PHAGOTROPHY IN PHOTOSYNTHETIC EUKARYOTIC MICROBES FROM POLAR ENVIRONMENTS

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ABSTRACT

Polar regions impose harsh conditions, including low temperatures, and prolonged periods of darkness on resident microbial communities. Despite these challenges, the conditions in these environments can also create opportunities for organisms utilizing combined trophic strategies (Mixotrophy). Only a limited number of studies have identified mixotrophic behavior in polar microbial eukaryotes, and even fewer studies have quantified the response of mixotrophs to likely environmental drivers of trophic behavior (light and nutrients). The goal of this work is to provide an identification of mixotrophic behavior and elucidate some of the factors that influence algae isolated from polar environments. First, a study of the Arctic prasinophyte, *Micromonas pusilla* is presented in the first species-specific identification of mixotrophy in a eukaryotic phytoflagellate of this size class. *M. pusilla* grazed on bacteria under all experimental conditions, responding to nutrient limitation with increased rates of bacterivory. *M. pusilla* also showed evidence of prey selection. In contrast to the phagotrophic response, photosynthetic production was decreased under low-nutrient conditions. In an additional study of microbial eukaryotes from the Antarctic environment, identification of phagotrophy in photosynthetic nanoflagellates representing multiple evolutionary lineages: Cryptophyceae (*Geminigera cryophila*) and Prasinophyceae (*Pyramimonas tychotreta* and *Mantoniella antarctica*), showed that mixotrophy is more widespread in the Southern ocean that previously thought. *G. cryophila* and *M. antarctica* increased ingestions in dark treatments, but did not respond to difference in nutrient concentrations. In contrast, no significant grazing activity was observed in *P. tychotreta* under high nutrient conditions. When nutrients were reduced, ingestion of bacteria by *P. tychotreta*
was observed and grazing increased in dark as compared to illuminated treatments. Finally, through a series of experimental assays, the competitive advantages of mixotrophic flagellates as opposed to monotrophic specialists were evaluated, using organisms isolated from the Southern Ocean. In these experiments, *G. cryophila* is emerged as a dominant competitor against two solely autotrophic diatoms (*Fragilaria* sp. and *Fragilariopsis* sp.). In contrast, *P. tychotreta* was outcompeted by the solely heterotrophic chrysophyte *Paraphysomonas antarctica*. These results show that mixotrophic ability can confer advantages to organisms in some cases, while in other interactions the cost associated with maintenance of multiple trophic strategies results in competitive exclusion by a specialist. These results present novel identification as well as rigorous investigation of mixotrophic behaviors in phototrophic flagellates from both polar (Arctic and Antarctic) environments representing two evolutionary lineages. This work provides a significant contribution to our understanding of the versatile nature of the physiology and trophic ecology of microbial eukaryotic organisms occupying polar marine ecosystems.
This work is dedicated to

My family,

My mother, Karen, and father, Barry,

My brother, Moshe,

Also to my late Grandmother, Rose Boatwright.

Without them this work would not be possible.
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First, I would like to thank my advisor Bob Sanders for this mentorship during this project. Bob introduced me to the world of microbial ecology as well as the study of extremophile organisms. Bob’s contributions to this work are irreplaceable and his efforts have allowed me to develop greatly as a scientific researcher. I would also like to thank Erik Cordes and Allen Nicholson for their contributions to this work. Erik’s willingness to provide advice and support throughout this work greatly improved the strength of this dissertation. Allen’s thoughtful critiques provided vital input and contributed to a more complete dissertation. I would also like to thank Rebecca Gast, my external committee member. Without her contributions, providing support and review of both the data and writing in this dissertation, this project would not have come together. The experience of being able to take part in the research collaborations headed by Rebecca and Bob is something that I will take with me for the rest of my scientific career.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>CHAPTER</td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Mixotrophy: The Use of Combined Trophic Strategies</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Mixotrophs and the Microbial Loop</td>
<td>3</td>
</tr>
<tr>
<td>1.3 Gradient of Mixotrophic Behaviors</td>
<td>4</td>
</tr>
<tr>
<td>1.4 Factors Influencing Phagotrophic Behavior in Phototrophs</td>
<td>6</td>
</tr>
<tr>
<td>1.4.1 Nutrients</td>
<td>6</td>
</tr>
<tr>
<td>1.4.2 Prey Selectivity in Protistan Predators</td>
<td>8</td>
</tr>
<tr>
<td>1.5 The Polar Environment</td>
<td>9</td>
</tr>
<tr>
<td>1.5.1 Northern Polar Latitudes</td>
<td>9</td>
</tr>
<tr>
<td>1.5.2 The Antarctic</td>
<td>10</td>
</tr>
<tr>
<td>1.6 Previous Observations of Mixotrophy in Polar Flagellates</td>
<td>11</td>
</tr>
<tr>
<td>1.7 Bacterivory in Photosynthetic Pico- and Nanoflagellates From Polar Regions</td>
<td>12</td>
</tr>
</tbody>
</table>
2. PHAGOTROPHY BY THE ICOEUKARYOTIC GREEN ALGA *MICROMONAS*:

IMPLICATIONS FOR ARCTIC OCEANS.................................................................14

2.1 Abstract.........................................................................................................14

2.2 Introduction....................................................................................................15

2.3 Materials and Methods..................................................................................17

2.3.1 Culture Origin, Maintenance and Sizing..................................................17

2.3.2 Microscopic Imaging of *Micromonas*......................................................18

2.3.3 General Grazing Experiment (2x2 factorial).............................................20

2.3.4 Size Selection Experiment.........................................................................21

2.3.5 Photosynthesis vs. Irradiance Responses..................................................22

2.3.6 Data and Statistical Analysis.....................................................................23

2.4 Results............................................................................................................24

2.5 Discussion......................................................................................................28

2.5.1 Bacterivory in *Micromonas*...................................................................29

2.5.2 Photosynthetic Responses in *Micromonas*............................................31

2.6 Conclusion.....................................................................................................32

3. PHYSIOLOGICAL RESPONSES OF THREE SPECIES OF ANTARCTIC

MIXOTROPHIC PHYTOFLAGELLATES TO CHANGES IN LIGHT AND

DISSOLVED NUTRIENTS.......................................................................................34

3.1 Abstract...........................................................................................................34

3.2 Introduction....................................................................................................35

3.3 Materials and Methods..................................................................................37

3.3.1 Culture Origin and Maintenance...............................................................37
3.3.2 Grazing Experiments (2x2 factorial).................................37
3.3.3 Photosynthesis vs. Irradiance Response Curves..................40
3.3.4 Dark Survival..................................................................41
3.3.5 Data and Statistical Analysis...........................................41

3.4 Results.................................................................................42
3.4.1 Grazing Rates in Mixotrophic Flagellates..........................42
3.4.2 Photosynthesis in Antarctic Nanoflagellates....................45
3.4.3 Dark Survival...................................................................47

3.5 Discussion............................................................................49

4. COMPETITION BETWEEN ANTARCTIC MIXOTROPHIC PROTISTS AND
PHOTOTROPHIC AND HETEROTROPHIC SPECIALISTS..................56
4.1 Abstract.............................................................................56
4.2 Introduction.........................................................................57
4.3 Materials and Methods........................................................60
4.3.1 Culture Origin and Maintenance......................................60
4.3.2 Experimental Setup........................................................60
4.3.3 Data and Statistical Analysis...........................................61

4.4 Results..................................................................................63
4.4.1 Experiment I: Mixotroph (G. cryophila) vs. Autotrophic
Competitors..............................................................................63
4.4.2 Experiment II: Mixotroph (P. tychotreta) vs. Heterotrophic
Flagellate..................................................................................65

4.5 Discussion............................................................................69
5. CONCLUSIONS AND FUTURE DIRECTIONS ............................................75

5.1 The Arctic Picoeukaryote: Micromonas pusilla.....................................76

5.2 Antarctic Flagellates.............................................................................78

5.3 Mixotrophy in Competition.................................................................79

BIBLIOGRAPHY ........................................................................................81
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Confocal and epifluorescence microscopic imaging if ingestion in <em>Micromonas pusilla</em></td>
<td>19</td>
</tr>
<tr>
<td>2.2. General grazing rates (2x2 factorial) by <em>M. pusilla</em></td>
<td>25</td>
</tr>
<tr>
<td>2.3. Size-selective grazing behavior in <em>M. pusilla</em></td>
<td>25</td>
</tr>
<tr>
<td>2.4. Photosynthesis vs. Irradiance curves for <em>M. pusilla</em></td>
<td>27</td>
</tr>
<tr>
<td>3.1. Epifluorescence micrographs of grazing in Antarctic mixotrophic nanoflagellates</td>
<td>39</td>
</tr>
<tr>
<td>3.2. Phagotrophic ingestion (2x2 factorial) in Antarctic mixotrophic nanoflagellates</td>
<td>44</td>
</tr>
<tr>
<td>3.3. Photosynthesis vs. Irradiance curves for Antarctic mixotrophic nanoflagellates</td>
<td>46</td>
</tr>
<tr>
<td>3.4. Cell abundances during dark survival experiments with Antarctic mixotrophs</td>
<td>48</td>
</tr>
<tr>
<td>4.1. Competition Experiment I: Mixotroph (<em>G. cryophila</em>) vs. autotrophic competitors</td>
<td>64</td>
</tr>
<tr>
<td>4.2. Competition Experiment II: Mixotroph (<em>P. tychotrema</em>) vs. heterotrophic flagellates</td>
<td>67</td>
</tr>
<tr>
<td>4.3. Bacterial abundances for Experiment II: Mixotroph (<em>P. tychotrema</em>) vs. heterotrophic flagellates</td>
<td>69</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Parameter of P vs. I curve for <em>M. pusilla</em> estimated by Eilers-Peeters function</td>
<td>27</td>
</tr>
<tr>
<td>3.1. Parameter of P vs. I curve for Antarctic nanoflagellates estimated by Eilers-Peeters function</td>
<td>47</td>
</tr>
<tr>
<td>4.1. Models of microbial growth used for competition experiments</td>
<td>62</td>
</tr>
<tr>
<td>4.2. Parameter estimates for curves estimated for Experiment I</td>
<td>65</td>
</tr>
<tr>
<td>4.3. Parameter estimates for curves estimated for Experiment II</td>
<td>68</td>
</tr>
</tbody>
</table>
1.1 Mixotrophy: The Use of Combined Trophic Strategies

Most organisms are thought to be either heterotrophic, deriving their energy requirement by consuming other organisms (or the organic by-products of other organisms) or, alternatively, as autotrophic, fixing carbon compounds for energy by utilizing an external energy source (light, for example) as a reducing agent. Although many organisms can be described sufficiently in this manner, there are still many organisms, particularly among protistan groups, that exhibit a much more complicated trophic ecology. Microbial eukaryotes exhibit a high degree of diversity in terms of form and function. The use of mixotrophy, the combined use of photosynthesis and phagotrophic modes of feeding within a single organism, represents an example of this complexity.

Some of the earliest observations of mixotrophic behavior were presented by Pfeffer (1897) whose observations described a single organism linking multiple trophic strategies. Pringsheim (1952) and Myers and Graham (1956) were among the first to describe the plastic nature of energy acquisition in the mixotroph Ochromonas, a chrysophyte. Almost 40 years later, increased inquiry into the significance and prevalence of mixotrophic behavior by researchers such as Boraas et al. (1988) and Sanders (1991) presented reviews on the prevalence of mixotrophy in several evolutionary lineages. Both studies noted the impact of, and the phagotrophic activity in, phototrophs. Also noted was the widespread occurrence of mixotrophic species in
aquatic systems. Over the next three decades this work led to increased research on alternative acquisition strategies in microbial eukaryotes. The results of this extended study of mixotrophy were a number of important scientific discoveries, including evidence for the mechanism of endosymbiotic theory proposed by Sagan (1967). According to this theory, organelles used for phototrophic energy production were originally foreign organisms phagotrophically ingested, and subsequently retained for endogenous functioning (Raven 1997). Although the specific definition of mixotrophy can include photosynthetic organisms that take up dissolved organic matter, for the purposes of this dissertation, I will use the strict definition of mixotrophy to include photosynthetic organisms that engage in phagotrophy (the ingestion of particles).

Mixotrophic behavior has been observed in organisms that belong to several distinct evolutionary groups of unicellular eukaryotes and includes members of all major photosynthetic groups, with the exception of the Bacillariophyceae (diatoms) (Boraas et al. 1988, Sanders 1991, Stickney et al. 2000, Sherr and Sherr 2002). This suggests that mixotrophic strategies are not simply a result of evolutionary conservation, but could also provide ecological niche opportunities for organisms of diverse evolutionary lineages to thrive where they would otherwise be excluded. The strategy of mixotrophy, it seems, is employed by organisms in response to multiple factors for different reasons (Stickney et al. 2000, Jeong et al. 2010, Hartmann et al. 2013). Mixotrophic eukaryotes are found in a variety of habitats including both marine and fresh water. Research into the conditions that facilitate the survival of mixotrophic protists is ongoing, but a full understanding as to the trophic complexity in microbial food webs will provide a more complete picture of ecosystem processes (Czypionka et al. 2011, Sanders 2011, Unrein et al. 2014).
1.2 Mixotrophs and the Microbial Loop

Despite strong competition for dissolved nutrients, the coexistence of phytoplankton and bacteria is believed to be maintained, in part, by the top-down control of bacteria via the grazing activity of heterotrophic flagellates and ciliates (Fenchel 1982, Azam et al. 1983, Hulot et al. 2001). These interactions of bacteria, algae and heterotrophic protists are responsible for much of the recycling of the soluble nutrient pools and the transfer of biomass to higher trophic levels, particularly in pelagic systems (Azam et al. 1991, Caron 1994). Just as algae, bacteria, and heterotrophic protists differ in their abilities to obtain specific resources, the composition, structure and abundance of organisms within microbial communities can affect the size and availability of nutrient pools within a system.

Grazing by hetero- and mixotrophic protists on bacteria and phytoplankton can have a significant effect upon bacterial production; this process can account for a substantial energy input at higher trophic levels (Sherr and Sherr 2002). When mixotrophic flagellates and ciliates are considered in these systems, the situation becomes particularly interesting. With a net C growth efficiency (amount of bacterial C retained in protistan biomass following the digestion of prey) of 60%, at best, heterotrophic flagellates are not as efficient in carbon transfer as most autotrophic organisms (Calow 1977, Fenchel 1982, Caron et al. 1990). The combination of photosynthesis and bacterivory in a single organism is thought to provide a more efficient use of nutritional substrates than is provided by their heterotrophic counterparts (Troost et al. 2005). In addition, reintroduction of nutrients (i.e. N, P, Fe) previously immobilized by bacteria
can be recycled back into the food web via grazing (Azam et al. 1983, Caron and Goldman 1988, Maranger et al. 1998).

In addition to the effect on bacterial biomass, bacterivorous activity by protist populations can have a substantial effect on the structure of bacterial communities (Simek et al. 1997, Boenigk and Arndt 2002). Microbial communities are responsible for important pathways linking the reintroduction of carbon and nutrients into biotic pathways of ecosystems (Azam et al. 1983, Pomeroy et al. 2007, Mitra et al. 2014). The effect of mixtrophic protists, contributing unknown rates of remineralization (by predation of immobilized bacterial production), as well as autotrophic fixation, is still unknown. Increased understanding of the mixtrophic portion of the food web in aquatic systems can provide insight into the ecosystem dynamics of the larger habitat.

