

MIXOTROPHY IN FRESHWATER FOOD WEBS

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## ABSTRACT

Environmental heterogeneity in both space and time has significant repercussions for community structure and ecosystem processes. Dimictic lakes provide examples of vertically structured ecosystems that oscillate between stable and mixed thermal layers on a seasonal basis. Vertical patterns in abiotic conditions vary during both states, but with differing degrees of variation. For example, during summer thermal stratification there is high spatial heterogeneity in temperature, nutrients, dissolved oxygen and photosynthetically active radiation. The breakdown of stratification and subsequent mixing of the water column in fall greatly reduces the stability of the water column to a vertical gradient in light. Nutrients and biomass that were otherwise constrained to the depths are also suspended, leading to a boom in productivity.

Freshwater lakes are teeming with microbial diversity that responds to the dynamic environment in a seemingly predictable manner. Although such patterns have been well studied for nanoplanktonic phototrophic and heterotrophic populations, less work has been done to integrate the influence of mixotrophic nutrition to the protistan assemblage. Phagotrophy by phytoplankton increases the complexity of nutrient and energy flow due to their dual functioning as producers and consumers. The role of mixotrophs in freshwater planktonic communities also varies depending on the relative balance between taxon-specific utilization of carbon and energy sources that ranges widely between phototrophy and heterotrophy. Therefore, the role of mixotrophy in the microbial food web is difficult to predict because functional types of mixotrophs along a gradient of nutritional strategies contribute differently to nutrient cycling and carbon sequestration.

The overall objective of this work was to advance existing knowledge of the abundance and activity of phagotrophy phytoplankton in lacustrine systems. The incorporation of mixotrophy into the microbial food web requires the complement of physiological studies in culture (as described in chapter 2) and quantification of activity (including abundance and bacterivory) in relation to strict phototrophs and heterotrophs *in situ* (as described in chapter 3 and 4). Information on the physiological ecology of mixotrophic protists is crucial to understanding their role in planktonic food webs and influence on the dynamic microbial community structure in lake ecosystems. An understanding of the ecological functioning of lakes has ultimate consequences for management of water resources, particularly in the face of global climate change.

To my mother,  
Who taught me not to fear the unknown, but challenge it.

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# CHAPTER 1

## INTRODUCTION

### 1.1 Mixotrophy in lacustrine planktonic food webs

Plankton dynamics in lake ecosystems are governed by a variety of physical and biological forces that contribute to well-established patterns in seasonality (Sommer *et al.*, 2012, Wetzel, 2001b). The PEG (Plankton Ecology Group) model introduced in 1986 predicts that in temperate, dimictic lakes plankton exhibit a unimodal pattern in abundance, reaching a pronounced peak in spring as a result of increased irradiance and renewed upward nutrient flux (Sommer *et al.*, 1986a). Although the PEG model has some validity, the recognition of mixotrophy as a prominent nutritional strategy challenges its assumptions in that the diverse metabolisms of phagotrophic phytoplankton may cause different responses to shifts in temperature and light that trigger the initial spring bloom. Mixotrophs combine phototrophy and phagotrophy to yield dual functional roles as primary producers and consumers, and are hypothesized to have different effects on food-web structure and carbon sequestration depending on their nutritional strategy (Wilken *et al.*, 2013b, Mitra *et al.*, 2016). A better understanding of how nutritionally diverse protists respond to abiotic variables that underlie plankton succession is imperative to the inclusion of mixotrophs into the food web structure of aquatic ecosystems. This is particularly important under global change scenarios where climate-induced changes in plankton dynamics and phenology are a reality (Sommer & Lengfellner, 2008, Domis *et al.*, 2013, Findlay *et al.*, 2001).

Protists that ingest bacteria, or bacterivores, are vital components of the aquatic food web as prey for zooplankton and top-down regulators of bacteria (Azam *et al.*, 1983). Grazing pressure by nanoflagellates can result in changes in abundance, morphology and taxonomy of the bacterial assemblage (Weisse, 2002). These organisms are vital to the transfer of carbon to higher trophic levels. Bacterivory by heterotrophic nanoflagellates is vital to the efficiency of the microbial loop by reintroducing previously unavailable resources to the classic food web (Sherr & Sherr, 2002). Grazing activity is also an important mechanism of nutrient regeneration because bacteria are more efficient in uptake of nitrogen and phosphorus by virtue of their high surface area to volume ratio (Kamjunke *et al.*, 2008, Tranvik, 1994, Rothhaupt, 1997). However, many bacterivores can function as mixotrophs by combining photosynthesis with ingestion of particulate matter and defy historic classification into discrete trophic modes.

Mixotrophy refers to a broad array of ecological strategies, including the osmotic uptake of dissolved organic matter by phytoplankton and retention of foreign plastids by phagotrophic predators (Stoecker *et al.*, 2009). The current work emphasizes mixotrophic organisms that are capable of obtaining energy and nutrients by photosynthetic fixation of carbon and phagotrophic ingestion of bacteria. Incidence of mixotrophic behavior has been identified in a variety of protistan lineages including chrysophytes, prymnesiophytes, cryptophytes, dinoflagellates and ciliates (Sanders & Porter, 1988, Sherr & Sherr, 2002). This suggests that mixotrophy is a successful nutritional strategy that allows organisms to occupy variable ecological niche space. Although mixotrophy has been documented within a range of aquatic habitats from estuaries to the open ocean, it seems to be a dominant strategy in oligotrophic environments because it affords an

advantage in acquiring limiting nutrients (Nygaard & Tobiesen, 1993a, Arenovski *et al.*, 1995a, Hartmann *et al.*, 2012b).

