the released faecal vesicles.

Table 4.5. Metabolic activity of uningested bacterial cells and bacterial cells in faecal pellets of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 examined microscopically after performing cell elongation assay for 4 Gram-negative bacteria

	Metabolic activity of bacterial cells			
Bacterial prey	Heat-treated control	Uningested	*Paramecium* sp. RB1	*Tetrahymena* sp. RB2
E. coli ATCC 8739	-	+	-	-
E. coli isolate EC33	-	+	-	-
S. Typhimurium ATCC 14028	-	+	-	-
Vibrio sp. strain KM1	-	+	-	-

(+) elongation of bacterial cells; (-) elongation of bacterial cells was not observed

After 24 hours of feeding, faecal pellets that were expelled from the two isolated ciliates contained no metabolically active bacterial cells. Nalidixic acid inhibits cell division, as evidenced by the presence of elongated cells with a cell length of up to 10 µm in the positive controls (Figure 4.1.), which was not observed in heat-treated cells. Uningested bacteria were still metabolic active as cell elongation was observed while no cell elongation of bacteria was observed in faecal pellets.

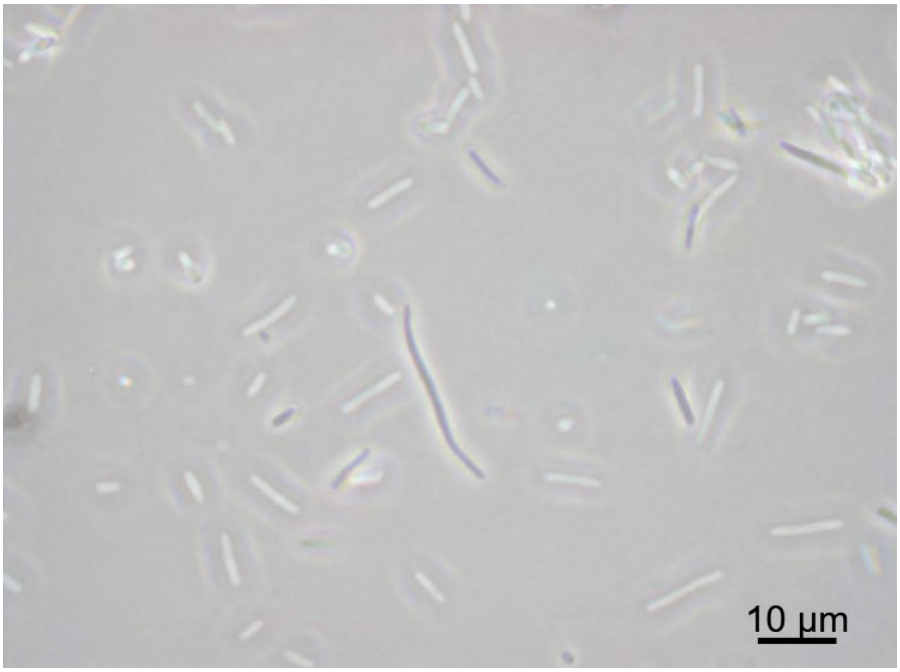

Figure 4.1. Representative micrograph showing the presence of elongated metabolically active cells of uningested *E. coli* ATCC 8739 after treatment with nalidixic acid.

4.3.4. Chlorine inactivation of ciliate isolates

Chlorine is widely used in the disinfection of water and various surfaces in food industries. It is regarded as one of the most effective antimicrobial substances, even at low concentrations. Therefore, a qualitative assessment was undertaken to assess microscopically the response of the three isolated ciliates to chlorine solutions at varying concentrations.

The response of *Coleps* sp. RB3 to chlorine

When the cells of the *Coleps* isolate were exposed to chlorine solutions of different concentrations, the cells were immobilized instantly upon the addition of disinfectant. While the cells remained intact at 0 mg/L free chlorine, all cells were lysed after 15 minutes of contact time at concentrations from 1 to 2.5 mg/L, while exposure at 5 and 10 mg/L caused the cells to lyse instantly upon exposure.

The response of *Paramecium* sp. RB1 to chlorine

Exposure of *Paramecium* sp. RB1 to chlorine solutions at concentrations of 1 to 2.5 mg/L reduced the swimming speed of the cells, though cells remained intact and active. A chlorine concentration of 5 mg/L immobilized the cells, and all cells were lysed after 30 minutes of contact time. Cells were lysed instantly upon contact with residual chlorine at 10 mg/L.

The response of *Tetrahymena* sp. RB2 to chlorine

Cells of *Tetrahymena* sp. RB2 remained intact and active, albeit with reduced swimming speed after exposure to 1 to 2.5 mg/L residual chlorine. Cells were immobilized instantly upon addition of 5 mg/L chlorine and were lysed after 15 minutes of contact time. At 10 mg/L of free Chlorine, cells were lysed instantly.

4.3.5. Survival of bacteria within the isolated ciliates after chlorination

Bacterivorous protists can act as a reservoir for bacteria. Once ingested by the ciliate, intracellular bacteria are protected from direct exposure to external environments. Studies have demonstrated that bacteria that can survive within protists are protected during treatments with disinfectants or biocides. Therefore, the potential of the isolated ciliates to protect bacteria during chlorination was evaluated.

4.3.5.1. Viability of bacteria in the food vacuoles *Paramecium* sp. RB1 during chlorine disinfection

As some of the tested bacteria survived and remained viable within the ciliate cells after 24 hours (Table 4.3.), chlorine disinfection assays were carried out to determine whether these bacteria are protected during chlorine exposure.