1.3 Gradient of Mixotrophic Behaviors

It is assumed that a combination of trophic strategies will incur a cost to the mixotrophic organism, such as the sacrifice of cell surface for phagotrophy that could otherwise house photosynthetic antennae machinery complex (Ward et al. 2011). Physiological adjustments are required in some mixotrophs before switching from sole heterotrophy to photosynthetic functioning (Sanders et al. 1990). In a study of *Ochromonas danica*, cultures grown in the presence of bacterial prey resulted in an increase in the PSI:PSII, a physiological shift that would increase cyclic photophosphorylation using photosynthetically derived NADPH for ATP production rather than for C-fixation (Wilken et al. 2014). In terms of microbial community
composition, the trade-offs associated with heterotrophy and/or autotrophy are likely drivers of realized niche occupancy of mixotroph populations.

A specific mixotrophic protist can exhibit differential reliance, either obligate or facultative, on either heterotrophy or autotrophy along a gradient of mixotrophic behavior unless high levels of dissolved organic matter are present (Stoecker 1998, Jones 2000, Stickney et al. 2000, Sanders et al. 2001). Several reviews of mixotrophy have discussed tradeoffs between autotrophic and heterotrophic inputs in mixotrophic organisms (Rothhaupt 1996b, Ward et al. 2011). Examples of mixotrophic flagellates tending toward the heterotrophic end of the spectrum include the nanoflagellate chrysophyte, *Ochromonas*, a chloroplast containing organism that is able to sustain high rates of growth while remaining in total darkness, solely sustaining itself through bacterial predation (Sanders et al. 2001). While variations in light and nutrients have little effect on its grazing rates, *Ochromonas* will not survive when grown in the dark, in the absence of bacteria (Andersson et al. 1989), unless high levels of dissolved organic matter are present (Sanders et al. 2001). Another related mixotrophic chrysophyte, *Poterioochromonas malhamensis*, showed no evidence of decreased ingestion of prey even when light levels showed photoinhibition of photosynthesis, (Sanders et al. 1990). In this mixotroph, photosynthesis was found to contribute, at most, 7% of its carbon budget and is considered to be on the phagotrophic end of the mixotrophic gradient.

Examples of mixotrophic protists which display more obligative autotrophic behavior include the chrysophyte, *Dinobryon* which is more phototrophic. In this case, studies have estimated that phagotrophic behavior can only account for 50% of carbon assimilation, while up to 100% of carbon can be derived from photosynthesis (Bird and
Kalff 1987, Bird and Kalff 1989, Raven 1997). The prymnesiophyte *Chrysochromulina brevifilum* is another example of a photosynthetic flagellate capable of prey ingestion, yet is unable to survive solely on this nutritional source in the absence of light (Jones 1997). Several dinoflagellate species also exhibit obligate phototrophy, ingesting prey to supplement limiting nutrients (Stoecker 1999). There are also mixotrophic flagellates that are more balanced in their hetero- vs. autotrophic capabilities. Photosynthetic response was reduced by up to 49% in the presence of prey in the mixotrophic dinoflagellate *Fragilidium subglobosum* (Skovgaard et al. 2000). *F. subglobosum* can survive as an obligate phototroph, but growth rates were significantly higher when ingestible prey were present. Included in this definition of mixotrophy are the kleptoplastidic organisms that feed on photosynthetic prey, sequestering the chloroplast for endogenous functions. This also includes organisms such as the ciliates *Laboea strobila*, *Mesodinium rubrum*, and dinoflagellates such as the Ross Sea Dinoflagellate (Stoecker et al. 1988, Gustafson et al. 2000, Gast et al. 2007, Sellers et al. 2014).

### 1.4 Factors Influencing Phagotrophic Behavior in Phototrophs:

#### 1.4.1 Nutrients

The strategy of mixotrophy can be utilized for various reasons in different groups of phytoplankton. For example, bacterivory (ingestion of bacteria as prey) in mixotrophic flagellates can be induced by multiple factors including: decreased light availability, prolonged darkness, and/or specific dissolved nutrient limitation (Stoecker et al. 2009, Sanders 2011, Flynn et al. 2013). These limiting resources can serve as cues for photosynthesizing mixotrophs to begin ingesting prey and/or cause changes in ingestion
rates by these protists (Nygaard and Tobiesen 1993, Keller et al. 1994). Ingestion of prey by photosynthesizing protists has been suggested as a mechanism for surviving prolonged periods of darkness, and could be a useful strategy for organisms that are required to survive the extended Austral and Boreal winters such as those which occur at polar latitudes (Jones et al. 2009). In some cases, mixotrophic activity may actually require light. For example, Keller et al. (1994) did not observe significant feeding rates in the mixotrophic chrysophyte *Ochromonas* until cultures were exposed to low-level light irradiances; maintaining *Dinobryon* sp. in the dark inhibited grazing (Caron et al. 1993).

Algal cells show diverse photoinhibitory responses, but it is likely that light levels exceeding the optimal irradiance of photosynthesis for a given organism are likely to elicit increases or, at least, maintenance of grazing responses in mixotrophic flagellates, particularly in species utilizing the macro- or micronutrients of prey biomass.

Other studies have shown that reduced concentrations of soluble nutrients led to increased phagotrophic activity in some mixotrophic protists, presumably as a mechanism for the acquisition of those substrates (Nygaard and Tobiesen 1993, Arenovski et al. 1995, Maranger et al. 1998). Species-specific studies include several evolutionarily distinct lineages of algae including the haptophytes *Chrysochromulina*, and *Prymnesium parvum*, the chrysophytes *Uroglena americana* and *Ochromonas minima*, responding to P limitation by increased rates of bacterivory (Nygaard and Tobiesen 1993, Horne and Goldman 1994, Rothhaupt 1997, Hitchman and Jones 2000). In addition, Stoecker et al. (1999) found the mixotrophic dinoflagellate, *Prorocentrum minimum*, engaged in predation of cryptophyte prey in response to inorganic nutrients or trace growth factor requirements. Phagocytosis in at least one mixotrophic genus, has
been linked to a requirement for certain phospholipids (Kimura and Ishida 1989).

Though the factors that control phagotrophic activity in mixotrophic organisms are diverse, light availability, access to nutrients and/or prey density are probably the largest driving forces for mixotrophic behavior (Keller et al. 1994, Jones et al. 2009).

1.4.2 Prey Selectivity in Protistan Predators

Foraging by microbial predators is governed by dynamics similar to those that govern their larger metazoan counterparts. Phagotrophic protist populations vary in terms of preferred food size, handling time, and recognition of nutritional content. Elemental stoichiometry of prey can modify growth rates and cell size of Ochromonas (Chrzanowski and Simek 1990), and mixotrophic flagellates have been found to operate under the optimal foraging paradigm, implying that they are much more than opportunistic predators (Stibor and Sommer 2003). Species-specific studies have shown the ability to select for particle size in obligate, solely phagotrophic flagellates and ciliates. However, grazing behavior of mixotrophic protists has received less attention (González et al. 1990, Holen and Boraas 1991, Epstein and Shiaris 1992). Previous studies have shown that some mixotrophic nanoflagellates, like the chrysophyte, Ochromonas, show a high level of selectivity for large sized bacterial prey (Andersson et al. 1986, Chrzanowski and Simek 1990, Pfandl et al. 2004). Identifying prey-size selectivity preferences in bacterivorous mixotrophs will help shape our understanding of how mixotrophic predator populations can influence bacterial prey community structure.
1.5 The Polar Environment

1.5.1 Northern Polar Latitudes

The Arctic marine environment is categorized by seasonally ice-free shallow shelf seas, as well as central deep basins, that are usually permanently ice covered throughout the year (Wheeler et al. 1997), though climate change has modified ice cover in recent years (Vincent 2010). This region can present challenges to organisms because of extreme variation in: light availability, ocean temperatures associated with the seasonal change, and fluctuations in salinity due to the influx of Pacific and Atlantic waters from temperate latitudes. Despite the contiguous appearance of marine ecosystems, intrusion of temperate waters due to ocean currents can form a gradient of environmental conditions. Molecular surveys of these regions have identified specific and distinct Arctic phylogenotypes of planktonic species endemic to these waters (Lovejoy and Potvin 2011). The Arctic marine environment has particular sensitivity to external forces, such as changes in greenhouse gas concentrations relative to temperate latitudes of the Northern Hemisphere. This results in the “polar amplification” of climate change and biological communities in the region are likely to be strongly affected (Walsh 2008).

These seas have a distinct lack of significant cyanobacteria blooms; the small size fraction of the phytoplankton community (0.2-2µm) is dominated by picoeukaryotes (Booth and Horner 1997, Tremblay et al. 2009). Increasing temperatures have been suggested to contribute to other changes in environmental conditions vital to microbial communities, such as decreased nutrient concentrations and salinity (Vincent 2010). These factors drive changes in bacterial production and may increase population sizes of in picoplanktonic eukaryotes (Tremblay et al. 2009). One of the most abundant
picoflagellates is the prasinophyte, *Micromonas pusilla* which was observed to be the most abundant picoeukaryotic mixotroph present during an autumn cruise to the Beaufort Sea, and is often at high concentration (Lovejoy and Potvin 2011, Sanders and Gast 2012). This is particularly important because, even with very low individual grazing rates, mixotrophic activity of abundant picoeukaryotes (such as *M. pusilla*), can make them important bacterivores in temperate oceanic systems and potentially dominant grazers of bacteria in the Arctic Ocean (Sanders and Gast 2012).

### 1.5.2 The Antarctic

The Southern Ocean comprises the largest open ocean marine environment on Earth. This region is particularly important in its potential influence upon the global cycling of nutrients, including carbon. The study of the central roles, dynamics and specific ecological strategies employed by members of the microbial communities in the Southern Ocean are integral to our understanding of these systems (Azam et al. 1991). The potential advantages of mixotrophic strategies in this extreme marine environment suggest that mixotrophy could play a critical role in survival of marine eukaryotes.

The topographic heterogeneity of the Antarctic environment allows for a diversity of microhabitats both adjacent to the terrestrial Antarctic continent and far offshore: open water, under ice, in brine channels and slush on floating ice. Algal communities in the Southern Ocean include large number of species of various evolutionary lineages, with some of the dominant open water blooms often comprised of diatom species, such as: *Fragilariopsis*, *Nitzschia*, and *Pseudonitzschia* and the haptophyte alga *Phaeocystis antarctica* (Dennett et al. 2001, Fiala et al. 2006); during
algal blooms these species make up the dominant proportion of the biomass in these systems. Although flagellates (other than *Phaeocystis*) often make up less than 5% of total cell abundance, they can play a crucial role in controlling bacterial production (Sherr and Sherr 2002, Fiala et al. 2006, Pearce et al. 2011). Indeed, phytoflagellates from lineages as diverse as Prasinophyceae and Cryptophyceae can form blooms and in the Antarctic and several members of these taxa are known mixotrophs (Arrigo et al. 1999, Daugbjerg et al. 2000, Kang et al. 2001, Gast et al. 2014).

### 1.6 Previous Observations of Mixotrophy in Polar Flagellates

To date, investigations of mixotrophy in polar microbial communities have been limited. Despite this, the available studies and recent field investigations suggest a major grazing impact on bacterial populations. Mixotrophic flagellates such as the Prymnesiophyte *Chrysochromulina* have been found to be present in the Arctic Seas (Hansen and Hjorth 2002). Sanders and Gast (2012) recently identified mixotrophic behavior in picoplanktonic phytoflagellates, tentatively identified as *Micromonas pusilla*. Eukaryotic picoplankton can at times comprise the majority of the phytoplankton biomass in this region, so impacts of mixotrophic behavior by these populations could be substantial (Zubkov and Tarran 2008, Lovejoy and Potvin 2011).

In the Antarctic environment, mixotrophs have been found to be present in both marine and lake habitats. In Antarctic marine waters, mixotrophs can be found in significant abundances (up to 42% of bacterivorous nanoflagellates) and can have a substantial grazing impact on bacterial standing stocks (Moorthi et al. 2009, Gast et al. 2014). In lake environments, bacterivory has been observed in the prasinophyte
Pyramidonas gelidicola, as well as in studies of unidentified cryptophytes in Antarctic saline lakes, with up to 16% of bacterial biomass removed per day (Roberts and Laybourn-Parry 1999, Bell and Laybourn-Parry 2003, Hammer and Pitchford 2006, Unrein et al. 2014). These previous studies show evidence that mixotrophic organisms are found in multiple habitats in these regions. This information will also provide insight with regard to the selection of candidate organisms for experiments presented in this work.

1.7 Bacterivory in Photosynthetic Pico- and Nanoflagellates From Polar Regions

The goal of this work is to identify the presence of mixotrophic behavior (combined strategy of autotrophy and phagotrophy) in algae isolated from both the Arctic and Antarctic environments, and to elucidate some of the factors that inhibit or promote mixotrophic activity. While there is direct evidence of the presence of mixotrophic organisms in Arctic and Antarctic microbial communities, the characteristics of phagotrophic behavior in photosynthetic flagellates (i.e., grazing rates or size-selectivity) have not been adequately quantified in a species-specific manner. In addition, how these ecological strategies are conserved within and between evolutionary lineages requires further study. In this dissertation I will present contributions to the understanding of the functioning of both the autotrophic and phagotrophic mechanisms in response to environmental factors relevant to the polar environment.

Specifically, in the first chapter, I present an investigation and description of mixotrophic behavior in the photosynthetic picoflagellate prasinophyte, Micromonas pusilla. In the second study, I explore the utilization of mixotrophy in the Antarctic
environment, identifying phagotrophy in photosynthetic nanoflagellates representing multiple evolutionary lineages: Cryptophyceae (*Geminigera cryophila*) and Prasinophyceae (*Pyramimonas tychotreta* and *Mantoniella antarctica*). A purely heterotrophic chrysophyte (*Paraphysomoas antarctica*) was also examined under identical conditions, for comparison. The photoautotrophic abilities of these mixotrophs and a purely autotrophic diatom (*Chaetoceros* sp.) were also measured by compiling photosynthesis versus irradiance response (P vs. I) curves, which display photosynthetic rates across a range of light intensities. Lastly, in Chapter three, in an effort to understand how trophic “generalists” like mixotrophs, fare in competitive interactions with monotrophic specialists, I examined the competitive dynamics of a mixotrophic flagellate (*P. tychotreta*), paired with a solely heterotrophic flagellate (*P. antarctica*). Cell populations of each competitor were monitored along with resident bacterial populations.