## **1.2 Influence of abiotic factors on the spectrum of mixotrophic behaviors**

Abiotic factors such as light, dissolved nutrient availability and temperature are predicted to influence particle ingestion in phagotrophic phytoflagellates (Sanders & Porter, 1988). Considerable work has been done to classify mixotrophic protists based on their nutritional requirements, but predicting the relative importance between phototrophy and heterotrophy in nature has proven difficult (Stoecker, 1998, Lones, 1997) Mixotrophs exist along a gradient of nutritional strategies that range from primary phototrophy to primary heterotrophy. Phytoplankton can supplement photosynthetic growth with facultative heterotrophy under light-limitation or to gain essential macro- and micronutrients, vitamins and growth factors (Li *et al.*, 2000, Caron *et al.*, 1993a, Nygaard & Tobiesen, 1993a). Conversely, primarily heterotrophic mixotrophs may utilize photosynthesis during periods of reduced prey concentration (Holen, 1999a, Sanders *et al.*, 1990b). In an attempt to quantify the costs of mixotrophy, Raven (1997) estimated that maintenance of photosynthetic machinery outweighs that of the phagotrophic apparatus. Therefore, despite the various benefits of facultative phototrophy, the ability to maintain phototrophic capabilities in a primary heterotroph is not without great energy demands (Raven, 1997a). Mixotrophic organisms must make physiological adjustments in the switch between trophic modes, and such trade-offs are often dependent on the unique strategy of the mixotroph (Sanders *et al.*, 1990b). For example, in a study of the nutritional flexibility of *Poteroiochromonas malhamensis*, a chrysophyte on the

heterotrophic end of the spectrum, Holen (1999) observed reductions in cell-specific chlorophyll *a* (chl-*a*) during phagotrophic growth. However, when bacterial concentrations limited phagotrophic growth in *P. malhamensis*, chl-*a* levels increased in illuminated cultures, indicating a switch from phagotrophy to phototrophy (Holen, 1999a). Similarly, *Ochromonas danica* exhibited reductions in chl-*a* content during mixotrophic growth that were accompanied by modifications to the photosynthetic apparatus (higher PS1:PSII) and declines in cellular Rubisco (Wilken *et al.*, 2014b). At the phototrophic end of the mixotrophic spectrum, *Dinobryon cylindricum* ceased growth under low light conditions despite a high density of bacteria, but did not survive in axenic culture conditions even with a favorable light regime (Caron *et al.*, 1993a). Regardless, alternative methods of carbon and energy uptake may allow protists to thrive and outcompete trophic specialists in environments that are otherwise inhospitable (Rothhaupt, 1996b).

### **1.3 Field studies of bacterivory by mixotrophic protists in freshwater lakes**

Lakes are ecologically and economically important as a source of biodiversity and array of ecosystem services, including the supply of a majority of Earth's fresh water (Williamson *et al.*, 2009). Mixotrophic algae can be numerically dominant and contribute significantly to bacterivory in freshwater ecosystems amongst heterotrophic flagellates, rotifers, ciliates and cladocerans (Sanders *et al.*, 1989a, Carrias *et al.*, 1996b). Grazing impact by mixotrophic flagellates have been reported to exceed that of heterotrophs, particularly in oligotrophic lakes where grazing by phagotrophic phytoflagellates may be enhanced by nutrient-limitation (Unrein *et al.*, 2007, Domaizon *et al.*, 2003b, Isaksson *et*

*al.*, 1999a). For example, pigmented flagellates accounted for 70% of the total bacterivorous community grazing impact in the epilimnion of Lake Alatsee (Germany), an alpine lake that receives little allochthonous input (Oikonomou *et al.*, 2014). Seasonal peaks in phagotrophic phytoflagellates have been observed throughout the year, most notably in winter (Berninger *et al.*, 1992a, Palsson & Graneli, 2003, Sanders *et al.*, 1989a), and the ability for primary phototrophs to ingest bacteria is hypothesized to be particularly advantageous in the harsh environment under ice (Roberts & Laybourn-Parry, 1999). For example, bacterivorous protists composed 32% of the phytoplankton biomass in oligotrophic Lake Skärnen during February (Palsson & Graneli, 2003).

Dimictic lakes that exhibit seasonal patterns of thermal stratification provide valuable arenas to study the influence of abiotic factors on bacterivory by mixotrophic protists. During summer stratification, solar heating of surface waters creates a warm, well-lit epilimnion that can often be nutrient-depleted due to compartmentalization into biomass and suppression of upward nutrient flux. In the absence of wind-driven mixing, many lakes exhibit a hypoxic hypolimnion. Habitat heterogeneity created by thermal stratification is known to play a key role in the distribution of phytoplankton by creation of vertical environmental gradients (Longhi & Beisner, 2009, Oikonomou *et al.*, 2014). Motile protists, including nanoflagellates, are able to select favorable conditions within the water column, which may confer a competitive advantage in heterogeneous environments (Jones, 1988b). The breakdown of thermal stratification in the fall weakens habitat heterogeneity and stability, as water column mixing restores dissolved nutrients previously restricted at depth to the surface (Wetzel, 2001b).

The alternation between stratification and mixis should also create temporal patterns in plankton abundance that may differ between phototrophs, heterotrophs and mixotrophs. Relationships between stratification patterns and phytoplankton distribution have been demonstrated in field and laboratory studies, but often fail to include mixotrophic organisms that possess different physiological traits. Although it is becoming generally accepted that mixotrophic organisms can contribute significantly to the biomass of freshwater ecosystems, quantitative data of grazing impact (in comparison to pure heterotrophs) across stratification gradients and season are lacking. Despite an abundance of field studies that describe dynamics of mixotrophic protists, few have assimilated the importance of mixotrophy across both season and depth. Even fewer have considered the role of the “mixotrophic gradient” in field examinations of spatial patterns in plankton distribution. The objective of this dissertation is to meld observations of mixotrophic behavior collected from work in culture to those in freshwater lakes in an effort to better describe mixotrophic plankton dynamics. Work such as this is vital to the inclusion of mixotrophic protists to models of food-web structure and carbon cycling in aquatic ecosystems (Mitra et al., 2016).

### *1.3.1 Lake Lacawac*

The seasonal study of plankton dynamics and diversity described here was carried out in Lake Lacawac (41°22.912'N, 75°17.543'W, 439 m altitude), an oligotrophic lake of glacial origin located in the Pocono Mountains of northeastern Pennsylvania, USA. This 21 ha, dimictic lake has a maximum depth of 13 meters. Lake Lacawac and its watershed are undeveloped and protected by the Lacawac Sanctuary, making it an

invaluable baseline for ecological study. Mixotrophic nanoflagellates have been reported to form up to 48% of the phototrophic population and contribute up to 90% of observed bacterivory in Lake Lacawac under ice during winter (Berninger et al., 1992a).