Table 4.6. Viable bacteria recovered from *Paramecium* sp. RB1 from 24-hour co-cultures with bacterial prey in Chalkley's medium at 25 ± 2ºC in the dark after 30-minute treatments with chlorine at different concentrations

	Packaged bacteria within ciliate (CFU x ciliate^{-1})							
	1 mg/L		2 mg/L		2.5 mg/L		5 mg/L	
Bacterial prey	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar
Gram-negative								
E. coli ATCC 8739	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
E. coli isolate EC33	2.00 (0 – 5)	1.25 (0)	0.50 (0 – 3)	1.00 (0 – 2)	0.50 (0 – 1)	0 (0)	0.50 (0 – 1)	0 (0)
S. Typhimurium ATCC 14028	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pseudomonas sp. strain B12	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Vibrio sp. strain KM1	16.00 (5 – 31)	1.00 (0 – 3)	2.00 (0 – 7)	0 (0 – 2)	2.00 (0 – 5)	0 (0)	2.00 (0 – 2)	0 (0)
Acinetobacter sp. strain S21	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gram-positive								
Staphylococcus sp. CPS23	100.00 (36 – 198)	20.00 (10 – 54)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. aureus ATCC 6053	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
E. faecalis ATCC 29212	3.00 (2 – 7)	3.00 (1 – 4)	4.50 (1 – 6)	1.00 (0 – 4)	5.00 (0 – 5)	4.00 (0 – 5)	3.00 (1 – 7)	1.00 (0 – 4)
E. durans ATCC 6056	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

The values (CFU x ciliate^{-1}) presented are the median of 20 randomly selected ciliate cells analysed from two independently performed experiments. The values in brackets represent the range of colony counts per each ciliate selected.

Undigested bacteria that were recovered from cells of *Paramecium* sp. RB1 after 24 hours feeding (Table 4.3.) were also recovered as viable bacteria from the cells of *Paramecium* sp. RB1 after chlorine treatment at concentrations of 1 to 2.5 mg/L where cells of ciliates remained intact. At chlorine concentration of 5 mg/L, which caused lysis of ciliates after 30 minutes of exposure and inactivated bacteria (Table 4.8.), viable bacteria were still recovered after chlorine disinfection of cells of *Paramecium* sp. RB1

Exposure of bacterial prey to different chlorine concentrations for 30 minutes demonstrated that chlorine disinfection was effective at reducing viable bacteria (Table 4.8.). Viable counts of tested bacteria decreased with an increase in chlorine from a concentration from 1 to 2.5 mg/L achieving up to 3 log reduction, while viable bacteria were not detected at a chlorine concentration of 5 mg/L.

Similarly, uningested bacterial cells from the 24-hour co-culture with *Paramecium* sp. RB1 with bacterial prey were further reduced after 30 minutes of exposure to chlorine concentrations from 1 to 2.5 mg/L (Table 4.7.), while no viable bacteria were detected at 5 mg/L.

Table 4.7. Inactivation of uningested bacterial cells from 24-hour co-culture of *Paramecium* sp. RB1 with bacterial prey in Chalkley's medium at 25 ± 2ºC in the dark after chlorine disinfection at different concentrations

Bacterial prey	Residual uningested bacteria (Log CFU x mL^{-1})									
	0 mg/L		1 mg/L		2 mg/L		2.5 mg/L		5 mg/L	
	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar
Gram-negative										
E. coli ATCC 8739	4.73	3.30	3.56	2.95	3.60	2.26	2.87	2.08	0	0
E. coli isolate EC33	4.51	4.00	3.46	3.08	3.43	3.00	2.73	2.00	0	0
S. Typhimurium ATCC 14028	4.78	2.41	3.77	2.15	2.04	2.65	2.00	0	0	0
Pseudomonas sp. strain B12	4.33	3.02	3.20	3.94	2.75	1.38	1.65	1.04	0	0
Vibrio sp. strain KM1	3.00	2.70	2.91	2.18	0	0	0	0	0	0
Acinetobacter sp. strain S21	3.21	2.62	2.89	2.95	2.38	0	0	0	0	0
Gram-positive										
Staphylococcus sp. CPS23	4.49	4.48	3.45	3.26	3.18	2.87	2.98	2.26	0	0
S. aureus ATCC 6053	4.88	4.50	3.16	3.01	2.87	2.76	2.19	2.46	0	0
E. faecalis ATCC 29212	3.84	3.59	2.53	3.45	2.84	2.20	2.83	1.32	0	0
E. durans ATCC 6056	4.95	3.79	3.90	3.66	3.15	3.00	2.38	0	0	0

The values presented are the average of two independently performed experiments.

Table 4.8. Inactivation of planktonic bacteria from the 24-hour culture without ciliate present in Chalkley's medium at 25 ± 2ºC in the dark after 30 minutes chlorine disinfection at different concentrations

Bacterial prey	Planktonic bacteria (Log CFU x mL^{-1})									
	0 mg/L		1 mg/L		2 mg/L		2.5 mg/L		5 mg/L	
	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar
Gram-negative										
E. coli ATCC 8739	9.40	8.47	6.86	5.32	5.74	4.26	5.69	3.56	0	0
E. coli isolate EC33	9.57	8.10	6.84	4.96	5.87	4.75	5.59	4.28	0	0
S. Typhimurium ATCC 14028	9.06	8.96	7.00	6.79	6.53	5.08	4.62	4.00	0	0
Pseudomonas sp. strain B12	8.98	7.82	7.98	6.90	6.32	5.00	4.89	4.91	0	0
Vibrio sp. strain KM1	8.93	8.00	6.43	7.93	5.96	4.39	5.31	3.64	0	0
Acinetobacter sp. strain S21	9.18	8.34	7.98	6.75	6.74	4.59	5.16	3.99	0	0
Gram-positive										
Staphylococcus sp. CPS23	9.31	8.70	7.98	6.05	5.46	4.68	5.81	2.91	0	0
S. aureus ATCC 6053	8.54	8.11	7.06	6.76	5.55	4.11	4.87	3.83	0	0
E. faecalis ATCC 29212	8.95	7.90	6.40	5.63	5.89	4.34	4.09	3.54	0	0
E. durans ATCC 6056	9.29	8.43	7.11	6.66	5.55	4.92	4.27	3.40	0	0

The values presented are the average of two independently performed experiments.