This dissertation presents novel identification as well as rigorous investigation of mixotrophic behaviors in phototrophic flagellates from both polar (Arctic and Antarctic), environments representing two evolutionary lineages. The results of this work indicate a diverse utilization of combined trophic strategies and provide vital descriptive information regarding the physiology of polar microbial eukaryotes.
CHAPTER 2

PHAGOTROPHY BY THE PICOEUUKARYOTIC GREEN ALGA

MICROMONAS: IMPLICATIONS FOR ARCTIC OCEANS

2.1 Abstract

Photosynthetic picoeukaryotes (PPE) are recognized as major primary producers and contributors to phytoplankton biomass in oceanic and coastal environments. Molecular surveys indicate a large phylogenetic diversity in the picoeukaryotes, with members of the Prymnesiophyceae and Chrysophyceae tending to be more common in open ocean waters and Prasinophyceae dominating coastal and Arctic waters. In addition to their role as primary producers, PPE have been identified in several studies as mixotrophic and major predators of prokaryotes. Mixotrophy, the combination of photosynthesis and phagotrophy in a single organism, is well established for most photosynthetic lineages. However, green algae, including prasinophytes, were widely considered as a purely photosynthetic group. The prasinophyte Micromonas is perhaps the most common picoeukaryote in coastal and Arctic waters and is one of the relatively few cultured representatives of the picoeukaryotes available for physiological investigations. In this study, we demonstrate phagotrophy by a strain of Micromonas (CCMP2099) isolated from Arctic waters and show that environmental factors (light and nutrient concentration) affect ingestion rates in this mixotroph. In addition, we show size-selective feeding with a preference for smaller particles, and determine P vs. I (photosynthesis vs. irradiance) responses in different nutrient conditions. If other strains have mixotrophic abilities similar to Micromonas CCMP 2099, the widespread
distribution and frequently high abundances of *Micromonas* suggest that these green algae may have significant impact on prokaryote populations in several oceanic regimes.

### 2.2 Introduction

Functional groups of marine plankton have long been grouped according to size, with the smallest fraction (0.2 to 2 µm) designated as picoplankton (Sieburth et al. 1978). Early studies of photosynthetic picoplankton recognized them as an important and widely distributed group across marine habitats, but assumed they were composed exclusively of bacterioplankton (e.g., Platt et al. 1983). Further research revealed that there are several clades of eukaryotic algae within this size-fraction (reviewed by Vaulot et al. 2008), especially within an extended size boundary ≤ 3 µm, which is considered a more natural delineation for picoeukaryotes (Massana 2011). Photosynthetic picoeukaryotes (PPE) contribute greatly to both biomass and primary production in various marine systems; in some regions with reduced cyanobacterial populations, small-sized eukaryotic phytoplankton can comprise up to 90% of this production (Worden et al. 2004, Jardillier et al. 2010, Grob et al. 2011). Evidence is also accumulating that mixotrophic PPE are major consumers of prokaryotes, with potential to dominate not only primary production, but also bacterivory, in ecosystems as diverse as subtropical gyres and Arctic seas (Zubkov and Tarran 2008, Hartmann et al. 2012, Sanders and Gast 2012).

Culture-independent molecular surveys, often coupled with flow cytometry, demonstrate high genetic diversity for PPE across a range of pelagic and coastal marine environments (Diez et al. 2004, Not et al. 2007, Lepère et al. 2009, Cuvelier et al. 2010, Kirkham et al. 2013). These phylogenetic analyses indicate that distinct clades of PPE
can be associated with different water masses. For example, prymnesiophyceae and chrysophyceae had complementary abundance patterns associated with N:P ratios in all major ocean biomes (Kirkham et al. 2013). Furthermore, Foulan et al. (2008) found that three clades of *Micromonas pusilla* had different relative contributions to total abundance along environmental gradients in tropical, temperate and polar environments suggesting niche partitioning. Despite this growing understanding of the diversity and the key roles that PPE play as primary producers and grazers of prokaryotes in marine waters, many of the major clades remain uncultured (Vaulot et al. 2008, Massana 2011). Consequently, there is relatively little information on their morphology and physiology, which could aid in understanding environmental conditions that affect their growth and relative abundances (DuRand et al. 2002, Foulon et al. 2008, Hartmann et al. 2013).

One PPE that has several strains in culture is *M. pusilla*, a small (< 2 µm) motile green alga with a single flagellum, mitochondria and chloroplast (Manton and Parke 1960). It has a global distribution and can be abundant in marine biomes from the Arctic to the ice-edge of Antarctica (Díez et al. 2004, Not et al. 2004, Lovejoy et al. 2007, Foulon et al. 2008), and has the potential to be the dominant contributor to PPE communities in coastal and nutrient-rich environments (Not et al. 2004, Lovejoy et al. 2007, Not et al. 2007, Li et al. 2009). Molecular data indicate that *M. pusilla*, long considered a single morphospecies, actually represents several lineages and analyses of complete genomes of two isolates support their classification as separate species (Šlapeta et al. 2006, Worden et al. 2009).

The aim of this study was to confirm mixotrophy in a strain of *M. pusilla* (hereafter identified as *Micromonas* CCMP2099) isolated from Arctic waters, which was
suggested to be a major bacterivore by Sanders and Gast (2012). A temperate strain identified as *M. pusilla* was previously shown to be bacterivorous (González et al. 1993), but there still is little eco-physiological information available for any of the clades (Foulon et al. 2008, Hartmann et al. 2013). To this end, we compared the effect of light and nutrients on rates of bacterivory for *Micromonas* CCMP2099, tested its ability to discriminate between prey sizes, and examined autotrophic functioning (P vs. I) in different light and nutrient conditions. To our knowledge, this study provides the first species-specific examination of factors potentially affecting mixotrophy in algae of this size class.

### 2.3 Materials and Methods

#### 2.3.1 Culture origin, Maintenance and Sizing

Cultures of *Micromonas* (CCMP2099), originally isolated from an Arctic polyna (76.283 °N, 74.75 °W) between Ellesmere Island and Greenland (Lovejoy et al. 2007) and designated as *M. pusilla* by CCMP, were maintained at 4°C in f/2 + Si media made with artificial seawater (ASW) at 32 PSU (Guillard 1975, Caron 1993). The cultures were non-axenic and grown under continuous irradiance from 50W cool-white fluorescent bulbs at ~50 µmol m⁻² s⁻¹.

Average cell size of *Micromonas* CCMP2099 in late exponential phase was determined by measurement of 50 Lugol’s iodine fixed cells using a calibrated ocular micrometer at 1000x magnification on a Zeiss Axiovert 10 inverted microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).
2.3.2 Microscopic Imaging of Micromonas

For confocal images, fixed primulin-stained samples of Micromonas were centrifuged at 16,000 G for 1 hr to separate uningested spheres from algal cells. Centrifuged Micromonas were then pipetted onto slides and air-dried overnight. Images were obtained using a Leica TCS-NT confocal microscope. Illumination was provided by a Krypton/Argon Laser with excitation wavelengths of 488nm and 633nm. Emission wavelengths were 503-550nm for the microspheres and 628-724nm to view chloroplast autofluorescence and primulin staining. Additionally, a DIC image was captured to combine with the confocal.

For epifluorescent micrographs, samples of Micromonas CCMP2099 inoculated with microspheres were fixed as previously described, stained with primulin (Sigma-Aldrich, St. Louis, MO, USA), concentrated on 0.8µm pore black Poretics PC filters and excess primulin cleared with 0.1M Tris (pH 4). Filters were mounted on slides with Vectashield with DAPI, and frozen until images were taken. Images of Micromonas CCMP2099 were captured with a Zeiss Axiovert 10 microscope equipped for epifluorescence and a Coolpix995 digital camera (Nikon, Tokyo, Japan). Images were post-processed to reduce overexposure of fluorescing particles in ImageJ (http://rsbweb.nih.gov/ij/). Images of a single field of view were taken with both filters and each image was separated into red, blue, and green channels, and adjusted for
Figure 2.1. (A-C) Confocal images of 3 *Micromonas* CCMP2099 cells, one with an ingested microsphere. (D) DIC image of the same *Micromonas* cells. Scale bar in (D) applies to panels A-D. (E) Epifluorescence micrograph of fluorescent microspheres only (dia=0.5µm), and (F) the same image showing microspheres and *Micromonas* cells, including an ingestion event. *Micromonas* cells stained with 4′,6-diamidino-2-phenylindole (DAPI) and primulin. Scale bar in panel E also applies to panel F. Images in panels A-D are from a separate feeding experiment than for panels E and F.
brightness and contrast. Channels with the strongest signal for tracer particles and algal cells were selected and used to create a merged image of a specific cell.

2.3.3 General Grazing Experiment (2x2 Factorial)

Replicate flasks with high- and low-nutrient concentrations were divided into dark or light (~50 µmol m⁻² s⁻¹) treatments for a 2x2 factorial design comparing light and nutrients on grazing activity (n=4 flasks per treatment). High-nutrient treatments were full strength f/2+Si media in artificial seawater (i.e., the maintenance media). The concentration of N and P in f/2 are 145 and 8 µM, respectively. Low-nutrient treatments were grown in f/2 + Si diluted 10-fold with ASW. These nutrient concentrations were generally greater than those reported for much of the Arctic summer, but phosphate concentrations in this range have been observed (Wheeler et al. 1997). Light was supplied in the same manner and level as for culture maintenance. Culture flasks for dark treatments were wrapped in aluminum foil and placed in a cardboard container lined with aluminum foil to exclude all light. Both light and dark treatments remained in the same incubator at 3°C. After 1 week of acclimation to experimental conditions, fluorescently labeled particles (0.55 µm diameter, Fluoresebrite, Polysciences, Inc., Warrington, PA, USA) were added to culture flasks at tracer levels (5-10% of bacterial abundance). Bacterial and total microsphere abundances were determined from samples collected on 2.4 mm, 0.2 µm pore-size Poretics polycarbonate filters (GE Infrastructure Water & Process Technologies, Trevose, PA, USA), mounted on glass slides using Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and enumerated at 1000x magnification using epifluorescence on a Zeiss Axiovert microscope. To determine ingestion, samples were taken from each flask immediately after addition of microspheres.
(T_0) and at 10 minute intervals thereafter for ~1 hour. Samples were fixed with Lugol’s iodine, cleared with Na_2S_2O_3, with final fixation in formalin (Sherr and Sherr 1993). Subsamples from each replicate and time point were collected onto 0.8µm polycarbonate filters, mounted using Vectashield with DAPI and examined as described above for bacteria. At least 100 Micromonas cells from each replicate slide through the time course were examined for ingested microspheres. For each replicate, an average number of ingested microspheres, based on all cells examined, was determined. Random coincidental overlap of microspheres and cells was accounted for by subtracting T_0 counts from other time points. Ingestion rates of microspheres were determined from the slope of a linear regression of average ingestion versus time using all replicates. Ingestion rates of bacteria were calculated assuming that microspheres and bacteria were ingested relative to their ratios at the beginning of the time course (Sherr and Sherr 1993).

2.3.4 Size Selection Experiment

Size selectivity by Micromonas CCMP2099 was examined with three treatments of added Fluoresebrite fluorescent microspheres: 1) small, 0.46 µm diameter; 2) large, 0.9 µm diameter; and 3) a combination of the two particle sizes. Microsphere sizes were chosen to give the largest size range that we believed to be ingestible by Micromonas based on previous field experiments (Sanders and Gast 2012). Additionally, the microspheres fluoresced at different wavelengths making them even easier to distinguish from each other in the combined treatment. Microspheres were presented at the same total particle concentration (3.5x10^6 ml^-1) for all treatments. These size selection experiments were carried out under the previously described low-nutrient conditions with
light. Ingestion rates were measured over a 1-hour time-course, sampling at 10-minute intervals from replicate flasks (n=5) using protocols for grazing experiments above.

2.3.5 Photosynthesis vs. Irradiance Responses

Carbon fixation by *Micromonas* CCMP2099 grown in high-nutrient (f/2 + Si) or low-nutrient conditions (5-fold dilution of f/2 + Si) were measured under a range of irradiances using the method of Macintyre et al. (1996), modified by the addition of centrifugation techniques used by Smith and Azam (1992). Photosynthesis vs. Irradiance (P vs. I) responses were established by incubating 1-ml of *Micromonas* CCMP2099 adapted to either high or low nutrients at different light levels (4 replicate incubations per light level/nutrient treatment) with sodium $^{14}$C bicarbonate (final specific activity per sample 0.5 µCi, 18.5 kBq). Samples were incubated at PAR light levels ranging from ~3 to 150 µmol m$^{-2}$ s$^{-1}$ for 2 hours. Following incubations, samples were centrifuged at 16,000 G for 30 minutes, followed by aspiration of the supernatant. The algal pellet was then resuspended in 32 PSU ASW solution and fumed with 6N HCL overnight to remove residual unassimilated $^{14}$C. The samples were neutralized with 6M NaOH and scintillation fluid added. Radioactivity of samples was measured in a scintillation counter (Beckman LS-3801, Irvine, CA, USA), and the average counts per minute converted to disintegrations per minute (DPM) using a quench correction curve. Rates of carbon fixation were determined by the equation:

$$C_{\text{fixed}} = \frac{\text{DIC}_{\text{sample}} \ast \text{DPM}_{\text{sample}}}{\text{DPM}_{\text{total}}} \ast \text{incubation time (h)}$$
where:

\[ C_{\text{fixed}} = \text{the amount of total carbon fixed by photosynthesis}, \]
\[ \text{DIC}_{\text{sample}} = \text{total dissolved organic carbon available for fixation in sample}, \]
\[ \text{DPM}_{14}\text{C}_{\text{sample}} = \text{disintegrations per minute for the algal pellet} \]
\[ \text{DPM}_{14}\text{C}_{\text{total}} = \text{disintegrations per minute of total }^{14}\text{C available for incorporation}. \]

DIC was calculated using the program CO2SYS in Excel available at http://cdiac.ornl.gov/ftp/co2sys/. Average \(^{14}\text{C}\) uptake of dark controls was subtracted from the light incubations to correct for incorporation not due to photosynthesis. Net primary production was normalized to a per cell basis from enumeration of Micromonas CCMP2099 using light microscopy fixed with Lugol’s iodine (3% final concentration).

### 2.3.6 Data and Statistical Analysis

General and size-selection grazing experiments were analyzed first using a repeated-measures regression to identify whether there was significant variance between the replicate flasks. Following this, ingestion per cell was regressed over time to obtain a grazing coefficient and related standard error parameters. Rates were compared using an ANCOVA of ingestions over time by treatment with pair-wise comparisons assessed using Tukey’s HSD test. The R statistical software program was used to fit P vs. I curves to the Eilers-Peeters light limitation curve, which defines photosynthesis as the product of the maximal photosynthetic rate and the light limitation at a given light irradiance, \(I\) (Eilers and Peeters 1988). Specifically,
Light Limitation = \frac{2 \cdot (1 + \beta) \cdot I/I_{\text{opt}}}{(I/I_{\text{opt}})^2 + 2 \cdot \beta \cdot I/I_{\text{opt}} + 1}

and

Photosynthesis = P_{\text{max}} \cdot \text{Light Limitation}

where,

$I_{\text{opt}}$ = light irradiance at optimal photosynthetic rate

$\beta$ = a dimensionless parameter which defines the degree of photoinhibition.

$P_{\text{max}}$ = maximum photosynthetic rate

Non-linear regression analysis of P vs. I data was done using nls function in the R statistical software program. Pairwise comparisons of grazing rates were performed with the multcomp package in R.

2.4 Results

*Micromonas* CCMP2099 ingested particles under all experimental conditions and the average number of ingestions increased at each time point over the course of the incubations (Fig. 2.1, Fig. 2.2). Bacterial ingestion rates based on the time courses ranged from 0.44 to 1.32 bacteria cell\(^{-1}\) h\(^{-1}\), and were unlikely to be influenced by prey density since bacterial abundances did not differ significantly in any of experimental light and nutrient conditions. The highest grazing rates were observed under low-nutrient/illuminated conditions and were 3 times greater than in other treatments. Ingestion rates for the remaining three treatments (low nutrient in darkness and high nutrient in light or dark) were not significantly different from each other. Light
Figure 2.2. Bacterivory rates of *Micromonas* CCMP2099 in 2x2 factorial design experiment including high (f/2 media, Hnutr) and low nutrients (1:9 dilution of f/2 media with ASW, Lnutr) in either dark or light (50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) conditions. Means and error bars (+/- SE, n = 4) are a result of the deviation of the data from the slope of the line predicted by the regression of ingestions over time. Letters indicate Tukey’s HSD comparisons; there are no significant differences between treatments with the same letter above the bars.