Lake Lacawac has been previously described as a “Chrysophycean” lake due to the dominance of chrysophyte flagellates (Siver & Chock, 1986a). Mixotrophic members of the Chrysophyceae have been documented to constitute a majority of the planktonic biomass in oligotrophic lakes due in part to their ability to thrive in phosphorus-deplete environments (De Hoyos *et al.*, 1998, Bird & Kalff, 1989a, Bennett *et al.*, 1990b, Domaizon et al., 2003b). In Lac Cromwell (Quebec), grazing by *Dinobryon* during peak abundances removed up to 92% of the bacterial standing stock (Bird & Kalff, 1989a). Mixotrophic grazing in Lake Annecy surpassed bacterial production in spring due to bacterivory by *Dinobryon* (Domaizon *et al.*, 2003b). Chrysophytes are important to the study of mixotrophy because its members exhibit a range of nutritional strategies and varying photosynthetic and phagotrophic abilities (Holen & Boraas, 1995, Rottberger *et al.*, 2013). In fact, one of the earliest observations of mixotrophy by Pascher (1943) was in the colonial Chrysophyte, *Dinobryon*.

## CHAPTER 2

### TEMPERATURE-DEPENDENT PHAGOTROPHY AND PHOTOTROPHY IN A MIXOTROPHIC CHRYSOPHYTE

#### 2.1 Abstract

The roles of temperature and light on grazing and photosynthesis were examined for *Dinobryon sociale*, a common freshwater mixotrophic alga. Photosynthetic rate was determined for *D. sociale* adapted to temperatures of 8, 12, 16 and 20 °C under PAR light irradiances of 25, 66 and 130  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , with concurrent measurement of bacterial ingestion at all temperatures under medium and high light (66 and 130  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Rates of ingestion and photosynthesis increased with temperature to a maximum at 16°C under the two higher light regimes, and declined at 20 °C. Although both light and temperature had a marked effect on photosynthesis, there was no significant difference in bacterivory at medium and high irradiances at any given temperature. At the lowest light condition (25  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), photosynthesis remained low and relatively stable at all temperatures. *D. sociale* acquired the majority of carbon from photosynthesis, although the low photosynthetic rate without a concurrent decline in feeding rate at 8 °C suggested 20-30% of the carbon budget could be attributed to bacterivory at low temperatures. Grazing experiments in nutrient-modified media revealed that this mixotroph had increased ingestion rates when either dissolved nitrogen or phosphorus was decreased. This work increases our understanding of environmental effects on mixotrophic nutrition. Although the influence of abiotic factors on phagotrophy and phototrophy in pure heterotrophs and phototrophs has been well studied, much less is known for mixotrophic organisms.

## 2.2 Introduction

Temperature is known to set limits to protistan metabolic processes including growth, photosynthesis and heterotrophic ingestion (Davison, 1991, Raven & Geider, 1988, Rose & Caron, 2007, Staehr & Sand-Jensen, 2006). The responses of these processes to increasing temperature have been well characterized for many protists and can often be represented by a bell-shaped curve (Eppley, 1972, Falkowski & Raven, 2007, Sherr *et al.*, 1988). What has not been well documented, however, is how combinations of environmental factors, including temperature, influence mixotrophic organisms that combine nutritional modes – phototrophy and phagotrophic ingestion of particles. A recent study investigating how the metabolic theory of ecology applied to these organisms suggested that mixotrophs may become more reliant on heterotrophic processes as temperature increases, thereby shifting the balance of their ecological roles from primary producers to consumers (Wilken *et al.*, 2013a). However, only a single species, *Ochromonas*, was tested and phagotrophy dominates its growth under all conditions. Mixotrophic nutrition has been identified in a diverse range of taxa (Sanders & Porter, 1988), so one expectation is that temperature may differentially affect the balance of nutrition in some of these species.

Species of the genus *Dinobryon* are a common component of freshwater phytoplankton communities and appear to be obligate phototrophs that are still capable of substantial rates of community bacterivory – often greater than that of co-occurring heterotrophic flagellates (Bird & Kalff, 1986a, Kamjunke *et al.*, 2007a, Sanders & Porter, 1988). Phagotrophic phytoplankton, including *Dinobryon* spp., have been reported to derive benefits in carbon and nutrient acquisition via bacterivory in suboptimal light or

nutrient poor environments (Caron *et al.*, 1993b, Jones & Rees, 1994b, Nygaard & Tobiesen, 1993b), which suggests that algal phagotrophy can be regulated by light , inorganic nutrient concentration or prey density (Granéli *et al.*, 1999).

The major objective of this study was to examine the combined effects of temperature and light on feeding and photosynthesis in a species of *Dinobryon*. We determined rates of bacterial ingestion and photosynthesis (via  $^{14}\text{C}$  fixation) under experimental treatments with temperature and light levels that this genus is likely to experience in nature. Photosynthesis vs. irradiance (PE) curves at different temperatures guided the choice of light levels utilized in other experiments. In order to examine the potentially changing contributions of phototrophy and heterotrophy, we converted the data from bacterial ingestion and carbon fixation into percent carbon contributed to the overall nutritional budget. Additional experiments focused on potential factors regulating phagotrophy in *D. sociale*, including exposure to continuous darkness, varying dissolved macronutrient concentrations, prey abundance and prey/tracer type.