4.3.5.2. Viability of bacteria in the food vacuoles of *Tetrahymena* sp. RB2 after chlorine disinfection

Chlorine disinfection assays were carried out to determine whether ingested bacteria present within cells of *Tetrahymena* sp. RB2 are protected from chemical treatments.

Table 4.9. Viable bacteria recovered from *Tetrahymena* sp. RB2 from 24-hour co-cultures with bacterial prey in Chalkley's medium at 25 ± 2ºC after 30 minutes chlorine treatment at different concentrations

	Packaged bacteria within ciliate (CFU x ciliate^{-1})							
	1 mg/L		2 mg/L		2.5 mg/L		5 mg/L	
Bacterial prey	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar
Gram-negative								
E. coli ATCC 8739	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
E. coli isolate EC33	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
S. Typhimurium ATCC 14028	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pseudomonas sp. strain B12	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Vibrio sp. strain KM1	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Acinetobacter sp. strain S21	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gram-positive								
Staphylococcus sp. CPS23	0.20 (0 – 1)	0 (0 – 1)	0 (1 – 2)	0 (0)	0 (0 – 1)	0 (0)	0 (0)	0 (0)
S. aureus ATCC 6053	0 (0 – 1)	0 (0 – 1)	0 (0 – 1)	0 (0 – 1)	0 (0)	0 (0)	0 (0)	0 (0)
E. faecalis ATCC 29212	2.00 (0 – 3)	0.80 (0 – 2)	0.50 (0 – 1)	0.40 (0 – 1)	0.20 (0 – 1)	0.20 (0 – 1)	0 (0 – 1)	0 (0)
E. durans ATCC 6056	3.00 (0 – 5)	1.00 (1 – 3)	1.00 (1 – 3)	1.00 (0)	1.00 (0 – 1)	0 (1 – 2)	1.00 (0 – 1)	0 (0)

The values (CFU x ciliate^{-1}) presented are the median of 20 randomly selected ciliate cells analysed from two independently performed experiments. The values in brackets represent the range of colony counts per each ciliate selected.

As shown in Table 4.9., only Gram-positive bacteria were recovered from the cells of *Tetrahymena* sp. RB2 after 30 minutes of exposure to free chlorine at concentrations of 1 to 5 mg/L. Similar to *Paramecium* sp. RB1, cells of *Tetrahymena* sp. RB2 remained intact at residual chlorine concentrations between 1 to 2.5 mg/L and were only lysed at 5 mg/L after 15 minutes of exposure.

Chlorine was effective at reducing viable bacteria from mono-cultures of bacterial prey, and viable counts decrease with an increase in chlorine concentrations from 1 to 2.5 mg/L achieving up to 2 log reductions (Table 4.11.). A concentration of 5 mg/L inactivated all bacterial species to below detectable levels.

Similarly, chlorine disinfection was effective at reducing uningested bacterial cells from the 24-hour co-cultures of *Tetrahymena* sp. RB2 with bacterial prey (Table 4.10.). Viable counts decreased with increased chlorine concentrations from 1 to 2.5 mg/L after 30 minutes of contact time, while chlorine concentrations of 5 mg/L reduced bacteria to a below detectable level.

Table 4.10. Inactivation of uningested bacterial cells from 24-hour co-culture of *Tetrahymena* sp. RB2 with bacterial prey in Chalkley's medium at 25 ± 2ºC in the dark after chlorine disinfection at different concentrations

	Residual uningested bacteria (Log CFU x mL^{-1})									
	0 mg/ L		1 mg/L		2 mg/L		2.5 mg/L		5 mg/L	
	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar
Gram-negative										
E. coli ATCC 8739	4.26	4.03	3.88	3.84	3.79	3.51	3.15	3.00	0	0
E. coli isolate EC33	4.81	4.71	3.78	4.67	3.93	3.00	3.40	3.04	0	0
S. Typhimurium ATCC 14028	4.77	3.81	4.61	3.23	3.00	3.32	3.00	2.62	0	0
Pseudomonas sp. strain B12	3.11	2.95	3.11	0	2.82	0	0	0	0	0
Vibrio sp. strain KM1	3.04	2.73	3.00	1.76	0	0	0	0	0	0
Acinetobacter sp. strain S21	3.48	2.30	3.46	2.92	2.87	0	0	0	0	0
Gram-positive										
Staphylococcus sp. CPS23	3.34	2.30	3.32	3.00	2.66	3.00	2.58	1.73	0	0
S. aureus ATCC 6053	3.70	2.90	3.59	2.90	2.11	2.04	2.15	0	0	0
E. faecalis ATCC 29212	2.86	2.86	2.85	2.81	2.67	2.45	0	0	0	0
E. durans ATCC 6056	3.02	3.80	2.75	2.61	2.49	1.99	1.98	0	0	0

The values presented are the mean of two independently performed experiments.