Figure 2.3. Ingestion rates of large (dia = 0.9 \( \mu \text{m} \)) and small (dia = 0.46 \( \mu \text{m} \)) microspheres when combined (Both/) or offered alone in separate treatments. Total microsphere abundance was identical in all treatments (n = 5, ns = not significant, error bars +/- SE). Letters indicate Tukey’s HSD comparisons, as in Fig. 2.2.
incubations did not increase ingestion rates as a single factor; higher grazing rates were
induced only when nutrient levels were reduced and light was present (Fig. 2.2).

*Micromonas* CCMP2099 exhibited an ability to discriminate between different
particle sizes, with ingestion rates of microspheres ranging from 2.8 to 5.4 spheres cell
\(^{-1}\) hr\(^{-1}\). Small (0.46 µm) and large (0.9 µm) particles were both ingested when either was
exclusively available, but small-sized particles were ingested at almost twice the rate
(91% higher) of large particles (Fig. 2.3). When both sizes of particles were offered
together, *Micromonas* CCMP2099 grazing of the large-sized particles was not
significantly different from background. Small particles were grazed at equivalent rates
in the treatment with a mixture of microsphere sizes and in the treatment with only small
particles (Fig. 2.3).

P vs. I determinations at the two nutrient levels showed similar distribution of
photosynthesis with increasing light (Fig. 2.4, Table 2.1). The maximum photosynthetic
output was produced at \(\sim 30 \, \mu\text{mol} \, \text{m}^{-2} \, \text{s}^{-1} \, (I_{\text{opt}}, \text{optimal irradiance})\) for both nutrient
treatments (Table 1) and photoinhibition occurred at higher irradiances in both treatments
(Fig. 2.4). However, the maximum photosynthetic rate was reduced by \(\sim 59\%\) in the lower
nutrient treatment; rates of photosynthetic C fixation ranged from 4.5 to 91 \(\mu\text{g} \, \text{C} \, \text{cell}^{-1} \, \text{hr}^{-1}\) in low-nutrient treatments, and from 4.4 to 169 \(\mu\text{g} \, \text{C} \, \text{cell}^{-1} \, \text{hr}^{-1}\) in high-nutrient
treatments (Fig. 2.4, Table 2.1). Thus, lower nutrient concentrations resulted in both an
increase in grazing activity (Fig. 2.2) and a decrease in photosynthetic output (Fig. 2.4).
Table 2.1. Parameters from high and low nutrient treatments of *Micromonas* CCMP2099 photosynthesis versus irradiance responses fit to the Eilers-Peeters curve by non-linear regression. *** = p<0.0001, ns = not significant.

<table>
<thead>
<tr>
<th></th>
<th>High Nutrient</th>
<th>Low nutrient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimate</td>
<td>Std. Error</td>
</tr>
<tr>
<td>$P_{max}$</td>
<td>157.89</td>
<td>8.46</td>
</tr>
<tr>
<td>$b$</td>
<td>-0.377</td>
<td>0.099</td>
</tr>
<tr>
<td>$I_{opt}$</td>
<td>31.74</td>
<td>1.64</td>
</tr>
<tr>
<td>$Df$</td>
<td>29</td>
<td></td>
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Figure 2.4. Photosynthesis vs. Irradiance curves for *Micromonas* CCMP2099 grown in high (black circles, solid line, full strength) or low (grey circles, dotted line, 5X dilution with ASW) f/2 media.
2.5 Discussion

Though microbial eukaryotes occupy complex and versatile ecological niches, most were thought to engage solely in photoautotrophic or heterotrophic nutritional strategies to fulfill their energy and nutrient requirements. Mixotrophy, the combination of phototrophic production and phagotrophic ingestion of prey has received increasing amounts of attention by ecologists as its prevalence in aquatic systems has become apparent. The majority of mixotrophy studies focused on nanoplanktonic (2-20 µm) flagellates and ciliates, which are known to sometimes have greater grazing impact than heterotrophic flagellates - the presumed dominant bacterivores of planktonic systems - in marine, freshwater and even sea ice ecosystems (Sanders 1991, Nygaard and Tobiesen 1993, Moorthi et al. 2009, Flynn et al. 2013). Recent studies, especially in oligotrophic waters, have demonstrated the importance of PPE with phagotrophic capabilities. PPE comprised high proportions of the bacterivores and were responsible for up to 95% of the bacterivory in some marine systems (Unrein et al. 2007, Zubkov and Tarran 2008, Sanders and Gast 2012). These experimental results and observations of the numerical dominance of PPE have led to the suggestion that mixotrophy sustains the functioning of oligotrophic ocean ecosystems (Hartmann et al. 2012). In oligotrophic systems, it seems likely that the mixotrophic PPE are using phagocytosis for increased access to limiting nutrients. However, there are a variety of advantages that a mixotrophic strategy offers to nanoplankton, and by extension, to PPE (Sanders 1991, Jones 1994, Unrein et al. 2014).
2.5.1 Bacterivory in Micromonas

Evidence of mixotrophic activity in green algal lineages has rarely been reported. However, Maruyama et al. (2013), showed ultrastructural evidence of bacterivory in a nanoplanktonic prasinophyte, *Cymbomonas*, and González et al. (1993) found that a temperate strain of *M. pusilla* was capable of ingesting fluorescently labeled bacteria. The current experiments with the pan-Arctic strain of *Micromonas* CCMP2099 confirms mixotrophy reported for a *Micromonas*-like alga from field experiments in the Beaufort Sea (Sanders and Gast 2012). Our calculations from the data presented in González et al. (1993) indicate an ingestion rate of 2.4 bacteria alga\(^{-1}\) h\(^{-1}\) for that *M. pusilla* strain at “room temperature,” which is in the same range as the Arctic strain investigated here.

These data together suggest that despite the divergence of *Micromonas* CCMP2099 from numerous other *M. pusilla* lineages (Šlapeta et al. 2006), other clades also may be phagotrophic. However, in experiments in the NW Mediterranean Sea, where *M. pusilla* dominated the PPE, the species was not observed to ingest fluorescently labeled bacteria (Unrein et al. 2014). These contrasting results could be clade-specific or environmentally determined, and highlight the potential of distinct mixotrophic strategies between species – even in the same genus (Worden and Not 2008, Unrein et al. 2014).

Nutrient limitation increased phagotrophic behavior in some mixotrophic nanoflagellates (Nygaard and Tobiesen 1993, Arenovski et al. 1995), and reduced nutrient concentration also altered feeding rates in *Micromonas* CCMP2099 (Fig. 2.2). The increased grazing rates of *Micromonas* in the lower nutrient conditions imply that mixotrophy functioned to obtain limiting nutrients required for physiological functions. Furthermore, increased rates of bacterivory were induced only in light treatments,
suggesting that ingestion supplemented N and/or P supply in order to allow for balanced
growth when photosynthesis was greatest. However, since lower nutrient conditions were
achieved by dilution of complete f/2 media, other components of the media such as
vitamins and trace metals were also lower and could have factored in the increased
feeding rate we observed. The increase in feeding in the light versus dark (Fig. 2.2) also
suggests that phagotrophy and photosynthesis are not substitutable as carbon sources for
Micromonas CCMP2099. The result of reduced grazing activity in the absence of light
was unexpected, although reduced feeding rates by mixotrophs in the dark were reported
for several freshwater species. Pålsson and Granéli (2003) found reduced bacterivory
rates during night for several cryptomonads, and light-dependent phagotrophy in a
mixotrophic chrysophyte was also observed in culture (Caron et al. 1993). We suggest
that the trophic strategy of phagotrophy in Micromonas CCMP2099 is to obtain some
limiting dissolved nutrients rather than a mechanism for survival of the boreal winter.

Size selective grazing by heterotrophic protists is well established (e.g., Verity
1991, Hahn and Höfle 2001). Less is known for mixotrophic nanoflagellates, though the
chrysophyte, Ochromonas, selected for larger bacteria in several studies (Andersson et al.
1986, Chrzanowski and Simek 1990, Pfandl et al. 2004). In contrast, Micromonas
CCMP2099 selected for small-sized prey, and ingested at lower rates than Ochromonas,
most likely due to its own small size. The predator/prey size ratios for Micromonas
CCMP2099 were only 2.4 for 0.5 μm microspheres, 2.6 for 0.46 μm microspheres, and
1.3 for 0.9 μm microspheres, based on the average diameter of 1.27±0.07 μm for
Micromonas CCMP2099. These results agree with the observation of Sanders and Gast
(2012) that the Micromonas-like PPE in the Beaufort Sea ingested 0.5 μm microspheres,
but never ingested larger (1.0 – 1.2 µm) fluorescently labeled bacteria. Size-selective feeding has several implications for the microbial food web; the morphology and species structure of prey bacterial populations can be altered (Andersson et al. 1986, Jürgens et al. 1999), and the potential contribution of PPE such as Micromonas to the total grazing impact on prokaryotes may be limited to the smallest prey in a mixed-size bacterial assemblage. It also implies that cyanobacteria, typically on the large end of the size spectrum of bacteria, are less likely to be ingested by Micromonas.

2.5.2 Photosynthetic Responses in Micromonas

Photosynthetic parameters observed for Micromonas CCMP2099 included a low optimal irradiance (I_{opt}) for maximal photosynthesis (~30 µmol m^{-2} s^{-1}, Table 1). This was expected based on field observations of M. pusilla in the Arctic where blooms were typically located under ice at < 20% of incident surface irradiance (Booth and Horner 1997, Sherr et al. 2003). Lovejoy, et al. (2007) found that the clone used in our experiments (CCMP2099) had pan-Arctic distribution and grew best at or below 10 µM photons m^{-2} s^{-1} (~10 µmol m^{-2} s^{-1}) in field experiments. Other autotrophic picoplankton also appear to be adapted to photosynthesize at lower light irradiances typical of deep chlorophyll maximum layers; small-sized phytoplankton also can have a greater potential for photodamage compared to their larger counterparts (Raven 1998). Carbon fixation by Micromonas CCMP2099 was reduced under low-nutrient compared to high-nutrient levels (Fig. 2.4), which may be due to decreased energy conversion efficiencies caused by nutrient limitation (Kolber et al. 1990). Conversely, grazing activity increased under reduced nutrient conditions in the light for Micromonas CCMP2099 (Fig. 2.2).
bacterivory in low nutrients has been noted for some other mixotrophs (Nygaard and Tobiesen 1993, Arenovski et al. 1995), with the assumption that ingested bacteria supplied limiting nutrients, but photosynthetic production was not determined in those experiments. It is possible that the reduced photosynthesis and increased feeding rate *Micromonas* CCMP2099 could be an effect of the mode of nutrient supply (dissolved versus particulate) affecting the efficiency of photosynthesis; alternatively some other tradeoff may exists for combining phototrophy and heterotrophy in mixotrophic organisms (Raven 1997).

### 2.6 Conclusion

Mixotrophy by photosynthetic picoeukaryotes (PPE) appears to be a widespread phenomenon, though most studies found bacterivory by Prymnesiophyceae, Chrysophyceae and Pelagophyceae – all of which have known mixotrophic species in larger size classes. Mixotrophic PPE are particularly important in oligotrophic oceans where they potentially sustain ecological functioning of the ecosystem (Hartmann et al. 2012). This study is the first to directly examine species-specific factors that affect feeding by a phototrophic picoeukaryote, in particular, of a prasinophyte member of the Chlorophyta. The chlorophytes are generally considered predominantly phototrophic, although the ability to ingest particulate food was previously shown for another strain of *Micromonas* and for *Cymbomonas* (González et al. 1993, Maruyama and Kim 2013). *Micromonas* may be the most widely dispersed genus of PPE and can dominate the picoplankton biomass in many coastal and more nutrient-rich environments, including the Arctic where they can be the dominant bacterivore (Not et al. 2005, Lovejoy et al. 2007,
Sanders and Gast 2012). In the Arctic, global climate change may be leading to a picoplankton-dominated system, in which *Micromonas* could play an increasingly important role (Li et al. 2009). Thus, understanding factors that affect phagotrophy and photosynthesis in this widespread group is increasing valuable (Foulon et al. 2008).
3.1 Abstract

Antarctic phototrophs are challenged by extreme temperatures, ice cover, nutrient limitation and prolonged periods of darkness. Yet this environment may also provide niche opportunities for phytoplankton utilizing alternative nutritional modes. Mixotrophy, the combination of photosynthesis and particle ingestion, has been proposed as a mechanism for some phytoplankton to contend with the adverse conditions of the Antarctic. We conducted feeding experiments using fluorescent bacteria-sized tracers to compare the effects of light and nutrients on bacterivory rates in three Antarctic marine photosynthetic nanoflagellates representing two evolutionary lineages: Cryptophyceae (Geminigera cryophila), and Prasinophyceae (Pyramimonas tychotreta and Mantoniella antarctica). Only G. cryophila had previously been identified as mixotrophic. We also measured photoautotrophic abilities over a range of light intensities (P vs. I) and used dark survival experiments to assess cell population dynamics in the absence of light. Feeding behavior in these three nanoflagellates was affected by either light, nutrient levels, or a combination of both factors in a species-specific manner that was not conserved by evolutionary lineage. The different responses to environmental factors by these mixotrophs supported the idea of tradeoffs in the use of phagotrophy and phototrophy for growth.
3.2 Introduction

Traditional conceptual models restrict the functionality of microbial eukaryotes in aquatic environments to either heterotrophy or autotrophy. However, mixotrophy, in this instance the combination of photosynthesis and ingestion of particles in individual protists, has become widely recognized as a common nutritional strategy and is beginning to be incorporated into ecosystem models (Sanders 1991, Unrein et al. 2007, Hartmann et al. 2013, Mitra et al. 2014). Mixotrophy has been identified in a diverse array of microbial eukaryotic taxa, is prevalent in aquatic microbial food webs, and can have significant effects on these systems (Zubkov and Tarran 2008, Moorthi et al. 2009, Sanders 2011, Sanders and Gast 2012, Unrein et al. 2014). Although mixotrophic eukaryotes are found in a variety of freshwater and marine habitats, including polar environments, the conditions that control their presence, or the extent of mixotrophic activity, are still poorly understood (Boraas et al. 1988, Sanders et al. 1990, Czyponka et al. 2011, Sanders 2011, Sanders and Gast 2012, Unrein et al. 2014).