## **2.3 Methods**

### *2.3.1 Culture Conditions and General Experimental Design*

Non-axenic cultures of *Dinobryon* sp. were obtained from the University of Texas at Austin culture collection (UTEX strain no. 2267). Though no longer available through UTEX, this strain is also held in the Canadian Phycological Culture Center (formerly University of Toronto Culture Collection) and was sequenced by R. A. Andersen as *D. sociale* (CPCC no. 392, GenBank EF165158.1). Cells were maintained at 12 °C under a 14:10 light:dark cycle in diluted (50%) DY-IV medium (Sanders *et al.*, 2001a). Light was

provided by fluorescent lamps at PAR irradiances between 60  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . All feeding and photosynthesis experiments were performed in either a walk-in cold room (experimental temperatures  $\geq 12$  °C) or temperature-controlled incubator (Thermo Scientific Precision Model 818). Cultures were never shifted between temperatures that differed by more than 4 °C and were allowed to acclimate to the experimental temperatures for a minimum of five days prior to starting experiments. Photosynthetically active radiation (PAR) was measured with a Biospherical Instruments Irradiance Meter, model QSL-100.

### 2.3.2 *Photosynthetic Activity in response to temperature and light*

Photosynthesis was measured at four temperatures and three irradiances by the uptake of  $^{14}\text{C}$  according to the method by MacIntyre *et al.* (1996) modified by the addition of centrifugation techniques of Smith and Azam (Smith & Azam, 1992). Five replicate flasks of *D. sociale* were incubated at “high” (130  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), “medium” (66  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and “low” (25  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) irradiances at four temperature regimes: 8, 12, 16 and 20 °C. Photosynthesis vs irradiance (PE) response curves were determined at 12, 16 and 20 °C at PAR irradiances ranging from 8  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  to 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Radiac Tungsten Halogen Lamp, 120V/300W). Estimates of photosynthetic rate were determined by incubating five replicate 1.5-mL aliquots of temperature-acclimated culture with sodium bicarbonate ( $\text{NaH}^{14}\text{CO}_3$ ) at a final specific activity of 0.5  $\mu\text{Ci}$  per sample for 2 hours. Immediately following addition of radioactive isotope, background samples were taken to quantify the total amount of  $^{14}\text{C}$  available for incorporation. Dark controls were included to correct for

incorporation not due to photosynthesis. After incubation, samples were centrifuged at 14,000 g for 15 minutes and the supernatant was removed by aspiration. The resulting algal pellet was resuspended in distilled water and fumed with 6 M HCl overnight to remove unassimilated  $^{14}\text{C}$ . Samples were then neutralized with 6 M NaOH and added to scintillation fluid and radioactivity of samples was measured using a scintillation counter (Beckman LS-3801). Average counts per minute were converted to disintegrations per minute using a quench correction curve. Dissolved inorganic carbon (DIC) of DY-IV media was calculated using the program CO2SYS in Excel available at <http://cdiac.ornl.gov/ftp/co2sys/> and primary productivity was normalized to cell abundance. Rates of carbon fixation were determined by the equation:

$$\frac{C_{\text{fixed}}}{\text{h}} = \frac{\text{DIC}_{\text{sample}} \times \text{DPM}_{^{14}\text{C}_{\text{Sample}}}}{\text{DPM}_{^{14}\text{C}_{\text{Total}}}} \div \text{incubation time (h)}$$

where,

$C_{\text{fixed}}$  = total carbon fixed by photosynthesis

$\text{DIC}_{\text{sample}}$  = naturally occurring total dissolved carbon available for fixation

in DY-IV media

$\text{DPM } ^{14}\text{C}_{\text{sample}}$  = disintegrations per minute of algae

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

### *2.3.3 Temperature and light effects on bacterial ingestion rate of Dinobryon sociale*

A series of grazing experiments were conducted under the four temperature regimes, but at only the “high” and “medium” PAR irradiances described from the experiments measuring carbon fixation. Fluorescent polycarbonate microspheres (0.55  $\mu\text{m}$  dia., Fluoresbrite, Polysciences, Inc.) were added as tracers of bacterial ingestion to each culture flask at a final concentration of  $\sim 5 \times 10^5$  microspheres  $\text{mL}^{-1}$ . In order to account for background coincidence, subsamples were taken immediately following tracer addition ( $T_0$ ). Additional subsamples were taken after 30 minutes. Previous experiments determined that the uptake of tracer particles was linear during this time period (DeVaul Princiotta, unpublished data, see also Kamjunke et al., 2007a). Samples were fixed using a Lugol's/Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>/Formalin method (Sherr & Sherr, 1993) that prevents egestion of particles.

A second grazing series was performed to analyze the influence of continuous darkness on ingestion rate. Replicate cultures were incubated at 16 °C under a 14:10 light cycle of 130  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  for 1 week, after which the cultures were covered in aluminum foil and fluorescent tracers were added to subsamples after 0.5, 6, 12, 24, 48 and 72 hours of darkness (final concentration  $\sim 5 \times 10^5$  microspheres  $\text{ml}^{-1}$ ). Feeding incubations were performed in the dark as previously described, and samples for cell abundance also were taken at each time point. Concurrently with the first dark incubation (0.5 h), grazing rate was determined in subsamples that had remained in the light.

#### 2.3.4 Temperature and Functional Response to Food Concentration

Additional grazing experiments were performed in order to analyze the functional feeding response of *D. sociale* under 3 temperature regimes: 6, 10 and 14 °C. All experiments in this series were performed under constant illumination of 33  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . Fluorescently labeled tracers were added as food proxies at 6 final concentrations ranging from  $5 \times 10^5$  to  $5 \times 10^7$  microspheres  $\text{mL}^{-1}$  and feeding rates were determined as described above in three replicates at each combination of microsphere concentration and temperature. Particle concentrations were chosen based on the range of natural bacterial abundances in aquatic ecosystems.