Table 4.11. Inactivation of planktonic bacteria from the 24-hour culture without ciliate present in Chalkley's medium at 25 ± 2ºC in the dark after 30 minutes chlorine disinfection at different concentrations

Bacterial prey	Planktonic bacteria (Log CFU x mL^{-1})									
	0 mg/L		1 mg/L		2 mg/L		2.5 mg/L		5 mg/L	
	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar	Nutrient agar	Selective agar
Gram-negative										
E. coli ATCC 8739	7.23	5.91	6.97	5.36	6.04	4.90	4.72	4.26	0	0
E. coli isolate EC33	7.20	5.22	6.08	4.32	5.73	4.99	4.88	3.38	0	0
S. Typhimurium ATCC 14028	6.96	6.14	6.56	4.98	5.90	3.83	5.34	3.63	0	0
Pseudomonas sp. strain B12	6.92	4.38	6.65	4.66	5.30	3.94	3.81	3.49	0	0
Vibrio sp. strain KM1	6.15	5.75	5.58	4.41	4.99	4.04	3.95	2.62	0	0
Acinetobacter sp. strain S21	6.68	4.91	5.98	3.83	4.90	4.59	4.63	3.08	0	0
Gram-positive										
Staphylococcus sp. CPS23	6.38	4.78	5.11	4.95	4.79	3.32	4.62	3.68	0	0
S. aureus ATCC 6053	6.93	5.11	5.89	4.67	5.15	3.77	4.93	3.46	0	0
E. faecalis ATCC 29212	7.27	5.58	6.64	4.00	5.43	3.95	4.92	3.81	0	0
E. durans ATCC 6056	6.91	4.80	5.97	3.74	4.57	3.26	4.00	2.81	0	0

The values presented are the mean of two independently performed experiments.

4.4. Discussion

Bacterial resistance to ciliate ingestion

The feeding studies carried out in Chapter 2 demonstrated that all bacterial species used in this study were consumed by the three isolated ciliates resulting in the reduction of bacterial cell numbers. Grazing and growth rates varied for each bacterial strain, with some bacteria such as *E. coli* isolate EC33 and *Staphylococcus* sp. CPS23 not supporting the growth of isolated ciliates. The bacterial strains (cell size range from 0.5 to1.5 µm x 1.5 to 5 µm for rod-shaped bacteria and 0.6 to 1.5 µm for cocci-shaped bacteria) (Table 2.1., Chapter 2) used were within the preferred size range determined for the isolated ciliates.

During the grazing of ciliates on bacterial prey, the formation of defensive morphotypes was not observed for any of the bacterial prey studied. Uningested bacterial cells remained freely suspended (planktonic) in the presence and the absence of a predator (Table 4.2.). Most bacteria in the environment can form microcolonies under conditions of environmental stress such as toxins, antibiotics, predation, or lack of nutrients (Trunk *et al.,* 2018). In response to protist predation, bacteria can express defensive traits that form inedible morphotypes. Thus, a change in cell size, filamentation, and aggregation are ways to evade protist ingestion as the size of the prey particle exceeds the prey size limit that can be ingested (Jürgens *et al.,* 1999; Carno and Jürgens, 2006; Blom *et al.,* 2010; Batani *et al.,* 2016). This response of prey to protist predation varies with predator species and depends on the morphological plasticity of each bacterial species (Pernthaler, 2005; Carno and Jürgens, 2006). Most bacterial species can form microcolonies, including bacteria from genera used as bacterial prey in this study. For example, species of the genus *Staphylococcus* are known to form aggregates or microcolonies (Haaber *et al.,* 2012; Trunk *et al.,* 2018). Other Gram-positive bacteria, such as a *Streptococcus* sp. mutant, a biofilm-forming bacterium, can express defense morphotypes and aggregates in response to protist predation (Welch *et al.,* 2012; Baumgartner *et al.,* 2016).

Defensive morphotypes are generally formed in nutrient-rich environments where energy supply is sufficient for both cell functioning and formation of different morphotypes at high bacterial density, which allows for sufficient quantities of signalling molecules to be present (Matz and Jürgens, 2003; Darch *et al.,* 2012; Friman *et al.,* 2013; Baumgartner *et al.,* 2016). The experiments in this study were carried out in Chalkley's medium, a chemically defined medium that does not contain any carbon and energy source to support bacterial growth. Therefore, as this is nutrient-limited, conditions were probably only sufficient for the maintenance of the bacterial cell by using carbon storage compounds or in the presence of ciliates, carbon compounds released by the predator but not for morphological changes.

Intracellular survival of bacteria within ciliates

Bacteria enter the ciliate cell through ingestion via the cytostome (oral cavity), where food particles are collected and enclosed in a membrane-bound food vacuole. The newly formed food vacuole is then transported to fuse with a lysosome through the endocytic pathway to form a degradative food vacuole, by releasing digestive enzymes such as acid phosphatase or proteases, where ingested food particles are broken down and consumed (Fenchel, 1980; 1987; Hausmann, 2002; Thao *et al.,* 2015). As demonstrated with the three isolated ciliates in Chapter 2 (Section 2.3.4.), when grazing on fluorescently labelled bacteria and fluorescently labelled microplastics, the three isolated ciliates formed food vacuoles containing ingested particles. The isolated ciliates consumed large amounts of bacteria (up to 10^3 bacteria x ciliate^{-1} x hour $^{-1}$ at a bacterial concentration of 10^8 bacteria x mL^{-1} and 10^5 at a bacterial concentration of 10^{10} bacteria x mL^{-1}) with up to 90% reduction in viable bacteria after 24 hours (Chapter 2, Section 2.3.3.).

Though the grazing activity of the isolated ciliates reduced the number of viable bacteria in the medium, some viable bacteria were recovered from food vacuoles within ciliate cells even after 24-hours of feeding. It was thus indicating possible digestion inefficiency in food vacuoles of the two isolated ciliates tested. This was observed for mostly Gram-positive bacteria as viable bacteria of most Gram-positive bacteria used in this study were recovered after ingestion from both *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2. For Gram-negative bacteria, only the antibiotic-