The extreme seasonality of the Southern Ocean environment, with prolonged periods of darkness during the austral winter, does impose constraints on the available niches that resident organisms can occupy (Laybourn-Parry 2009). As inhospitable and barren as the Southern Ocean appears at the surface, aquatic polar communities are among the most productive on the planet. And though microbial communities in Antarctic waters have been proposed as distinct from those at temperate latitudes (Azam et al. 1991), the proportion of phytoflagellates that are mixotrophic and their grazing impact on bacterial populations are similar to those reported for lower latitudes, at least

The Southern Ocean is a High Nutrient Low Chlorophyll (HNLC) region, where concentrations of soluble macronutrients such as nitrogen are high enough to sustain phytoplanktonic blooms, but densities of algal populations are not as great as expected (Fitzwater et al. 2000). While macronutrient limitation may not generally impose constraints on the growth of photosynthetic flagellates in this region, large phytoplankton blooms can exhaust local soluble nutrient concentrations (Arrigo et al. 1999, Arrigo et al. 2000), thus providing a situation that could promote mixotrophic behavior as a strategy for obtaining limiting nutrients. Kang et al. (2001) noted this draw-down of nutrients in well stratified surface waters, where low nitrate and silicate had an inverse relationship to Chl a concentrations. Additionally, micronutrient deficiencies, particularly iron, have been hypothesized as factors controlling phytoplankton growth in the Southern Ocean and could be a major resource targeted by the phagotrophic activity of mixotrophic flagellates (Maranger et al. 1998, Boyd et al. 2000). Mixotrophy has even been proposed as a strategy that phytoflagellates could use to survive the austral winter to enter spring as actively growing populations (Laybourn-Parry 2002, Jones et al. 2009).

To address factors potentially modifying feeding behavior and photosynthesis in Antarctic mixotrophs, cultured marine nanoflagellates from different evolutionary lineages, Cryptophyceae (*Geminigera cryophila*) and Prasinophyceae (*Pyramimonas tychotreta* and *Mantoniella antarctica*) were examined in 2x2 factorial experiments comparing the effects of light and nutrient concentrations on feeding. For comparison, feeding by a purely heterotrophic flagellate (*Paraphysomonas antarctica*) was examined
under identical conditions. We also determined the photoautotrophic abilities of these mixotrophs and a purely autotrophic diatom (*Chaetoceros* sp.) and conducted dark survival experiments for the three mixotrophic flagellates.

### 3.3 Materials and Methods

#### 3.3.1 Culture Origin and Maintenance

Cultures used in this study were from the Antarctic Protist Collection, which is maintained at Woods Hole Oceanographic Institution as well as at Temple University ([http://www.whoi.edu/science/B/protists/](http://www.whoi.edu/science/B/protists/)), and from the National Center for Marine Algae and Microbiota ([http://ncma.bigelow.org/](http://ncma.bigelow.org/)). Experimental cultures of the prasinophytes *Pyramimonas tychotreta* (I-9 Pyram), *Mantoniella antarctica* (SL-175), and the cryptophyte *Geminigera cryophila* (CCMP2564) were maintained at 4°C in f/2 + Si media made with artificial seawater (ASW) at 32 PSU (Guillard 1975, Caron 1993). We also included Antarctic isolates of the heterotrophic chrysophyte, *Paraphysomonas antarctica* (RS-4-2), and the diatom *Chaetoceros* sp. (RS-19) for comparison, both of which were cultured under the same conditions as the mixotrophic flagellates. The cultures were uni-protistan and non-axenic, and were grown under continuous irradiance from 50W cool-white fluorescent bulbs at ~50 µmol m⁻² s⁻¹.

#### 3.3.2 Grazing Experiments (2x2 Factorial)

Replicate flasks of the three mixotrophic flagellates *P. tychotreta*, *M. antarctica*, *G. cryophila*, and an Antarctic strain of the heterotrophic chrysophyte *Paraphysomonas antarctica* were grown in either high- or low- nutrient concentrations and divided into
dark or light (~50 µmol m⁻² s⁻¹) treatments for a 2X2 factorial design comparing light and nutrient effects on grazing activity (40ml total volume in 50ml culture flasks, n=6 per treatment/species). High-nutrient treatments were full strength f/2+Si in artificial seawater (i.e., the maintenance media) while low-nutrient treatments were a 5-fold dilution of f/2 + Si culture media with ASW (artificial sea water). Light was supplied in the same manner and level as for culture maintenance. Culture flasks (50ml) for dark treatments were wrapped in aluminum foil and placed in a cardboard container lined with aluminum foil to exclude all light and placed in the same incubator with light treatments at 4°C. After 1 week of incubation at experimental conditions, fluorescently labeled microspheres (0.55 µm diameter, Fluoresbrite, Polysciences) were added to culture flasks at tracer levels (5-10% of bacterial abundance). Microsphere size was selected to represent the cell size of the mostly coccoid-shaped resident bacterial populations. The flasks were sampled at 5 minute intervals for ~30 min. Samples were fixed with Lugol’s iodine, cleared with Na₂S₂O₃, with final fixation in formalin (Sherr and Sherr 1993, Sanders and Gast 2012). Subsamples from each replicate were collected onto 0.8µm polycarbonate (PC) filters, mounted using Vectashield with DAPI and examined by epifluorescence microscopy to determine abundances and ingestion rates. At least 100 cells from each replicate through the time course were examined for ingested microspheres (Fig. 3.1). Bacterial and total microsphere abundances in all treatment replicates were determined by epifluorescence microscopy using the fixation protocols reported previously, except subsamples were collected on 0.2 µm PC filters. Ingestion of bacteria was determined assuming that microspheres and bacteria were ingested relative to the ratio of the microspheres and bacteria (Sherr and Sherr 1993).
Figure 3.1. Epifluorescence micrograph of A-B) *G. cryophila*, C-D) *M. antarctica*, E-F) *P. tychoatra* showing ingestion of fluorescently label microspheres (dia = 0.5mm). Panels A,C,E are overlays of light microscopy and fluorescence images of the same cell. Panels B,D,F are fluorescence images only, showing ingested beads within algal cells. Cells fixed in 0.5% glutaraldehyde, Scale bar in panel (F) applies to (A-F).
3.3.3 Photosynthesis vs. Irradiance Response Curves

Carbon fixation by cell cultures grown in high-nutrient (f/2 + Si) or low-nutrient conditions (5-fold dilution of f/2 + Si) were measured under a range of irradiance using the method of Macintyre et al. (1996), modified by the addition of centrifugation techniques used by Smith and Azam (1992). Photosynthesis vs. Irradiance (P vs. I) Response curves were established by incubating 1-ml of cell cultures adapted to either high or low nutrients in 7ml glass vials at different light levels (4 replicates per light level/nutrient treatment) with sodium $[^{14}\text{C}]$bicarbonate (final specific activity per sample 0.5 µCi, 18.5 kBq). Samples were incubated at PAR light levels ranging from ~20 to 150 µmol m$^{-2}$ s$^{-1}$ for 2 hours. Following incubations, samples were centrifuged at 16,000 G for 30 minutes, followed by aspiration of the supernatant. The algal pellet was then resuspended in 32 PSU ASW solution and fumed with 6N HCL overnight to remove residual unassimilated $^{14}$C. The samples were neutralized with 6M NaOH and scintillation fluid added. Radioactivity of samples was measured in a scintillation counter (Beckman LS-3801), and the average counts per minute converted to disintegrations per minute (DPM) using a quench correction curve. Rates of carbon fixation were determined by the equation:

$$\frac{C_{\text{fixed}}}{h} = \frac{\text{DIC}_{\text{sample}} \times \text{DPM}_{14\text{C}}}{\text{DPM}_{14\text{C}}_{\text{total}}} \times \text{incubation time}^{-1} \text{ (h)}$$

where:

$C_{\text{fixed}} = $ the amount of total carbon fixed by photosynthesis,

$\text{DIC}_{\text{sample}} = $ total dissolved organic carbon available for fixation in sample,
DPM$_{14}^{\text{Csample}}$ = disintegrations per minute for the algal pellet

DPM$_{14}^{\text{Ctotal}}$ = disintegrations per minute of total $^{14}$C available for incorporation.

DIC was calculated using the program CO2SYS in Excel available at http://cdiac.ornl.gov/ftp/co2sys/. Average $^{14}$C uptake of dark controls was subtracted from the light incubations to correct for incorporation not due to photosynthesis. Net primary production was normalized to a per cell basis using light microscopy to enumerate algal cells fixed with Lugol’s iodine (3% final concentration).

3.3.4 Dark Survival

To measure the growth response of Antarctic mixotrophic protists to short-term continuous darkness, we incubated 40 ml samples of $G.\ cryophila$, $M.\ antarctica$, and $P.\ tychotreta$ for 22 days in complete darkness ($n=5$ for $P.\ tychotreta$ and $G.\ cryophila$, $n=4$ for $M.\ antarctica$). Culture flasks (50ml) were wrapped in aluminum foil and placed in a cardboard container lined with aluminum foil to exclude all light and placed in the same incubator with light treatments at 4°C. All experimental replicate cultures were grown in full strength f/2 media. Population abundances were monitored at least every 3-4 days. Samples (3 ml) were fixed with Lugol’s iodine (3% final concentration), settled in PhycoTech chambers (PhycoTech Inc., St. Joseph, MI) and enumerated with light microscopy.
3.3.5 Data and statistical analysis

Grazing experiments were analyzed first using a repeated-measures regression to identify whether there was significant variance between replicates. Following this, ingestion per cell was regressed over time to obtain a grazing coefficient and related standard error parameters. Rates were compared using an ANCOVA of ingestions over time by treatment with pair-wise comparisons assessed using Tukey’s HSD test. Cell biovolumes were calculated using geometric shapes identified for protist species as in Olenina et al. (Olenina et al. 2006) and were compared using a Wilcoxon rank sum test. The R statistical software program was used to fit P vs. I curves to the Eilers-Peeters light limitation curve, which defines photosynthesis as the product of the maximal photosynthetic rate and the light limitation at a given light irradiance, I (Eilers and Peeters 1988). This model was chosen because it allows for estimation of photosynthetic activity in organisms that have a limited peak irradiance, and significant photoinhibitory response (Eilers and Peeters 1988). Specifically,

\[
\text{Light Limitation} = \frac{2 \times (1 + \beta) \times I / I_{opt}}{(I / I_{opt})^2 + 2 \times \beta \times I / I_{opt} + 1}
\]

and

\[
\text{Photosynthesis} = P_{max} \times \text{Light Limitation}
\]

where,

\[I_{opt}\] = light irradiance at optimal photosynthetic rate

\[\beta\] = a dimensionless parameter which defines the degree of photoinhibition.

\[P_{max}\] = maximum photosynthetic rate
Non-linear regression analysis of P vs. I data was estimated using the nls function in the stats package of the R statistical software program, using a Gauss-Newton nonlinear least-squares algorithm. Models were not forced through the origin. All models achieved convergence in fewer than 24 iterations, and convergence tolerance was \( < 8.5 \times 10^{-6} \) for all models. Pairwise comparisons of grazing rates were performed with the multcomp package in R.

3.4 Results

3.4.1 Grazing Rates in Mixotrophic Flagellates

The three Antarctic photosynthetic nanoflagellates in this study were found to be mixotrophic, i.e., particles were ingested under some of the experimental conditions (Fig. 3.1). Grazing rates ranged from 0.5 to 1.8, 0.85 to 3.1, and undetectable to 0.6 bacterial cells ingested cell\(^{-1}\) hour\(^{-1}\) for \( M. \) antarctica, \( G. \) cryophila, and \( P. \) tychotreta respectively (Fig. 3.2). \( M. \) antarctica and \( G. \) cryophila showed significant increases in ingestion rates in the dark as compared to illuminated treatments. Within a specific light treatment, however, nutrient concentration had no effect on bacterial grazing rates in either of these mixotrophs. In contrast, \( P. \) tychotreta in the dark and low nutrients grazed at a rate 71% higher in the low nutrient treatments (Tukey HSD, \( p < 0.001 \)), and ingestion was undetectable in nutrient replete conditions regardless of light levels. The purely heterotrophic flagellate, \( P. \) antarctica, ingested bacteria at similar rates under all experimental conditions, except the ingestion rate in low nutrient, where illuminated treatments were significantly greater than in low nutrient, dark treatments (Tukey HSD, \( p < 0.002 \)) (Fig. 1).
Figure 3.2. Grazing rates of 3 Antarctic mixotrophic nanoflagellates (*G. cryophila*, *M. antarctica*, *P. tychotreta*), and the heterotrophic flagellate *P. antarctica* in a 2x2 factorial design experiment including high (*f/2* media) and low nutrients (1:4 dilution of *f/2* media with ASW) in either dark or light (50 µmol m$^{-2}$ s$^{-1}$) conditions (n=6, error bars +/- SE). Letters indicate Tukey’s HSD comparisons; there are no significant differences between treatments with the same letter above the bars.
3.4.2 Photosynthesis in Antarctic Nanoflagellates

P vs. I curves yielded characteristic relationships over all measured light levels (Fig. 3.3). Distributions of photosynthetic rates in the nanoflagellates conformed to the Eilers-Peeters model, and gave significant parameter estimates of maximal photosynthetic activity (P\textsubscript{max}) and, in most cases, significant estimates of irradiances (I\textsubscript{opt}) that yielded maximal photosynthetic rates (Table 3.1). Under the range of light irradiance tested, \textit{P. tychotreta} did not demonstrate significant photoinhibition in high nutrient treatments (Fig. 3.3). Therefore, neither photoinhibition (\(\beta\)) nor peak irradiance (I\textsubscript{opt}) could be significantly estimated from the curve for this species (Table 3.1). Peak irradiances (I\textsubscript{opt}), the light levels that generate the maximal photosynthetic production rates, were estimated at between \(\sim 140-215 \ \mu\text{mol m}^{-2}\ \text{s}^{-1}\) for \textit{P. tychotreta} (in the low nutrient treatments only), \textit{G. cryophila}, \textit{M. antarctica} and \textit{Chaetoceros} sp., the purely photosynthetic positive control (Table 3.1).

\textit{G. cryophila} and \textit{M. antarctica} showed a similar characteristic response with photosynthetic rate increasing with irradiance to a peak value, followed by inhibition at higher light levels (Fig. 3.3). Trends in photosynthetic rates for \textit{P. tychotreta} were markedly different, with photosynthetic fixation increasing to a peak, followed by little or no photoinhibition; and the reduction in photosynthetic output at higher irradiances in low nutrient treatments was not statistically significant (Fig. 3.3). For \textit{P. tychotreta}, photosynthetic rates on a per cell basis were about an order of magnitude higher than for \textit{G. cryophila}, \textit{M. antarctica}, or \textit{Chaetoceros}; P\textsubscript{max} of \(\sim 13 \ \text{pg C fixed cell}^{-1}\ \text{hr}^{-1}\) by \textit{P. tychotreta} in high nutrient vs. \(\sim 0.5-0.9 \ \text{pg C fixed cell}^{-1}\ \text{hr}^{-1}\) by \textit{G. cryophila},

45
*M. antarctica* and *Chaetoceros* sp. (Table 3.1). The diatom, *Chaetoceros* sp., exhibited photosynthetic responses to increasing light that were similar to *G. cryophila* and *M. antarctica* (Fig 3.3).