#### 2.3.5 Role of bacterivory in nutrient acquisition

In order to further elucidate the effect of nitrogen and phosphorus on bacterivory in *D. sociale*, a feeding experiment was conducted with 4 modifications of DY-IV media: a nutrient replete medium (50% DY-IV, modified from Sanders et al. 2001) with a molar ratio of nitrogen to phosphorus (N:P) of approximately 16:1; a decreased nitrogen medium (DNM) with a molar N:P of 5:1; a decreased phosphorus medium (DPM) with a molar N:P of 20:1 and a dilute nutrient- medium (DNPM) with concurrent decrease of nitrogen and phosphorus at a molar N:P of 16:1. Nutrient ratios were chosen according to the general trophic classification of lakes in relation to phosphorus and nitrogen content (Wetzel, 2001a). The DY-IV media described in Sanders et al. (2001) includes the organic buffer 2-[N-morpholino] ethanesulfonic acid (MES), which contributes considerable nitrogen and stimulated growth, even in the dark, of axenic cultures of another mixotroph, *Ochromonas* sp. (Sanders et al., 2001a). Consequently, we excluded

the buffer and adjusted the N:P ratios by adding different amounts of stock solutions of  $\text{NH}_4\text{NO}_3$ ,  $\text{NaNO}_3$  and  $\text{Na}_2\text{HPO}_4$ . The pH was adjusted to that of the original DY-IV and did not vary significantly during the experiment. To achieve an approximate Redfield ratio of N:P in the 50% DY-IV, the  $\text{Na}_2\text{HPO}_4$  was decreased to  $3.275 \text{ mg L}^{-1}$ , and  $\text{NH}_4\text{NO}_3$  and  $\text{NaNO}_3$  decreased to  $7.5$  and  $15 \text{ mg L}^{-1}$ , respectively giving an N:P molar ratio of 15.74:1. For the DNM, nitrogen, but not phosphorus was decreased from this 50% "Redfield formulation." Conversely phosphorus, but not nitrogen was decreased for the DPM. Finally for the DNPM, N and P were decreased, to  $2.55 \text{ mg total N L}^{-1}$  ( $182 \mu\text{M}$ ) and  $0.356 \text{ mg total P L}^{-1}$  ( $11.5 \mu\text{M}$ ). Other components of the media were maintained at the concentrations found in DY-IV. Four replicates for each treatment were incubated at  $16 \text{ }^\circ\text{C}$  under continuous illumination of  $66 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  ("medium") for 4 days of acclimation. Feeding rates were then determined using fluorescently labeled tracers as previously described.

### 2.3.6 Feeding Selectivity

A final grazing experiment was conducted in order to evaluate potential feeding selectivity by *D. sociale* between fluorescently labeled bacteria and fluorescent microspheres. For each treatment, four replicates were incubated at  $16 \text{ }^\circ\text{C}$  under continuous illumination of  $66 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  ("medium"). Grazing experiments were conducted as previously described with fluorescently labeled microspheres alone ( $0.6 \mu\text{m}$  diameter), heat-killed DTAF-labeled bacteria alone, and a mixture of equal amounts of both tracers. The concentration of total tracer particles in the experiments was approximately  $10^5 \text{ particles ml}^{-1}$ . DTAF-labeled bacteria of a comparable size to the

fluorescently labeled particles (0.5 – 0.7  $\mu\text{m}$ ) were prepared according to Sherr et al. (1987) and had a fluorescent signal distinct from the microspheres.

### *2.3.7 Microscopic Enumeration*

Abundances of bacteria and microspheres were determined by epifluorescence on a Zeiss Axiovert microscope at 1000x magnification. Subsamples were collected onto 25 mm black polycarbonate filters (GE Water and Process Technologies) with a pore size of 0.2  $\mu\text{m}$  and mounted onto slides with DAPI medium (Vector Laboratories, Inc.).

Ingestion of microspheres by *D. sociale* was determined from subsamples using counting chambers (PhycoTech, Inc.). Ingestion was based on recognition of tracer particle(s) located within the boundary of the cell with background (T0) subtracted. Background was always very low. Ingestion of bacteria was calculated by multiplying the microsphere ingestion rate by the ratio of bacterial abundance to microsphere abundance.

### *2.3.8 Statistical Analyses and Curve Fitting*

All statistical analyses were performed using the JMP® Pro 10 software package and R statistical software program. Differences in photosynthetic rate, ingestion rate, total carbon acquired and percent carbon acquired by photosynthesis or bacterivory across all treatments were determined using two-way ANOVA followed by pair-wise comparisons analyzed using a Tukey HSD test. Values for carbon fixation and total carbon acquired were log transformed prior to statistical analysis; percent carbon data were arcsine transformed. The effects of particle concentration and temperature on ingestion rate also were tested with two-way ANOVA and subsequent Tukey HSD test. Differences in

ingestion rate between modifications of nutrient media and over time in continuous darkness were analyzed with a one-way ANOVA.

Carbon incorporation from bacterivory for the first grazing series was calculated using the product of the average carbon content of a single bacterium (20 fg C bacteria<sup>-1</sup>, Lee & Fuhrman, 1987), the rate of bacterial ingestion (bacteria cell<sup>-1</sup> h<sup>-1</sup>) and the gross growth efficiency of *D. sociale* (54%, Bird & Kalff, 1989b). Because bacterial ingestion rate was not determined under the “low” irradiance (25 μmol photons m<sup>-2</sup> s<sup>-1</sup>) in our experiments, bacterivory rates determined by Heinze et al. (2013) for that light level were used in calculations (the strain was identified as *Dinobryon* sp. in that report). Using these assumptions, carbon incorporation by bacterivory was converted into percent of total carbon acquisition including that from photosynthesis.

PE curves were fit to the non-linear least-square regression model by Eilers-Peeters with the R statistical software program. This light-limitation model expresses photosynthesis as a function of maximum photosynthetic rate and light limitation at a given irradiance (Eilers & Peeters, 1988) where,

Photosynthesis = P<sub>max</sub> x Light Limitation, and

$$\text{Light Limitation} = \frac{2 \times (1 + \beta) \times \frac{I}{I_{\text{opt}}}}{2 \times \left(\frac{I}{I_{\text{opt}}}\right) + 2 \times \beta \times \frac{I}{I_{\text{opt}}} + 1}$$

The following parameters were estimated from this model:

I<sub>opt</sub> = Irradiance at optimal photosynthetic rate

P<sub>max</sub> = Maximum photosynthetic rate

β = dimensionless parameter that estimates photoinhibition

Non-linear regression analysis of functional response feeding data was fit to the Holling's Type III equation (Holling, 1959) that describes feeding rate ( $\omega$ ) as a function of prey density. This creates a sigmoidal response of predator-prey dynamics where consumption increases at intermediate prey densities, specifically,

$$f(\omega) = \frac{\gamma N^2}{\chi^2 + N^2} \text{ where,}$$

$\gamma$  = Maximum feeding rate

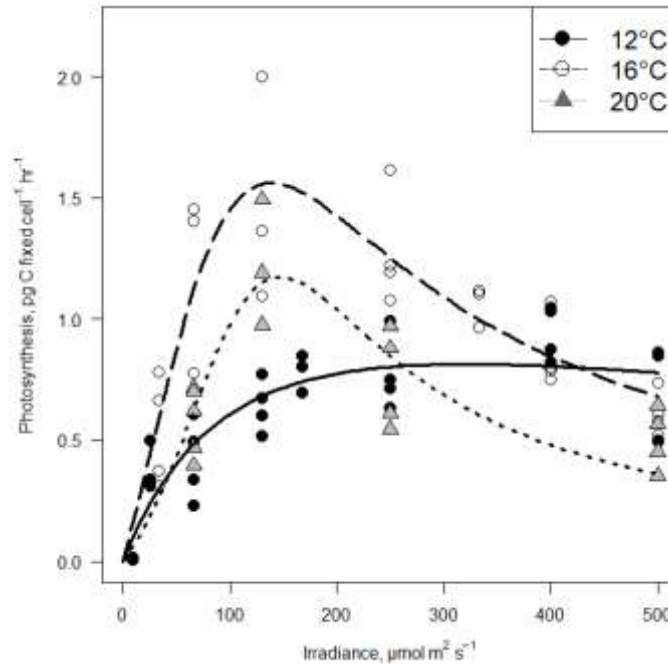
$N$  = prey abundance

$\chi$  = Prey density at half –maximum feeding rate ( $\gamma/2$ )

## 2.4 Results

### *Photosynthesis*

Data from the PE curves indicated that *D. sociale* photosynthetic rates varied significantly with irradiance and temperature (Fig. 2.1, ANOVA,  $p=0.0002$ ). The maximum photosynthetic rate ( $P_{\max}$ ) for cultures adapted to 16 °C was significantly greater than cultures kept at either 12 °C or 20 °C (Tukey HSD,  $p=0.0002$  and 0.0317 respectively). At 16 °C the  $P_{\max}$  was 1.56 pg C fixed cell<sup>-1</sup> h<sup>-1</sup> with an optimal irradiance ( $I_{\text{opt}}$ ) of 138  $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ , while at 12 °C, the maximum photosynthetic rate was only 0.81 pg C fixed cell<sup>-1</sup> h<sup>-1</sup> at a much higher optimal irradiance of 322  $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$  (Table 2.1). The apparent photoinhibition at higher light intensities (Fig. 2.1) was not statistically significant except at 20 °C, which had an intermediate maximum photosynthetic rate of 1.17 pg C fixed cell<sup>-1</sup> h<sup>-1</sup>, with an optimal irradiance of 147  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (Table 2.1).



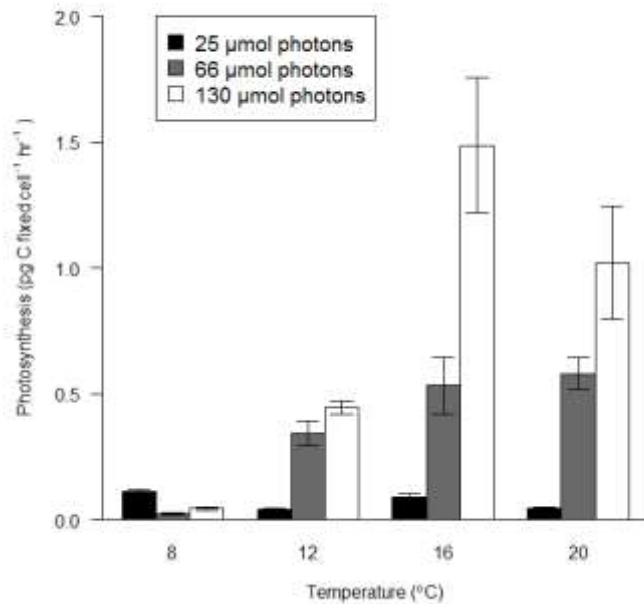
**Figure 2.1:** PE curves for *Dinobryon sociale* adapted to 12 °C (solid line, closed circles), 16 °C (dashed line, open circles), and 20 °C (dotted line, grey triangles)

In the experiments testing temperature and light effects on concurrent grazing and photosynthesis, the photosynthetic rates of *D. sociale* followed the same trend noted in the P vs. I determinations with significant light (ANOVA,  $p < 0.0001$ ), temperature (ANOVA,  $p < 0.0001$ ), and interactive effects (ANOVA,  $p < 0.0001$ ). Under high ( $130 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and medium ( $66 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) light conditions, photosynthesis increased at each temperature level between 8 °C to 16 °C (Fig. 2.2), but were not significantly different at 16 °C compared to 20 °C (Tukey HSD,  $p > 0.96$ ). In contrast to the other light conditions, maximum photosynthetic rate for *D. sociale* in low light occurred at 8 °C and remained low as temperature increased. Changes in

photosynthetic rates with increasing irradiance were small and not significant at reduced temperatures (Fig. 2.2).