resistant strain *E. coli* isolate EC33 and *Vibrio* sp. strain KM1 were recovered after ingestion from cells of *Paramecium* sp. RB1 and *E. coli* ATCC 8739 and the antibiotic-resistant *E. coli* isolate EC33 were recovered from *Tetrahymena* sp. RB2 (Table 4.3. and 4.4.). The antibiotic-resistant strain *E. coli* isolate EC33 even caused some degree of cell lysis of *Paramecium* sp. RB1 and enabled only poor growth of *Tetrahymena* sp. RB2 (Chapter 2, Section 2.3.3.3.). Some strains of *E. coli* are known to produce toxic metabolites that can act as a defense against protist predation (Lainhart *et al.,* 2009; Stolfa and Koudelka, 2012). Matz and Kjelleberg (2005) suggested that the build-up of toxic metabolites in food vacuoles leads to lysis of the protist cell, thereby causing expulsion from the food vacuole before all bacterial cells are digested. *E. coli* isolate EC33 presumably produced a metabolite that was toxic to *Paramecium* sp. RB1 as this strain caused lysis of this ciliate. In a study carried out by Steinberg and Levin (2007), Shiga toxigenic bacteria produced a metabolite that was lethal to *Tetrahymena thermophila* and increased the rate of survival of this bacterium within food vacuoles, which was not observed in non-Shiga toxin encoding bacteria.

The protist grazing pressure has resulted in bacteria developing mechanisms that resist digestion and allow survival within the cell of the protists (Jürgens, 2006; Gong *et al.,* 2016). Intracellular survival in protists has been reported for various Gram-positive and Gram-negative bacteria. They achieve this by secreting proteins that allow bacteria to evade digestion in food vacuoles by either disrupting the maturation pathway of food vacuoles or modulating the environment within the transformed degradative food vacuoles (Matz *et al.,* 2004; Jousset, 2012; Vaerewijck and Houf, 2015; Best and Kwaik, 2018). Consequences of intracellular survival are bacteria that persist in the environment and maintain their population in high numbers (Denoncourt *et al.,* 2014; Lambrecht *et al.,* 2015).

Intracellular survival in amoebae and ciliates has been reported for *Escherichia*, *Vibrio*, *Staphylococcus*, and *Enterococcus* (Anacarso *et al.,* 2012). Furthermore, these bacteria were reported to manipulate the host cell to multiply within the protist cell without causing cell lysis (Greub and Raoult, 2004). For example, *Vibrio cholerae* can escape from the food vacuole to the contractile vacuole in the amoebae *A. castellanii,* and *Vibrio harveyi* survives in the cytoplasm of the obligate parasitic marine ciliate, *Cryptocaryon irritans*, using a major outer membrane protein, OmpU, which offers resistance to phagosomal conditions required for digestion and virulence factors

related to hemolytic activity to disrupt the phagosome (Abd *et al.,* 2007; Van der Henst *et al.,* 2016; Espinoza-Vergara *et al.,* 2020). This could have contributed to the survival of *Vibrio* sp. strain KM1 in *Paramecium* sp. RB1, which was not observed in *Tetrahymena* sp. RB2 (Table 4.3. and 4.4.).

A study by Anacarso *et al.* (2012) did not observe any difference in population growth of *A. polyphaga* without bacterial prey and with bacteria that intracellularly survived within these amoebae. A similar study by Abd *et al.* (2005) did not observe any difference in growth rates of *A. castellanii* in the absence of bacterial prey and the presence of *V. cholerae* O139, which survived and multiplied within the amoebae cells. Bacteria with the ability to intracellularly survive can either cause host cell lysis, multiply without causing cell lysis or multiply and cause host cell lysis (Brandl *et al.,* 2005; Matz and Kjelleberg, 2005; Gong *et al.,* 2016). The growth of protists in the presence of bacteria infers that bacterial cells are either completely or partially digested, providing sufficient nutrients to support cell replication. Poor growth of *Paramecium* sp. RB1 was observed with the Gram-positive *E. durans* ATCC 6056 and *S. aureus* ATCC 6053 and poor growth of *Tetrahymena* sp. RB2 was observed with the Gram-negative *E. coli* isolate EC33, suggesting that these prey bacteria did not provide sufficient nutrients to support growth (Chapter 2, Section 4.3.3.). Recovery of these bacterial prey as viable bacteria from the food vacuoles of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 (Table 4.3. and 4.4.) suggests that ciliates were most likely unable to digest enough bacterial cells ingested to provide sufficient nutrients for growth.

Bacterial resistance to metal toxicity has enabled some bacteria to survive the digestion process within the phagosome of protists (Sun *et al.,* 2018). Survival of metal resistant bacteria in protist phagosomes was observed by Hao *et al.* (2016), reporting that bacteria with genes encoding copper translocating ATPases led to an increase in the intracellular survival of bacteria within amoebae and the recovery of intracellular bacteria without a gene encoding copper translocating ATPases from the cells of amoebae, *Dictyostelium discoideum* was reduced by 30%. Metals such as copper (Cu) and zinc (Zn) play a crucial role in the killing of ingested prey in mature phagolysosomes in amoebae (German *et al.,* 2013; Sun *et al.,* 2018). The P80 gene, which encodes a copper transporter protein localised in endocytic compartments and involved in the maturation of phagosomes, was induced by the presence of ingested