![Figure 3.3. Photosynthesis vs. Irradiance Response curves for Geminigera cryophila, Mantoniella antarctica, Pyramimonas tychotreta, and Chaetoceros sp. grown in high (black circles, full strength) or low (grey circles, 5X dilution with ASW) f/2 media.](image)
3.4.3 Dark Survival

Short-term exposure (22 days) to 24-hour darkness resulted in variable responses between the three mixotrophic flagellates. Both of the prasinophytes, *P. tychotreta* and *M. antarctica* responded to dark treatments with an initial increase in cell population followed by a consistent reduction in abundance for the duration of the time series (Fig. 3.4). The initial increase in cell abundance was accompanied by about a 62%

<table>
<thead>
<tr>
<th>Nutrient Treatment</th>
<th>$P_{max}$ (pg C fixed cell$^{-1}$ hr$^{-1}$)</th>
<th>$\beta$</th>
<th>$I_{opt}$ (umol m$^{-2}$ s$^{-1}$)</th>
<th>df</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G. cryophila</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.52(0.06)</td>
<td>$-0.71(0.1)$</td>
<td>213.10(17.26)</td>
<td>26</td>
</tr>
<tr>
<td>High</td>
<td>0.72(0.10)</td>
<td>$-0.62(0.09)$</td>
<td>161.29(11.94)</td>
<td>36</td>
</tr>
<tr>
<td><strong>M. antarctica</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.32(0.02)</td>
<td>$-0.97(0.006)$</td>
<td>148.06(3.96)</td>
<td>34</td>
</tr>
<tr>
<td>High</td>
<td>0.89(0.06)</td>
<td>$-0.69(0.08)$</td>
<td>161.94(11.21)</td>
<td>18</td>
</tr>
<tr>
<td><strong>P. tychotreta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>8.33(0.95)</td>
<td>$-0.053(0.42)^{ps}$</td>
<td>197.71(29.57)</td>
<td>30</td>
</tr>
<tr>
<td>High</td>
<td>12.80(2.61)</td>
<td>5.127(16.44)$^{ps}$</td>
<td>638.22(1659.53)$^{ps}$</td>
<td>21</td>
</tr>
<tr>
<td><strong>Chaetoceros sp.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0.38(0.03)</td>
<td>$-0.90(0.02)$</td>
<td>139.14(7.04)</td>
<td>27</td>
</tr>
<tr>
<td>High</td>
<td>0.53(0.04)</td>
<td>$-0.95(0.02)$</td>
<td>147.78(8.94)</td>
<td>24</td>
</tr>
</tbody>
</table>

Estimates of maximal photosynthetic rate ($P_{max}$), degree of photoinhibition (a non-dimensional parameter, $\beta$), and the optimal photosynthetic irradiance ($I_{opt}$) are given along with associated standard errors. All parameters, significant and non-significant are included for completeness. Values in bold indicate significance at the p < 0.0001 level, “ns” = not significant.
decrease in cell volume (38 µm$^3$ vs. 14 µm$^3$, p>0.001, n=40). *P. tychotreta* also declined in cell volume during the experiment. In contrast, *G. cryophila* maintained their population densities and cell size throughout the time-course (Fig. 3.4).
3.5 Discussion

There are only limited reports of species-specific grazing behaviors by phagotrophic algae from polar regions. Sometimes a single easily-identifiable mixotroph can dominate natural populations, and studies in lakes of the McMurdo Dry Valleys and especially in Ace Lake, Vestfold Hills, eastern Antarctica have taken advantage of relatively simple communities to examine feeding in individual species (Roberts and Laybourn-Parry 1999, Bell and Laybourn-Parry 2003, Moorthi et al. 2009). But the present study provides the first details of environmental factors affecting mixotrophy in three algal isolates, *P. tychotreta*, *M. antarctica* and *G. cryophila*, that were identified as co-occurring in spring and summer samples from the Ross Sea (Gast et al. 2014). *G. cryophila*, as well as an Antarctic species related to *P. tychotreta* were previously known to engage in phagotrophy, but to our knowledge, our work is the first description of mixotrophy in the genus *Mantoniella*. All three mixotrophs studied here fed on bacteria at rates equal to or greater than a heterotrophic nanoflagellate, *P. antarctica*, also isolated from the Ross Sea (Fig. 3.2). These experiments demonstrated contrasting effects of light and nutrient levels on feeding and photosynthetic rates in the flagellates, indicating that mixotrophy is a versatile strategy with different species-specific benefits for different algal species. Furthermore, these data are consistent with reports of major predation impacts of mixotrophs on bacterial assemblages in several polar environments (Roberts and Laybourn-Parry 1999, Bell and Laybourn-Parry 2003, Moorthi et al. 2009, Sanders and Gast 2012, Thurman et al. 2012, Gast et al. 2014).

The Achaerplastida, including the Chlorophyceae, have long been considered a clade consisting primarily of non-phagotrophic “true algae”. While studies such as
Unrein et al. (2014) found no evidence of mixotrophy in organisms from the Chlorophyceae in oligotrophic temperate waters, there is a growing body of evidence indicating some trophic plasticity in green algae. Several species belonging to one of the most ancient clades of Chlorophyceae, the prasinophytes, ingest prey. In addition to the prasinophyte species studied here, *M. antarctica* and *P. tychotreta*, mixotrophic behavior was recently demonstrated in *P. gelidicola* (Bell and Laybourn-Parry 2003), in Arctic *Micromonas pusilla* (McKie-Krisberg and Sanders 2014) and also in *Cymbomonas* (though ingestion was not quantified) (Maruyama and Kim 2013, Raven 2013).

Bell and Laybourn-Parry (2003) determined ingestion rates by *Pyramimonas gelidicola* in the brackish Ace Lake, Antarctica that were mostly equivalent to < 1 bacteria hour$^{-1}$, though ingestion exceeded 10 bacteria hour$^{-1}$ in one of their experiments. The highest feeding rate that we noted for *P. tychotreta* was 0.6 bacteria hour$^{-1}$ in the lower nutrient treatments; no ingestions were observed in high nutrient treatments (Fig. 3.2). The effect of lower nutrients on *P. tychotreta* feeding suggests that grazing rates of *P. gelidicola* may be partly driven by the permanent stratification of Ace Lake, which imposes severe nutrient limitation in the upper euphotic zone (Laybourn-Parry et al. 2002). However, later experiments in Ace Lake found insignificant bacterivory by *P. gelidicola* and a greater grazing impact by cryptophytes (Laybourn-Parry et al. 2005).

The other prasinophyte investigated here, *M. antarctica*, had a higher maximum ingestion rate than *P. tychotreta* under the same experimental conditions. Furthermore, the concentration of dissolved nutrients had no significant effect on ingestion by this organism, while incubation in darkness resulted in nearly a threefold increase in the rate
of bacterivory. This was a much different response than noted for the Arctic prasinophyte *Micromonas pusilla* (McKie-Krisberg and Sanders 2014), which belongs to the same order as *M. antarctica* (Mamiellales). Ingestion rates by *Micromonas* were significantly higher in low nutrient conditions, but only in the light. Thus, the effects of light and nutrients on bacterivory by *M. antarctica* were more similar to that of the cryptophyte *G. cryophila* than to either of the other mixotrophic prasinophytes that we have investigated. Light has been found to increase grazing rates in heterotrophic protists as well (Strom 2001), and this was observed in our experiments with *P. antarctica*.

*G. cryophila* in our study grazed bacteria at rates similar to the mixotrophic cryptophytes (possibly *Chroomonas lacustris*) from slightly saline and freshwater lakes in the McMurdo Dry Valleys (1999) (0.2-3.6 bacteria ingested cell⁻¹ hour⁻¹ vs. 1.6-3.6 bacteria ingested cell⁻¹ hour⁻¹). These high rates of bacterivory by Antarctic cryptophytes and a suggestion of a major contribution to the carbon budgets (2002) are in contrast to early reports of low ingestion by temperate freshwater cryptophytes (Tranvik et al. 1989). However, cryptophyte ingestion rates in the range determined for *G. cryophila* were observed in the NW Mediterranean Sea by Unrein et al. (2014), and very high rates of ingestion were estimated for cryptophytes in Lake Biwa, Japan (Urabe et al. 2000).

There is little to suggest that the non-polar cryptophytes would ingest bacteria as a darkness survival strategy. However, Thurman et al. (2012) found that bacterivory by mixotrophic flagellates, including three cryptophyte species, tended to increase as winter darkness approached in two Dry Valley lakes. Furthermore, Marshall and Laybourn-Parry (2002) suggested that cryptophytes in Dry Valley lakes were unable to survive without grazing bacteria due to low light levels under permanent ice cover. This
supposition implies that ingested organic carbon is substitutable for photosynthetically derived carbon and that bacterivory constitutes a major contribution to the carbon budget of the cryptophytes. While all three mixotrophic species in our lab studies had greater ingestion rates in the dark than in the light (Fig. 3.2), G. cryophila was the only strain that maintained a steady population size over 22 days of darkness (Fig. 3.4). Clearly this time frame only begins to approach the length of the Antarctic winter, but it does indicate a better dark survival of active cells in this Antarctic cryptophyte, and suggests that phagotrophy could contribute to population maintenance by phytoflagellates through at least the beginning of the austral winter. Longer-term dark survival experiments are needed to confirm the hypothesis.

The results from our laboratory grazing experiments, as well as the field experiments discussed, support the idea that mixotrophy can be employed by photosynthetic organisms for different reasons. Our data indicate that P. tychotreta physiology may be more like the “phagocytic algae” type of mixotrophy as defined by Stickney et al. (2000) than an organism substituting ingested carbon for photosynthesis-derived carbon. These "phagocytic algae" are obligate autotrophs that ingest prey to obtain limiting nutrients, though a supplement to carbon requirements is not precluded (2000). In the present study, bacterivory was observed in cultures of P. tychotreta only when soluble nutrients were decreased, irrespective of light treatment (Fig. 3.2), which suggests a potential for substitution of ingested organic nutrients for dissolved inorganic nutrients. Mixotrophs in this group are considered to be unable to survive in the dark on heterotrophy alone (2000); they require some amount of light. The steady decline of population size over 22 days of darkness (Fig. 3.4) suggests this to be the case for P.
 Likewise, the dark survival incubations indicate that *M. antarctica* is also an obligate phototroph, and although it grazes at rates higher than *P. tychotreta*, it does not appear to increase feeding rates when dissolved nutrients are decreased, suggesting a different driver for ingestion of particles between the species.

Photosynthetic flagellates utilizing phagotrophy for long-term dark survival was previously suggested (Laybourn-Parry 2002, 2009). As noted above, this is consistent with observations of *G. cryophila* and other Antarctic cryptophytes that engage in autotrophic energy production, but may not require photosynthesis for survival. Many mixotrophic and non-mixotrophic protists are also capable of taking up colloidal or dissolved organic matter (Marchant and Scott 1993, Sanders et al. 2001, Laybourn-Parry et al. 2005), which also could contribute to dark survival. *P. gelidicola* was shown to take up fluorescently labeled dextrans (Laybourn-Parry et al. 2005) suggesting that mixotrophic flagellates have access to a wider range of alternative carbon sources than ingestion of bacterial prey. Although the use of dissolved organics and phagotrophy are not mutually exclusive, the degree to which protists can take up dissolved organics at concentrations typically found in nature is unclear (Marchant and Scott 1993). In the Ross Sea, the levels of DOC are relatively low (Carlson et al. 1998), and its quality and the ability of phytoplankton to compete with bacteria for that DOC are unknown. We suggest that DOC would not support growth of mixotrophs during winter in the Ross Sea, and its contribution to population maintenance is equivocal. An additional strategy for winter survival is encystment, as suggested by Stoecker et al. (Stoecker et al. 1998) for dinoflagellates in sea ice. *P. tychotreta* produces cysts containing starch grains and lipid droplets (Daugbjerg et al. 2000), which could allow survival without photosynthesis or
phagotrophy during the austral winter darkness. Cyst production is unknown for *M. antarctica* and *G. cryophila*, but immobile palmelloid stages have been observed for *Geminigera* (Hill 1991).

In the grazing experiments presented here we used microspheres as opposed to fluorescently-labeled bacteria (FLB) as tracer particles for monitoring ingestion rates. Preference for tracer particles does exist among microbial predators, but responses to particle choice in feeding experiments is variable. For example, in a study examining grazing in seven protists, Sanders et al. (1989) found feeding rates of FLB and microspheres were not statistically different in four protists, two showed "preference" for microspheres, and only one large heterotrophic flagellate had significantly higher feeding rates on FLB. Previous grazing experiments with polar mixotrophic flagellates have included both microspheres and FLBs, and results showed lower ingestion rates of FLBs as compared to microspheres (Sanders and Gast 2012). Small picoplankton-sized mixotrophic grazers did not even ingest FLBs, but did graze on smaller microsphere particles in that study. McKie-Krisberg & Sanders (2014) observed size selection for smaller microsphere particles in the Arctic prasinophyte, *Micromonas*, with small (0.46 μm) microspheres at twice the rate of large (0.9 μm) microspheres.

One overall impression from this study is a lack of conserved responses in terms of grazing and primary productivity among algae from similar evolutionary lineages. The different responses between the two prasinophytes, *P. tychotreta* and *M. antarctica*, is notable for both feeding and photosynthesis (Fig. 3.2 and 3.3). The data suggest one similarity in that nutrition from phagotrophy does not appear to sustain large populations for long periods in darkness, a trait also noted for another phagotrophic prasinophyte,
"Cymbomonas" (Maruyama and Kim 2013). And though there are similarities between *G. cryophila* and other Antarctic cryptophytes noted in the field experiments of Laybourn-Parry and colleagues (Roberts and Laybourn-Parry 1999, Laybourn-Parry 2002, Marshall and Laybourn-Parry 2002), these species seem to have different mixotrophic strategies than non-Antarctic cryptophytes. The results of these experiments provide insight into the dynamic nature of mixotrophic behaviors, and indicate that they are not governed by a single factor, nor are the responses unilateral among mixotrophic organisms residing in the Antarctic, and by implication elsewhere in aquatic environments.
CHAPTER 4.

COMPETITION BETWEEN ANTARCTIC MIXOTROPHIC PROTISTS AND PHOTOTROPHIC AND HETEROOTROPHIC SPECIALISTS

4.1 Abstract

Competitive interactions provide a major biological filter through which natural selection can act on microbial populations. Microbial eukaryotes are involved in a complex network of interactions important for energy transfer, nutrient retention, and recycling. Previous experiments have shown at least one organism using a combined trophic strategy (Mixotrophy) to have competitive advantages over organisms confined to a single trophic mode. Here we present results of experimental competitive assays of two Antarctic mixotrophic flagellates paired with monotrophic competitors. In the first experiment, the cryptophyte *Geminigera cryophila* was grown with two solely phototrophic Antarctic diatoms of contrasting sizes (*Fragilaria* sp., and *Fragilariopsis* sp.). In the second experiment, the prasinophyte *Pyramimonas tychotreta* is grown with the solely heterotrophic chrysophyte *Paraphysomonas antarctica*. Results of these experiments show that the competitive advantage of mixotrophs over some monotrophic competitors is not always consistent. Under the experimental conditions, *G. cryophila* was a dominant competitor, regardless of the size of the autotrophic competitor. *P. tychotreta* was found to be competitively inferior to the *P. antarctica* in the second experiment. In this case the heterotrophic flagellate able to reduce prey density to an effective concentration below the critical density for survival of the mixotrophic flagellates. These experiments show that in some cases, mixotrophs are able to access alternative resources to their advantage, while in other cases, the combination of
acquisition strategies result in a loss of competitive ability when paired with a trophic specialist.