**Table 2.1:** Parameters from temperature treatments of *Dinobryon sociale* photosynthesis vs irradiance curves fit to the Eilers-Peeters model by nonlinear regression.  $P_{\max}$  represents the maximum photosynthetic rate at the optimal irradiance ( $I_{\text{opt}}$ ).  $\beta$  is a dimensionless term that estimates photoinhibition. \*\*\* $P < 0.001$ , \*\* $P < 0.01$ , n.s. = not significant

	12 °C			16 °C			20 °C		
	Estimate	Std Error	Pr (>  t )	Estimate	Std Error	Pr (>  t )	Estimate	Std Error	Pr (>  t )
$P_{\max}$	0.81	0.04	***	1.56	0.117	***	1.17	0.116	***
$I_{\text{opt}}$	322	91.0	**	138	9.68	***	147	10.9	***
$\beta$	1.27	1.23	n.s.	-0.236	0.177	n.s.	-0.621	0.121	***

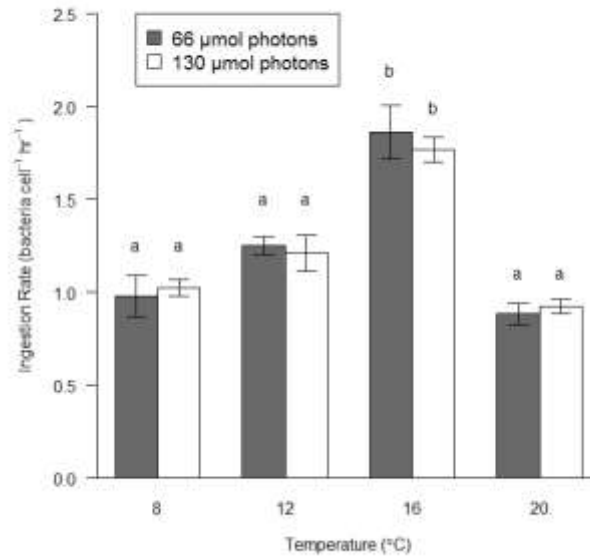


**Figure 2.2:** Rates of photosynthesis (carbon fixation) by *Dinobryon sociale* adapted to different temperatures and irradiances. Black, gray and white bars indicate experiments under “low” ( $25 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ), “medium” ( $66 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and “high” ( $130 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) PAR irradiances, respectively.

#### *Bacterivory by Dinobryon sociale in temperature and light treatments*

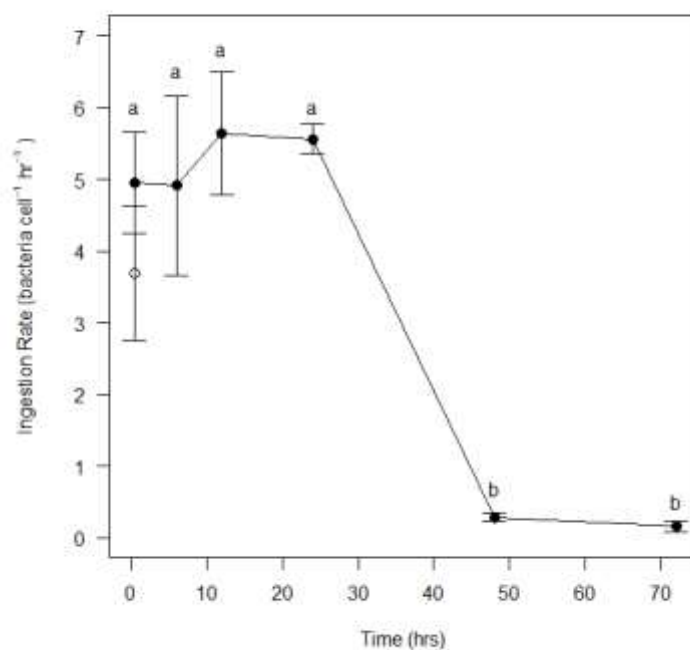
There was a significant effect of temperature (ANOVA,  $p < 0.0001$ ) on ingestion rate by *D. sociale*, but there was no effect of light for the light levels utilized in this experiment (ANOVA,  $p = 0.8311$ ). Under both light regimes, maximum ingestion rate occurred in populations adapted to  $16 \text{ }^{\circ}\text{C}$  (Fig. 2.3). Ingestion was 48% and 52% lower at

20 °C for high and medium light, respectively. Bacterial ingestion rates ranged from 0.88 bacteria cell<sup>-1</sup> h<sup>-1</sup> to 1.86 bacteria cell<sup>-1</sup> h<sup>-1</sup>.



**Figure 2.3:** Rates of bacterivory by *Dinobryon sociale* adapted to different temperatures and light regimes. Gray and white bars indicate experiments conducted under either “medium” (66 μmol photons m<sup>-2</sup> s<sup>-1</sup>) or “high” (130 μmol photons m<sup>-2</sup> s<sup>-1</sup>) irradiance.

When cultures were moved to total darkness, the amount of time without light had a significant effect on bacterivory (ANOVA,  $p < 0.001$ ). Ingestion rate in the dark at the 0.5 h time point was not significantly different from that for the remaining in the light ( $p = 0.351$ ). There was no change in the feeding rate during the first 24 h in darkness; however, ingestion was nearly undetectable by the 48 h in continuous darkness ingestion (Fig. 2.4). The abundance of *D. sociale* had declined by 43% after 72 hours in darkness (data not shown).



**Figure 2.4:** Bacterial ingestion rate over a period of 72 hours in continuous darkness (mean  $\pm$ SE). Open circle indicates feeding rate after 0.5 hours exposure to “high” irradiance ( $130 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ).

#### *Carbon acquisition by photosynthesis and bacterivory*

*D. sociale* acquired a maximum of  $1.49 \text{ pg C cell}^{-1} \text{ h}^{-1}$  by photosynthesis (Table 2.2), compared to ingestion of  $0.02 \text{ pg C cell}^{-1} \text{ h}^{-1}$  from grazing as determined concurrently in the medium and high light treatments and assuming a gross growth efficiency of 54%. There was a significant effect of temperature and light, as well as an interactive effect, on summed carbon acquired from grazing and bacterivory (ANOVA,  $p < 0.0001$ ), but *D. sociale* was primarily phototrophic in all treatments. With the

exception of the 8 °C treatment, the percentage of carbon acquired via bacterivory was appreciably less than that fixed photosynthetically (Table 2.2). The proportion of carbon acquired by grazing was significantly affected by temperature ( $p < 0.001$ ), but not light. The moderate reduction in grazing at the lowest temperature (8 °C) compared to the proportionally larger effect of the lower temperature on photosynthetic rates resulted in the much larger contribution of heterotrophic nutrition at that temperature (Table 2.2).