bacteria in the cells of *D. discoideum* (German *et al.,* 2013; Hao *et al.,* 2016). Hanna *et al.* (2021) reported that Zn is delivered to the phagosome by fusion with zincosomes of endosomal origin immediately after ingestion of prey. Bacteria initially evolved mechanisms to resist metal intoxication due to protist grazing pressure (Adiba *et al.,* 2010; Sun *et al.,* 2018). Though metal ions are essential in bacterial metabolism, high concentrations of metal ions can be toxic to bacterial cells. Bacteria limit the damage caused by a sudden influx of metal ions through sequestration of metals in storage proteins, cytosolic buffering, and the expression of efflux pumps (Chandrangsu *et al.,* 2017). The effect of metal resistance on the digestion of ingested bacterial prey and survival through food vacuole passage time has so far only been described in amoebae. Grazing on metal-resistant bacteria can influence the digestion efficiency of protists, as the growth of the amoebae *D. discoideum* and the flagellate *Paracercomonas crassicauda* was lower when feeding on copper-resistant bacteria than growth with copper-susceptible bacteria (Hao *et al.,* 2016). The mechanism of killing and digesting ingested bacteria in food vacuoles is similar in all protists; it is, therefore, reasonable to assume that grazing on bacteria with metal resistance may similarly reduce digestion efficiency in ciliates. Moreover, antibiotic-resistant bacteria are known to frequently exhibit metal resistance. The presence of heavy metals has been described to contribute to the promotion of antibiotic resistance in bacteria, and the co-existence of heavy metal and antibiotic resistance genes have been reported in various bacteria including strains of *E. coli* and *Pseudomonas* (Nguyen *et al.,* 2019; Glibota *et al.,* 2020). Metal ions are present naturally, and metal resistance is common in environmental bacteria. Therefore, it would be of great interest to determine if these bacteria have mechanisms providing resistance to metal ions and if that contributed to their survival within the cells of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2.

Protists are regarded as a training ground for bacterial virulence and resistance to some antimicrobial compounds. Many of the intra-phagosomal mechanisms to kill and digest bacteria expressed by the innate immune system as the first line of defense against bacterial pathogens are highly conserved between eukaryotic cells (Espinoza-Vergara *et al.,* 2020). Micronutrients such as zinc (Zn), manganese (Mn), copper (Cu), and iron (Fe) are essential in the antimicrobial activity and also in innate immune system in defense against invading microbial pathogens (Samanovic *et al.,* 2012; Stafford *et al.,* 2013). The mechanisms of killing invading bacteria in macrophages are

similar in amoebae, i.e., nutrient deprivation, oxidative burst from reactive oxygen and nitrogen species, ATPase related acidification of the phagosome, use of metal transporters for Cu and Zn influx, and release of antimicrobial proteins and lysosomal hydrolases (Dupont *et al.,* 2011; Sun *et al.,* 2018). Therefore, resistance to digestion by protists potentially enhances the virulence and infection potential of bacteria.

Constant exposure to metal ions has driven the evolution and selection of homeostatic mechanisms that regulate the metabolism of metal ions while limiting their toxicity (Bondarczuk and Piotrowska-Seget, 2013). Thus, survival through food vacuole passage can enhance bacterial resistance to metal ions when used as a disinfection agent. Copper is widely used as an active agent in chemical formulations in healthcare and agriculture due to its antimicrobial properties (Casey *et al.*, 2010; Ballo *et al.,* 2016). Studies have demonstrated that hospital surfaces coated with solid copper sustained a lower microbial burden compared to surfaces without copper (Montero *et al.,* 2019). Copper resistance is most prevalent in bacteria found in copper-rich environments such as acid mine drainage (Tarrant *et al.,* 2019). Equally so, bacteria from these environments may have higher resistance to protist digestion and intracellular survival within food vacuoles as they are well adapted and have well-developed homeostasis to high levels of metal ions.

The survival of bacteria within protist cells is not only attributed to intracellular survival mechanisms. Some bacteria do not need an elaborated mechanism to survive intracellularly in the food vacuoles of ciliates. Digestion efficiency is also influenced by the type of prey ingested and the number of particles contained per food vacuole (Thurman *et al.,* 2010). For Gram-positive bacteria, lower digestion rates were shown to be due to the thick cell wall (15 to 80 nm thickness) with several layers of peptidoglycan compared to Gram-negative bacteria, which have a single peptidoglycan layer of 10 nm thickness (Gonzàlez *et al.* 1990; First *et al.,* 2012; Mai-Prochnow *et al*., 2016). This may have contributed to incomplete digestion as viable bacteria were recovered for most Gram-positive bacteria tested (Table 4.3. and 4.4.). Thurman *et al.* (2010) observed that when the number of *Synechococcus* sp. cells was more than 6 in food vacuoles of *Tetrahymena* sp., digestion was less efficient while all particles were digested when prey cell numbers per food vacuole were below the threshold ($\leq$6). The isolated ciliates ingested large numbers of bacteria per hour; therefore, some bacterial cells may be shielded from the degradative enzymes

released into the food vacuole, thus remaining intact throughout the vacuole passage in the ciliate cell.

Once ingested, food particles are digested in the food vacuole, and the nutrients are absorbed; the food vacuole is released from the lysosome and transported to the cytoproct where its content is egested to the outside of the cell (Nilsson, 1977; Gray *et al.,* 2012). The release of vesicles out of the cell signifies the end of a food vacuole process in the ciliate, and the vacuole processing time in *Paramecium* spp. and *Tetrahymena* spp. was reported to last for 2 to 4 hours, depending on the physicochemical conditions (i.e., temperature and oxygen) (Smith *et al.,* 2012). Faecal pellets or vesicles were expelled from the cells of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 and were detected after 24 hours of feeding. If undigested bacterial cells are present in the food vacuole, vesicles released from ciliates can be a dispersal vehicle for bacteria. Gourabathini *et al.* (2008) observed viable *S. enterica* in faecal pellets of *Tetrahymena* sp., and some of the faecal pellets analysed contained more than 20 bacterial cells. The faecal pellets of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 did not contain any metabolically active Gram-negative bacteria tested based on the cell elongation assay, indicating that the Gram-negative bacteria ingested by the two ciliates *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 were completely digested in food vacuoles prior to being released outside the cell (Table 4.5.).