4.2 Introduction

Species interactions are the foundation for community ecology. Phototrophic and heterotrophic compartments of the biological community are maintained through a network of trophic interactions (Sherr and Sherr 2002, Holyoak and Lawler 2005, Jürgens et al. 2008). In aquatic systems, most of the energy and material moves through protists as the major primary producers and as predators of algae and bacteria. Grazing of bacteria by heterotrophic protists can reduce competition for dissolved nutrients between algae and heterotrophic bacteria (Hulot et al. 2001), and at the same time grazing can modify the structure of bacterial populations (Simek et al. 1997, Hahn and Höfle 2001). This portion of the food web, collectively referred to as the microbial loop, can account for significant reintroduction of energy and nutrients to higher trophic levels (Azam et al. 1983, Pomeroy et al. 2007). The net cumulative effect of these organismal interactions can affect the productivity, retention and residence time of nutritional elements within a given habitat (Rothhaupt 1996b, Azam and Malfatti 2007) and in oceanic systems, these effects can modify the biological pump, i.e., the sequestration of carbon from surface to deeper water (Mitra et al. 2014). By studying the outcomes of both predatory and competitive interactions we can gain insight into the mechanisms that stabilize biological communities. Microbial systems provide an ideal framework for the rigorous quantitative investigation of these ecological phenomenona through the interactions between bacteria, algae, heterotrophic flagellates and ciliates. In particular, the scale of
these communities lends itself to the constraints of experimental design (multiple treatments and controls, and replication).

Monod (1949) adapted substrate mediated growth and negative density dependence to a model of quantitative dynamics of microbial populations. Early models aimed at predicting interactions between competing populations assuming logistic growth were proposed by Lotka and Volterra (1925). Mechanistic studies of algal competition by Tillman (1977, 1980) extended these previous models by employing a graphical approach to predict the outcomes of competitive interactions of organisms surviving osmotrophically on soluble nutritional substrates. In addition, predator-prey interaction models, such as that developed by Rozenzweig and Macarthur (1963), predict a linear growth response of the predator in relation to prey availability, though predators often exhibit a Hollings-type response, conforming to an asymptotic maximal predation rate at high densities.

As yet, the competitive abilities of mixotrophic organisms as compared to their monotrophic counterparts remain unclear. The potential advantages of a combined trophic strategy could create additional opportunities for energy and nutrient acquisition, but maintenance of multiple machinery can come at a cost to the organism (Raven 1997, Ward et al. 2011). In the aquatic environment, a close association between bacteria and phytoplankton is often observed. This phenomenon, described as “pestering”, could provide a strategy whereby bacterial cells can derive organic C necessary for growth, but may also include a variety of more nuanced interactions (Azam and Malfatti 2007). Theoretical and empirical studies have shown that mixotrophic organisms can survive in communities with trophic specialists by occupying conditions on the margins of the
trophic ability of their specialist competitors (Troost et al. 2005, Crane and Grover 2010). Thingstad (1996) hypothesized that mixotrophs may use phagotrophy to acquire nutrients, and reduce competition from bacteria that have superior capabilities for the uptake of dissolved nutrients. Previous competition experiments with a heterotrophic or autotrophic flagellate paired with the mixotrophic chrysophyte, *Ochromonas*, illustrated that this mixotroph was able to gain a competitive edge by utilizing the alternative resource not accessible to the specialist competitor (Rothhaupt 1996a).

Here, we present competition experiments measuring population abundance changes of two Antarctic mixotrophic nanoflagellates grown with monotrophic competitors also isolated from the Antarctic. The mixotrophic cryptophyte *Geminigera cryophila* was grown in separate co-cultures with Antarctic diatoms in the genera *Fragilaria* and *Fragilariopsis*. In addition, the mixotrophic prasinophyte *Pyramimonas tychotreta*, was grown with the heterotrophic chrysomonad *Paraphysomonas antarctica*. In all experiments, population abundance of monocultures each protist species were also followed in along with competitive treatments. The results of this study show one mixotroph as a dominant competitor (*G. cryophila*), while the other (*P. tychotreta*) was unable to maintain populations with a heterotrophic specialist. These results provide insight into the interactions of mixotrophs, monotrophic specialists, and their bacterial communities in Southern Ocean microbial food webs.
4.3 Materials and Methods

4.3.1 Culture Origin and Maintenance

Cultures used in this study were from the Antarctic Protist Collection, which is maintained at Woods Hole Oceanographic Institution and at Temple University (http://www.whoi.edu/science/B/protists/). Experimental cultures of the mixotrophic prasinophyte *Pyramimonas tychotreta* (strain “I-9Pyram”), the mixotrophic cryptophyte *Geminigera cryophila* (strain “I-55Yam”) a heterotrophic chrysophyte *Paraphysomonas antarctica* (strain “RS-4-2”), the diatoms *Fragilaria* sp. (strain “SL-149#2Moon”) and *Fragilariopsis* sp. (strain “SL-64/78Cheetos”) were maintained at 4°C in f/2 + Si made with artificial seawater (ASW) at 32 PSU (Guillard 1975, Caron 1993). The cultures were non-axenic and grown under continuous irradiance from 50W cool-white fluorescent bulbs at ~50 µmol m⁻² s⁻¹.

4.3.2 Experimental Setup

To examine competition of a mixotroph versus phototrophic specialists, replicate flasks containing the mixotroph *G. cryophila* and either *Fragilaria* sp. or *Fragilariopsis* sp. were grown in f/2 media under continuous illumination supplied in the same manner and level (~50 µmol m⁻² s⁻¹) as for culture maintenance (n=3). Monocultures of each competitor species were grown alongside co-culture experiments. Population abundances were monitored with light microscopy at least bi-weekly for 8 weeks using 3 ml samples fixed with Lugol’s iodine (2.5-3% final concentration). Initial abundances of *Fragilaria* sp. and *Fragilariopsis* sp. were $4 \times 10^4 \pm 1.58 \times 10^3$ and $4.2 \times 10^3 \pm 2.1 \times 10^3$ cells ml⁻¹,
respectively. Initial abundances of *G. cryophila* were ~$5.0 \times 10^4 \pm 7.2 \times 10^4$ cells ml$^{-1}$ in both co-culture experiments.

A similar set of co-culture experiments to investigate competition between the mixotroph *P. tychotreta* and the heterotrophic nanoflagellate *Paraphysomonas antarctica* were run in a 5-fold dilution of f/2 media along with replicate monocultures of each competitor (n=4). Light level, sampling and enumeration of protists were identical to those above, but population abundances were monitored for 30 days, sampling at 5-7 day intervals. In addition, to gain insight into the population dynamics of prey in these experiments, bacterial populations were monitored. Bacterial subsamples were fixed with Lugol’s iodine, cleared with Na$_2$S$_2$O$_3$, with final fixation in formalin (1% final concentration). Subsamples from each replicate were collected onto 0.2µm polycarbonate filters, stained with 0.01% Acridine Orange (3,6-Acridinediamine) for 3-5 minutes and enumerated using epifluorescence microscopy.

4.3.3 Data and Statistical Analysis

For experiment I, examining competition between a mixotroph and autotrophs, data were evaluated with several models of microbial growth dynamics using Matlab (Table 1). Gompertz and Logistic models were chosen as standard microbial growth models (Zwietering et al. 1990). The Ricker function was chosen as an alternative growth model characterizing “struggle competition” (Ricker 1954, Brännström and Sumpter 2005). Linear and exponential models were also included for organisms in culture which exhibit a stationary response or decline in cell abundance due to competition pressure. Selection was determined through minimization of Root Mean
Square Error (RMSE). Parameters were then estimated using nls with R statistical software. For experiment II, with the heterotrophic competitor, parameters were estimated using either an exponential growth model or through linear regression, using nls function in the stats package. Where applicable, growth functions were evaluated using ANOVA comparing residuals of nonlinear regression models of the same organism growing in competition.

Table 4.1. Models used for selection of growth of organisms grown alone, or in competition in Experiment I.

<table>
<thead>
<tr>
<th>Function</th>
<th>Equation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gompertz</td>
<td>$a_e \cdot e^{-e^{(t-x_0)}}$</td>
<td>Richards (1959)</td>
</tr>
<tr>
<td>Logistic</td>
<td>$\frac{a_t}{1 + e^{-\frac{b_t-x}{c_t}}}$</td>
<td>Nelder (1961)</td>
</tr>
<tr>
<td>Ricker</td>
<td>$a_p x e^{-b_p x}$</td>
<td>Brännström &amp; Sumpter (2005)</td>
</tr>
<tr>
<td>Exponential</td>
<td>$a_{ex} x^{b_{ex}}$</td>
<td>Monod (1949)</td>
</tr>
<tr>
<td>Linear</td>
<td>$a_{Lin} x + b_{Lin}$</td>
<td>--</td>
</tr>
</tbody>
</table>
4.4 Results

4.4.1 Experiment I: Mixotroph (G. cryophila) vs. Autotrophic Competitors

In monocultures, G. cryophila, Fragilaria sp. and Fragilariopsis sp. reached maximum densities of $5.22 \times 10^5$, $2.3 \times 10^4$, and $1.7 \times 10^4$ cells ml$^{-1}$, respectively (Fig. 4.1). However, in co-culture, G. cryophila exhibited a competitive dominance over the two obligate phototrophs. When G. cryophila was paired with Fragilaria sp., the diatom rapidly declined to minimal population densities (Fig. 4.1D, Table 4.2). By week 4, the average abundance of Fragilariopsis sp. had dropped from initial abundance of $5.2 \times 10^3$ cells ml$^{-1}$ to $3.5 \times 10^3$ cells ml$^{-1}$ and by week 8, only $\sim 6.5 \times 10^2$ Fragilariopsis sp. cells ml$^{-1}$ were found in co-cultures (Fig. 4.1D). In contrast, G. cryophila abundance increased from $5.0 \times 10^4$ cells ml$^{-1}$ to $5.92 \times 10^5$ cells ml$^{-1}$ by week 6, which was similar to its abundance in monoculture. In the second pairing, G. cryophila outcompeted the larger phototroph Fragilaria sp., again reducing it to extremely low densities by the end of the time series (Fig. 4.1B). As expected in a true competitive interaction, co-culture with the diatoms did have a negative effect on G. cryophila, as seen by the reduction in G. cryophila cell abundance by week 8 in both experimental co-cultures (Fig. 4.1).

Model selection indicated the best descriptions of growth patterns were species-specific in the organisms tested. Comparisons of Gompertz and Logistic models resulted in near identical RMSE values, with a slight favor toward the Logistic function. G. cryophila conformed most to the logistic growth model (Fig. 4.1, Table 4.2). Fragilariopsis sp. and Fragilaria sp. displayed similar characteristic growth response that was best categorized by the Ricker function in both mono- and co-culture treatments (Fig. 4.1, Table 4.2).
Figure 4.1. Experiment I - Population abundances of the mixotroph *G. cryophila* (black, solid lines) and 2 diatoms *Fragilariopsis* “SL-64/78Cheetos” (grey, dashed lines) and *Fragilaria* “SL-149#2Moon” (grey, dotted lines) in competition for 8 weeks. Panels A,C,E are organisms grown as monocultures. Panels B,D are co-cultures (n=3, error bars +/- SE).
4.4.2 Experiment II: Mixotroph (P. tychotreta) vs. Heterotrophic Flagellate

Initial population abundances of protists were similar in monoculture and co-culture treatments, although P. tychotreta started at a slightly lower density in the co-culture (initially $9.2 \times 10^3$ cells ml$^{-1}$ P. tychotreta vs. $1.26 \times 10^4$ cells ml$^{-1}$ P. antarctica, Fig. 4.2). When grown alone, or with the mixotrophic competitor, cell densities of P. antarctica decreased slightly over the course of the experiment ($0.7 \times 10^5$ cells day$^{-1}$).
alone vs. -1.2×10^2 cells day^{-1} in co-culture, Fig. 4.2), with no significant difference between monoculture and co-culture treatments. *P. tychotreta* population increased in characteristic exponential fashion when grown in monoculture, reaching a density of ~3.0×10^4 cells ml^{-1} by day 30 (Fig 2A, Table 3). In contrast to the monoculture incubation, the mixotroph *P. tychotreta* was outcompeted and driven to very low abundances when grown together with *P. antarctica*; by day 30 of the competitive incubation, *P. tychotreta* cells were undetectable (Fig. 4.2B).

Initial bacterial abundances in all treatments ranged from 3.07×10^5 cells ml^{-1} to 1.7×10^5 cells ml^{-1}. The highest concentrations of bacteria were found in *P. tychotreta* monocultures, with abundances ranging from 3.07 - 2.8×10^5 cells ml^{-1} and no significant change in cell abundance over the time course. Bacterial concentrations in the monocultures of *P. antarctica* were initially ~45% lower than in the *P. tychotreta* monoculture replicates, ranging from 1.7×10^5 cells ml^{-1} - 0.84×10^5 cell ml^{-1}, with the highest abundances in the initial observations followed by a consistent decline of -3.27×10^3 cells day^{-1}, presumably due to grazing pressure (p<0.001) (Fig. 4.2). Though initially higher when the mixotroph and heterotroph were incubated together, bacterial abundances were closer to *P. antarctica* alone treatments (T_{initial}=1.86×10^5 cells ml^{-1}). However, during the time-course bacteria in co-culture were grazed to levels similar to those in the *P. antarctica* alone treatments, with a final concentration of 1.12×10^5 cells ml^{-1}. 
Figure 4.2. Experiment II - Population abundances of the mixotroph *P. tychotreta* (black, solid lines) and the heterotroph *P. antarctica* (grey, dotted lines) in competition for 30 days. Panels A and C are organism grown as monocultures, panel B is co-culture (n=4, error bars +/- SE)
Table 4.3. Parameter estimates for curves estimated for *P. tychotreta*, *P. antarctica* in Experiment II. Subscript labels identify co-culture treatment or monoculture (alone) designation. *** = p<0.001, ** = p<0.01, * = p<0.005, df=22.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>a&lt;sub&gt;EX&lt;/sub&gt;</th>
<th>b&lt;sub&gt;EX&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. tychotreta</em></td>
<td>alone</td>
<td>7.4459(2.45)**</td>
<td>0.044(0.01)**</td>
</tr>
<tr>
<td><em>P. tychotreta</em></td>
<td>para</td>
<td>10.57311(0.99)**</td>
<td>−0.093(0.02)**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>a&lt;sub&gt;Lin&lt;/sub&gt;</th>
<th>b&lt;sub&gt;Lin&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. antarctica</em></td>
<td>alone</td>
<td>−0.07(0.03)*</td>
<td>10.78(0.57)**</td>
</tr>
<tr>
<td><em>P. antarctica</em></td>
<td>pyram</td>
<td>−0.12(0.04)**</td>
<td>11.37(0.69)**</td>
</tr>
</tbody>
</table>
4.5 Discussion

The polar environment imposes challenging constraints on photosynthetic organisms in terms of prolonged darkness and limitations in micronutrients such as iron (Clarke 1988, Boyd et al. 2000, Sedwick et al. 2000). Microbial interactions between competitors, predators and prey will dictate the presence and abundance of specific
species in a particular community. As well, conditions in polar ecosystems also may offer unique opportunities for mixotrophic microbial eukaryotes to maintain populations. The reduction in prokaryotic competitors can also benefit larger sized autotrophic or mixotrophic competitors, which are generally inferior to bacteria at attaining soluble nutrients (Azam et al. 1983, Thingstad et al. 1996). These predatory and competitive interactions can affect larger scale processes such as nutrient regeneration via bacterivory (Caron 1994, Pomeroy et al. 2007).