Protection of ingested bacteria in ciliates from biocides

Ciliates can serve as a vector for the transmission of bacteria surviving intracellularly within ciliates. Moreover, ciliates can provide protection for undigested bacteria within the cell against biocides (Barker and Brown, 1994; Denoncourt *et al.,* 2014). Viable bacteria were recovered from cells of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 after exposure of isolated ciliates to chlorine solutions up to 5 mg/L (Table 4.6. and 4.9.). Chlorine is one of the most effective antimicrobial substances, which oxidizes various proteins and damages the bacterial cell membrane. It is widely used for microbial control in wastewater and drinking water treatments, in food industries for the reduction of the microbial population in food processing facilities, and on surfaces of fruits and vegetables (Virto *et al.,* 2005; Owoseni *et al.,* 2017; Azuma and Hayashi,

2021). The concentration of chlorine that is typically used in drinking water disinfection ranges from 0.5 to 2 mg/L, and the concentration of chlorine used in the disinfection of fresh produce ranges from 50 to 200 mg/L (Zhou *et al.,* 2015). Chlorine is extremely effective for inactivating Gram-negative bacteria, as a concentration of 0.2 mg/L with a contact time of 50 minutes and a concentration of 1.1 mg/L with a contact time of 1 minute inactivated 99.9% of *E. coli* O157: H7 cells (Rice *et al.,* 1999; Denisova *et al.,* 2014; Owoseni *et al.,* 2017).

However, chlorine has been shown to have limited success when used for inactivating protists, particularly cysts, as these are less susceptible to chlorine inactivation than vegetative cells (Cursons *et al.,* 1980; Mogoa *et al.,* 2011). *Hartmannella vermiformis* cells were sensitive to chlorine concentrations of 2 to 4 mg/L, while its cysts sensitivity to chlorine was only evident at 4 to 10 mg/L after 30 minutes of contact time, with 10 mg/L being lethal to both (Kuchta *et al.,* 1993). King *et al.* (1988) observed that the cells of *T. pyriformis* remained active and motile at lower concentrations (0.5 to 2 mg/L chlorine) after 30 to 60 minutes and that the ciliate cells survived exposure of residual chlorine at a concentration of 4 mg/L for 30 minutes and were rejuvenated following inoculation of fresh medium. The three isolated ciliates were sensitive to chlorine concentrations of 1 to 10 mg/L. Although cells remained intact and active as indicated by the ciliatory beating, the swimming speed of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 was reduced at chlorine concentrations of 1 to 2.5 mg/L. The cells of *Paramecium* sp. RB1 were lysed after 30 minutes of contact time while those of *Tetrahymena* sp. RB2 were lysed after 15 minutes of contact time at 5 mg/L free chlorine. The cells of *Coleps* sp. RB3 were the most sensitive of the three ciliates; cells were immobilized instantly and lysed after 15 minutes of contact time at a concentration of only 1 mg/L of free chlorine. Thus, the effectiveness of chlorine in the inactivation of ciliates depends on the concentration of chlorine and the ciliate species.

Generally, the disinfection efficiency of biocides such as chlorine depends on the concentration and contact time (LeChevallier *et al.,* 1988; Galal-Gorchev, 1996; West *et al.,* 2018). Luo *et al.* (2011) reported that a concentration of 0.5 mg/L free chlorine was effective for inactivating *E. coli* O157:H7 to below the detection level, but at 5 mg/L chlorine residual, *E. coli* O157:H7 was not detected in the wash solution after 30 seconds of contact time. Shen *et al.* (2013) demonstrated that some bacterial species are more susceptible to chlorine residual and may require shorter exposure to chlorine

to achieve maximal inactivation than other bacterial species. The inactivation assay of different bacteria in the present study demonstrated the effectiveness of chlorine treatment on freely suspended bacteria and that the efficiency of chlorine disinfection varied with each bacterial species. A chlorine concentration of 2.5 mg/L achieved up to 3-log reduction of viable counts of all tested bacteria after 30 minutes of exposure (Table 4.8. and 4.11.). Likewise, more than 2-log reduction of viable bacteria of uningested bacterial cells from co-cultures with ciliates was observed at a chlorine concentration of 2.5 mg/L after 30 minutes contact time (Table 4.7. and 4.10.). At this concentration (2.5 mg/L), ciliate cells were still intact; thus, undigested bacterial cells present in the food vacuoles are protected from the external environment and were recovered as viable bacteria from cells of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 after chlorine exposure (Table 4.6. and 4.9.). A higher concentration of chlorine, 5 mg/L, caused lysis of the isolated ciliates at 30 minutes exposure, thus, releasing and exposing undigested bacteria in food vacuoles to chlorine residuals. However, some viable bacteria were still recovered from ciliates after exposure of *Paramecium* sp. RB1 (*E. coli* isolate EC33, *Vibrio* sp. strain KM1, and *E. faecalis* ATCC 29212) and *Tetrahymena* sp. RB2 (*E. faecalis* ATCC 29212 and *E. durans* ATCC 6056) to a chlorine concentration of 5 mg/L for 30 minutes (Table 4.6. and 4.9.). The food vacuole membrane provides an additional barrier reducing contact of chlorine to packaged bacterial cells. As disinfection is not instantaneous, a longer contact time is required to inactivate undigested bacteria as they are released from membrane-bound food vacuoles of ciliates. Additionally, the efficiency of chlorine is influenced by the size of the cell. Noszticzius *et al.* (2013) reported that the diffusion and killing time of chlorine is a factor of body size. Thus, chlorine diffusion rate into a larger ciliate cell is most likely slower, i.e., *Paramecium* sp. RB1 than in the smaller ciliate cell of *Tetrahymena* sp. RB2. As expected, higher viable counts of bacteria were recovered from cells of *Paramecium* sp. RB1 than from *Tetrahymena* sp. RB2 (Table 4.6. and 4.9.).

Disinfection of fresh produce uses high free chlorine concentrations (50 - 200 mg/L) that are effective in inactivating both ciliates and bacteria. However, in wastewater and drinking water treatments, the two isolated ciliates, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 have the potential to protect bacteria present within the cells. A high concentration of chlorine (e.g., $\geq$ 5 mg/L and a longer contact time (> 30 minutes) is required to lyse ciliates instantaneously, releasing ingested bacteria from

the cells, thus exposing bacteria to chlorine. The use of high concentrations of chlorine, however, increases the risk of the formation of undesirable hazardous by-products that may decrease the quality of water (Richardson *et al.,* 1998).

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CHAPTER 5

Conclusion

Ciliates are an integral part of aquatic ecosystems. Therefore, in the present study three freshwater ciliates were isolated from the Blackborough stream in Pietermaritzburg, KwaZulu-Natal (South Africa) and identified as species of the genera *Coleps*, *Paramecium*, and *Tetrahymena* highlighting the presence of bacterivorous ciliates in South African natural freshwater environments. The uptake of bacteria as prey and reduction of bacterial counts demonstrates that the three freshwater ciliates can effectively remove bacteria from the aquatic environment. Grazing studies provided an insight into the predator-prey interaction of the three isolated bacterivorous ciliates with various bacterial species in view of growth. Their grazing impact on bacterial numbers is however influenced by numerous factors, including prey size, prey availability, and temperature. Additionally, the reduction of bacteriophage particles in the presence and absence of susceptible bacterial prey indicates that the grazing activity of *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 can contribute to the inactivation of viruses, thus indicating their potential in the removal of viruses particularly in wastewater treatment plants where pathogenic viruses are most abundant and are a major concern for public health (Gibson, 2014; Corpuz *et al.,* 2020). All three isolated ciliates ingested polystyrene microbeads of varying sizes that were detected in food vacuoles, thus demonstrating that their inability to discriminate between nutritious and non-nutritious particles might contribute to the transfer and bioaccumulation of microplastics and adsorbed pollutants via the aquatic food web. The recovery of viable bacteria from food vacuoles of the two isolated ciliates, *Paramecium* sp. RB1 and *Tetrahymena* sp. RB2 indicates that the interaction of ciliates with bacteria, both after ingestion and after chlorine treatment, may contribute to the persistence of bacteria in the environment. Ciliates therefore may contribute to the survival of ingested bacteria even during biocide treatments at concentrations typically used in wastewater treatment. If not digested within food vacuoles, the survival of pathogenic bacteria within ciliates may present a potential risk to public health, enabling the persistence and possible transmission of ingested pathogenic bacteria.

Ciliates are one of the main bacterial predators and account for 50% of the total protozoal population in freshwater environments (Beaver and Crisman, 1989; Hisatugo *et al.,* 2014; Weisse, 2017). Their crucial role as bacterial predators and an integral part of the food web has long been recognized in various aquatic environments. However, current studies on ciliates in South African aquatic ecosystems are lacking. Thus, the present study provides an insight into the presence of ciliates and their potential ecological role in South African natural aquatic systems. The contribution of ciliates to the possible survival and persistence of bacteria is now being acknowledged and confirmed by the present study. Therefore, this study contributes information to understand the role of ciliate and bacteria interaction in the persistence and transmission of bacteria in aquatic environments. Moreover, the potential role of ciliates in the bioaccumulation and transfer of microplastics in the food web was highlighted for fresh environments.

Limitations of the study

A limitation of this study is that the potential interaction of mixed ciliate assemblages was not assessed. Assessing predator-prey interaction with a single species of ciliates and bacteria is essential to demonstrate species-specific interaction with bacterial prey, however, it does not accurately depict more complex predator-prey interactions taking place in natural environments. In the natural environment, ciliates exist in a mixed consortium of different ciliates species and other protists including species feeding on ciliates, that may synergistically or antagonistically interact and affect each other due to competition for bacterial prey. The composition of protist communities is important for understanding the effect of protists grazing on bacterial communities. Additionally, the predator-prey interaction was evaluated with three ciliates isolated from the Blackborough stream in Pietermaritzburg under defined conditions. However, as ciliates are a diverse group of protists, are ubiquitous and a crucial component of microplankton communities in many aquatic ecosystems, more research is necessary to evaluate the species-specific interaction of different ciliate species with different bacterial species under environmental conditions to improve our understanding of the contribution of different ciliates to the survival of bacteria.

Outlook and future studies

The present study demonstrated that local ciliate isolates can feed on bacteria and even certain eukaryotes. In addition, it was demonstrated that ciliates can graze on microplastic particles and affect the number of phage particles. Finally, it was shown that bacteria might survive inside ciliate cells and are protected to some degree from biocide treatments.

However, based on the limitation of the present study, the following would be worth pursuing:

- Symbiotic relationship of *Tetrahymena* sp. RB2 with algae

Endosymbiosis of algae with ciliates has mostly been studied with *Paramecium* species. Grazing of *Tetrahymena* sp. RB2 on *Parachlorella* sp. strain AA1 resulted in the formation of presumptive perialgal vacuoles in the cytoplasm of the ciliates, therefore the interaction of *Tetrahymena* sp. RB2 with algae warrants further analysis.

- Grazing of ciliates on viral particles at environmentally relevant concentrations

The study demonstrated that ciliates are important in the removal of not only bacteria but can also contribute to the elimination of viruses. Viruses targeting bacteria are present at greater concentrations than bacteria in aquatic environments. Thus, studying the interaction of ciliates with bacteria and their viruses at proportions that naturally occur in the environment to evaluate the grazing impact of ciliates on both bacteria and viruses in the environment would be important.

- Uptake of microplastic particles by ciliates at environmentally relevant concentrations.

Microplastics were used in this study to determine the preferred prey size for ingestion of the isolated ciliates. Microplastics are a major concern in the environment as they can be ingested by aquatic organisms and accumulate in the food webs. The concentration of microplastics used in the current study was higher than concentrations commonly detected in freshwater and marine environments. Studying

the uptake of microplastics including varying plastic materials and shapes by ciliates would provide a better understanding of the possible impact of microplastics on the growth of ciliates and their role in microplastics bioaccumulation along the food webs.

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