The two mixotrophic flagellates investigated in this study were previously shown to differ physiologically from one another in terms of heterotrophic and photosynthetic functioning. The cryptophyte *G. cryophila* was demonstrated to graze bacteria at rates similar to solely heterotrophic flagellates from polar environments (Fig. 3.2). Previous studies of a saline Antarctic lake found the resident mixotrophic cryptophytes were obligate bacterivores (Marshall and Laybourn-Parry 2002). In contrast, *P. tychotreta* was found to possess a maximum photosynthetic rate an order of magnitude higher than several other mixotrophs and an autotrophic diatom, all isolated from the Antarctic (Fig. 3.3). In contrast to the Antarctic cryptophytes, *P. tychotreta* ingested bacteria only under conditions of low nutrients (Fig. 3.2). The experiments performed here show contrasting competitive abilities of these two mixotrophic organisms in the presence of autotrophic or heterotrophic competitors.

Microbial eukaryotes can exhibit complex life cycles, including 4 phase growth cycles, periods of encystment, and/or synchronized asexual and sexual reproduction (Anderson 2010). For this reason, it is difficult to predict the pattern of growth that would be observed in the protist species selected for these experiments. The contrasting
growth models between different species found in Experiment I illustrate this complexity. *G. cryophila* exhibited logistic-growth-like population dynamics, while the growth of *Fragilariopsis* sp. and *Fragilaria* sp. were best represented by the Ricker function. These differences may be accounted for by the rapid rate of growth in the diatoms species. Diatoms are known to form blooms through rapid cell division under conditions of high nutrient concentrations (Smetacek 1985), and under the experimental conditions and starting cell densities of the present study, *Fragilariopsis* sp. and *Fragilaria* sp. reached maximum densities within the first two sampling time points (Fig. 4.1). Despite this, the rapid rate of cell populations growth did not allow either of the diatoms to outcompete the *G. cryophila* and while monocultures *Fragilariopsis* sp. and *Fragilaria* sp. were able to maintain cell populations, in co-culture both diatom species decline by the end of the experiment.

The results from Experiment I identify *G. cryophila* as a superior competitor compared to the two diatoms, *Fragilaria* and *Fragilariopsis*. It is often assumed that smaller cells, with larger surface-to-volume ratios, will outcompete larger organisms for soluble nutrients, although this is not always the case (Grover 1989). The size of these diatoms (6μm and 4μm diameter for *Fragilaria* and *Fragilariopsis*, respectively) suggest differential ability for the uptake of dissolved nutrients and therefore show the comparative dominance of the mixotroph (6-9 μm diameter for *G. cryophila*) against competitors with different smaller cell sizes. Indeed, *G. cryophila* reached high abundances when grown in co-culture with either size of diatom competitor. As the only bacterivore in the system, *G. cryophila* had exclusive access to all suitable prey, which could provide *G. cryophila* an alternative resource pool not available to the solely
autotrophic competitors. In addition, the bacterial communities in these incubations may actually have functioned as competitors to the diatoms for dissolved nutrients, further challenging their abilities to outcompete the mixotroph. These results differ from those by Rothhaupt (1996a), who examined the freshwater mixotrophic flagellate *Ochromonas* in competition with a solely autotrophic *Cryptomonas*. In those experiments the mixotroph and phototroph coexisted in nutrient limiting conditions. However, prior to the inoculation of the mixotroph, the autotrophs were permitted to reach stationary population size. The heterotroph was able to "invade" the *Cryptomonas* culture, but the autotroph population remained stable for the remaining 10 days of the experiment while *Ochromonas* grew on bacterial prey (Rothhaupt 1996). Conversely, in the experiments with *G. cryophila* the diatoms population growth was suppressed (Fig. 4.1).

Predation pressure has been suggested as the most influential factor in controlling bacterial populations (Pernthaler 2005), and reduced abundance of these prey could lead to competition between a purely phagotrophic bacterivore and a mixotroph. In experiment II, *P. tychotreta* appeared to be competitively inferior to the heterotrophic *P. antarctica* under the experimental conditions. Specifically, *P. antarctica* appeared to be able to exist at a lower prey encounter rate (lower prey abundance), than the mixotroph. Bacterial prey were still available at day 30 of the incubation, implying that the *P. tychotreta* was displaced through a reduction in prey concentration below the level critical for its survival. The bacterial populations decreased in cultures of *P. antarctica* alone due to the activity of a heterotrophic predator. In contrast, bacterial densities in *P. tychotreta* monocultures did not significantly change by day 30 (Fig. 4.3).
Studies of predation of bacterial populations by heterotrophs have shown reduction and modification of prey community structure (Corno and Jürgens 2006). Mixotrophic flagellates, the chrysophyte *Ochromonas* for example, also can exhibit prey selectivity (Pfandl et al. 2004). Rothhaupt (1996a) showed the ability of *Ochromonas* to outcompete heterotrophic flagellates like the kinetoplastid *Bodo* sp. and the chrysophyte *Spumella* sp. only under illuminated conditions. Ochromonads are located toward the heterotrophic end of the mixotrophic gradient and can even survive in the dark with sufficient bacterial food (Sanders et al. 1990). However, *P. tychotreta* is an obligate autotroph that grazes under specific conditions and at relatively low rates, which may explain its poor competitiveness with the heterotroph *P. antarctica*. Although ingestion increased in *P. tychotreta* in non-illuminated conditions, populations still rapidly decreased in the dark (Fig. 3.4). This makes unlikely that the reduction in illumination would change the outcome for *P. tychotreta* competing with *P. antarctica*.

Carbon fixed photosynthetically and released as dissolved organic carbon by *P. tychotreta* most likely accounts for the higher bacterial populations in the cultures where the mixotroph was present (Fig. 4.3). Bacterial populations were at highest concentrations in the single protist cultures of *P. tychotreta*, most likely due to a higher concentration of DOC release during photosynthetic production coupled with the relatively low grazing impact by the mixotroph. This is also consistent with the lowest bacteria abundance in *P. antarctica* monocultures and intermediate bacterial abundance in co-cultures of *P. tychotreta* and *P. antarctica*. Previous experiments show that *P. tychotreta* will ingest prey only when nutrient concentrations are relatively low, as in the case of these competition experiments (Fig. 3.2). When *P. tychotreta* was grown alone, population size
increased in characteristic exponential fashion. However, as bacterial abundance
decreased, *P. tychotreta* was unable to maintain its populations when grown in co-culture.
The heterotroph *P. antarctica* exhibited similar growth characteristics when grown alone
or in treatments with *P. tychotreta*. The results here provide support for the idea as
suggested by Thingstad (1996), that the residual DOC from photosynthesis may allow
mixotrophic organisms to “garden” resident bacteria. However, in the presence of high
abundances of the heterotrophic flagellate, bacterial populations declined, perhaps to
levels below an ingestion threshold for *P. tychotreta*. This could potentially limit access
to a micronutrient that the mixotroph acquired via ingestion of bacteria.

Mixotrophic flagellates, monotrophic competitors and their bacterial prey interact
in complicated ways and comprise the foundation of the larger food web architecture.
The results of this chapter illustrate that the extent of a mixotrophic organism’s reliance
on a particular trophic mode can affect its competitive abilities. In the first experiment, *G. cryophila*
was able to outcompete phototrophs expected to be competitively superior in
acquiring dissolved nutrients based on their morphologies (cell size). In the second
experiment, the ability to combine different trophic modes was not able to provide an
advantage to the mixotrophs under the experimental conditions. The outcomes of these
experiments show that different mixotroph species combine phototrophy and
phagotrophy in different ways. More experimentation investigating the environmental
conditions where combined trophic strategies yield advantages over monotrophic
competitors is needed to gain a deeper understanding as to the role that mixotrophy plays
in the ecological success of microbial eukaryotes.
CHAPTER 5.
CONCLUSIONS AND FUTURE DIRECTIONS

Polar aquatic habitats provide unique environmental factors that lead to surprisingly diverse microbial communities in these extreme environments. The habitats present niche opportunities as well as constraints on survival that are absent at temperate or equatorial latitudes. In this dissertation, I have examined ecological strategies employed by several algal groups that reside in polar habitats. The objectives of this dissertation were to identify and describe phagotrophic behavior in microbial eukaryotes previously identified as purely heterotrophic. The data regarding phagotrophic ingestion of particulate matter by *M. pusilla* in Chapter 2 and *P. tychotreta* and *M. antarctica* in Chapter 3, represent novel identification of mixotrophic behavior in these polar flagellates. The second goal of this work was to describe the effect of light and nutrients on grazing behavior in these polar flagellates. This level of detail, with regard to phagotrophic behavior, has not previously been performed for any of the species investigated in this work, including the cryptophyte *G. cryophila*. In Chapter 4, competitive assays of mixotrophs and trophic specialists demonstrated that the strategy of mixotrophy can confer advantages to mixotrophic organisms over competitors that rely on a single trophic strategy. However, the strategy does no guarantee competitive superiority and can potentially inhibit competitive ability in other cases. This work provides a foundation for additional studies of mixotrophy in these organisms, as well as other eukaryotic microorganisms from these regions.
5.1 The Arctic Picoeukaryote: Micromonas pusilla

Recognition of the importance of picophytoplankton populations has steadily increased over the past decade. The progression of research efforts focused on this fraction of the microbial consortia have brought to light the important role that small-sized photosynthetic and heterotrophic picoflagellates play in marine environments. *Micromonas pusilla* is an organism that is well characterized and is found to be present in many marine environments. In the Arctic Seas, *M. pusilla* has been found to be particularly abundant, yet their ecological functioning, their impact in the global cycling of elements such as carbon, as well as, their interactions with marine bacterial communities is still largely unknown (Stockner 1988, Lovejoy et al. 2007). Mixotrophic behavior by the Arctic clade of *M. pusilla* identified in this dissertation adds to a growing list of prasinophyte algae (three in this dissertation alone) that have been identified as possessing phagotrophic ability. This trophic plasticity can provide potential advantages, particularly in extreme marine environments such as the polar regions. The results in this dissertation suggest that mixotrophy could play a critical role in the dynamic of Arctic marine communities.

The contrasting photosynthetic vs. heterotrophic response to illuminated treatments presented here has provided important insights into the physiology of these picoprasinophytes. Chapter 2 presents the first species-specific identification of mixotrophy in a microbial eukaryote of this size class. The increase in grazing under illuminated, low-nutrient conditions is a marked difference as compared to the Antarctic prasinophyte algae studied in Chapters 3 and 4. These results suggest that bacterivory in these species may function to scavenge limiting nutrients required for physiological
functions, including photosynthesis. Sanders et al. (1990), in a study of the mixotrophic chrysophyte, *Poterioochromonas malhamensis*, found that even when light reached levels that induced a photoinhibitory response, the flagellate did not exhibit a decrease in ingestion of prey. Different algal cells can exhibit a diverse array of photoinhibitory responses, but it is likely that light levels exceeding $I_{opt}$, will elicit increases or, at least, maintenance of grazing responses in mixotrophic flagellates.

The results from Chapter 2 indicate that, based on environmental conditions, relative heterotrophic and autotrophic activities are likely optimized independently in *M. pusilla*, but may still serve a compensatory function to some extent. Future grazing experiments with *M. pusilla* should include additional light and nutrient levels in order to create a functional response curve for this organism (Holling 1959). These experiments would build on the work presented here and provide further insight into the phagotrophic responses of this strain of *M. pusilla* to relevant environmental conditions encountered over the extreme seasonal changes experienced by polar organisms.

The cultured isolates of *M. pusilla* represent several evolutionarily distinct clades (Šlapeta et al. 2006). These clades have an extensive geographic range, that present different environmental conditions, some that may be conducive to alternative resource acquisition strategies, and some that may not. Phagotrophic ability in the Arctic strain of *M. pusilla* (CCMP 2099) used in these experiments may not be ubiquitous across all clades of this picoeukaryote. Changes in ocean temperatures and currents due to climate change have the potential to extend the geographic range of some clades, which alter the community impact of *M. pusilla* in the Arctic, particularly if differences in trophic behavior exist between particular clades of *M. pusilla*. Further studies should focus on
identifying the ability of ingestion in other isolates of *M. pusilla* representing additional clades.

### 5.2 Antarctic Nanoflagellates

The results reported here illustrate that mixotrophy occurs more prevalently among flagellated protozoa in the Southern Oceans than previously thought. The organisms chosen for this portion of the dissertation allow for comparison within genera as well as across different evolutionarily distinct lineages. In addition, the subject organisms utilized in Chapter 3 were isolated from different local habitats within the Antarctic environment. The diversity of responses between the mixotrophs tested here indicates that trophic plasticity is complex. It also appears that the combination of trophic modes is utilized in response to different environmental conditions.

Further experimentation with these Antarctic flagellates could include a continuation of grazing experiments similar to those recommended for the Arctic *M. pusilla* in Chapter 2, investigating phagotrophic behavior at additional light or nutrient conditions. Results of experiments such as these could provide further insight into the factors that control the balance between heterotrophic and photosynthetic machinery. Specifically, these experiments could identify the presence of a gradual decline in ingestion rates as light decreases in *M. antarctica*, and *G. cryophila*. Alternatively, further experimentation could pose the question: Is there existence of a threshold light irradiance after which there is a sharp reduction of phagotrophic foraging? In the experiments presented here, *M. antarctica*, and *G. cryophila* did not exhibit a complete cessation of ingestion in response to any factor. However, feeding did cease in the
experiments with *P. tychotreta*. In the case of this prasinophtye, a contrasting response in terms of regulation of the trophic strategies, as compared to the other two flagellates, is expected.

Dark survival experiments resulted in patterns of population abundances that were conserved among evolutionarily related groups. The two prasinophtyes *P. tychotreta* and *M. antarctica*, declined in the dark while the cryptophyte *G. cryophila* maintained population abundances. The duration of the dark-survival experiments presented here was only 22-days. This does not begin to approach the length of the Austral winter that these organisms are exposed to in their natural environment. Additional experiments should include longer dark incubations to observe ecological and/or morphological mechanisms that these photosynthetic flagellates may use to survive in the absence of light for this extended period of time.

### 5.3 Mixotrophy in Competition

Competitive assays of these mixotrophic protists contrast with the limited number of similar studies that exist from the Southern Ocean. In the experiments presented here, the diverse nature of mixotrophic abilities was highlighted. *G. cryophila* out-performed autotrophic competitors, while *P. tychotreta* was unable to out-compete or, at least, coexist with a heterotrophic competitor. The few laboratory studies of competition by any mixotrophic species have focused on a single genus (the chrysophyte, *Ochromonas*). This organism represents one functional form of mixotrophic behavior that cannot be generalized for all microbial eukaryotic mixotrophs. Additional information, such as the
data presented here, begins to inform our understanding as to the advantages mixotrophy provides as well as the costs associated with this strategy.

In conclusion, the experiments presented here provide a rigorous investigation of the trophic ecology of several species of microbial eukaryotes from polar regions. Through robust experimentation, the results provide novel identification of mixotrophy and a description of these activities in the form of grazing and photosynthetic rates under contrasting conditions. The species-specific identification of mixotrophy and the description of some of the factors that can influence grazing and photosynthetic rates in several microbial eukaryotes provide a significant contribution to the understanding of mixotrophy in aquatic microbial communities. The results presented here provide an initial determination of, and inquiry into, mixotrophy at high latitudes, and will prompt additional experimentation with these microbial eukaryotes there, as well as with others from these distant dynamic regions.
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