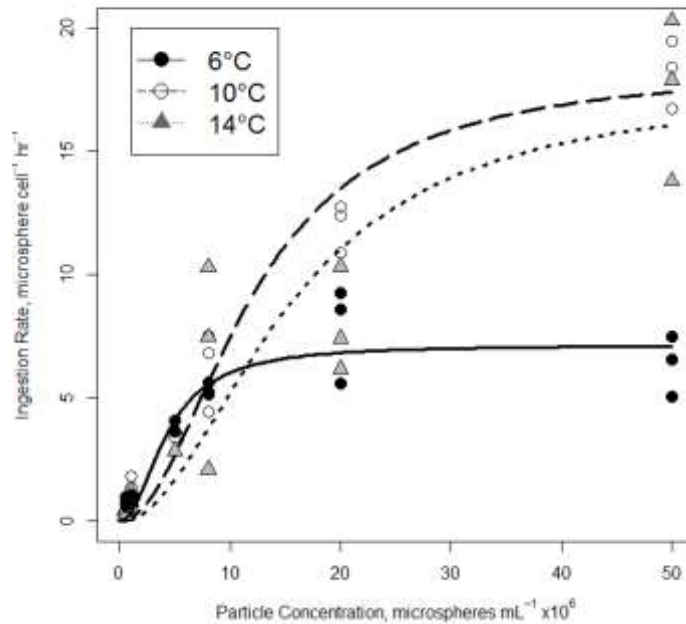
**Table 2.2:** Percent carbon acquired from grazing by *Dinobryon sociale* (mean  $\pm$ SE) under varying light and temperature conditions. Mean ( $\pm$  SE).

Temp (°C)	High	Medium
	130 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$	66 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$
8	21.2 (3.17)	30.1 (2.02)
12	2.9 (0.26)	4.0 (0.40)
16	1.4 (0.21)	4.1 (0.78)
20	1.2 (0.31)	1.7 (0.28)

*Feeding responses to particle abundance, prey type and dissolved nutrient concentration*

Change in ingestion rate based on food particle abundance (functional response) of *D. sociale* fit the Holling Type III function at all three temperatures examined (Fig. 2.5). The maximum ingestion rates at 6, 10 and 14 °C were 7.2, 18.4 and 17.6 microspheres  $\text{cell}^{-1} \text{ h}^{-1}$ , respectively (Table 2.3). At the highest concentration of tracer

particles tested ( $5 \times 10^7$  microspheres  $\text{ml}^{-1}$ ), the ingestion rate at 6 °C was significantly lower than that at 10 °C and 14 °C. There was a significant effect of temperature (ANOVA,  $p=0.0068$ ), particle concentration (ANOVA,  $p<0.0001$ ) and interactive effect of the variables (ANOVA,  $p=0.0035$ ) on particle ingestion rate.



**Figure 2.5:** Functional response curves for *Dinobryon sociale* across a range of concentrations of bacterial surrogates ( $0.6 \mu\text{m}$  microspheres) when adapted to 6 °C (solid line, closed circles), 10 °C (dashed line, open circles) and 14 °C (dotted line, grey triangles). Curves were fit to Hollings Type 3 by nonlinear regression.

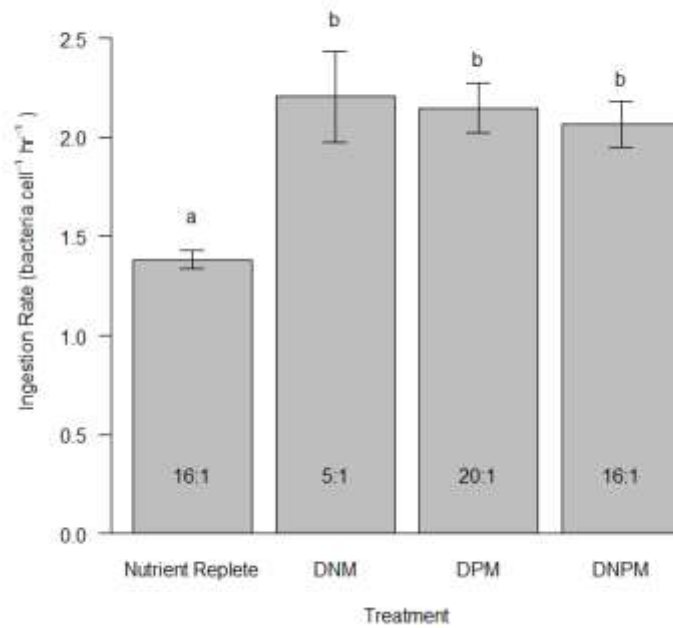
**Table 2.3:** Parameters from temperature treatments for functional response curves fit to Hollings Type 3. Maximum feeding rate is represented by  $\gamma$  and the density of food particles at  $\gamma/2$  (half-maximum) is represented by  $\chi$ . \*\*\* P<0.001

	6 °C			10 °C			14 °C		
	Estimate	Std Error	Pr (>  t  )	Estimate	Std Error	Pr (>  t  )	Estimate	Std Error	Pr (>  t  )
$\gamma$	7.2	0.50	***	18.4	0.88	***	17.6	2.2	***
$\chi$	4.3 x10 <sup>6</sup>	0.9 x10 <sup>6</sup>	***	12.1 x10 <sup>6</sup>	1.1 x10 <sup>6</sup>	***	15.4 x10 <sup>6</sup>	3.5 x10 <sup>6</sup>	***

*D. sociale* ingested both offered tracer particles in the feeding selectivity experiments. There was no difference in feeding rates when fluorescently labeled bacteria (FLB) or microspheres were offered separately at the same concentrations (ANOVA, p=0.22). In the experiment where FLB and microspheres were offered together at equal concentrations, Jacobs Selectivity Index (Jacobs, 1974) was -0.03, indicating nonselective feeding amongst the two types of tracer particles.

When grown in modifications of 50% DY-IV media, ingestion rate by *D. sociale* was significantly greater under conditions where nutrient concentrations were reduced (ANOVA, p=0.01). In comparison to nutrient replete media, ingestion rates increased by similar amounts in both modifications of the media in which nitrogen was decreased (DNM, N:P of 5:1) or phosphorus was decreased (DPM, N:P of 20:1). Decreased concentrations of both nitrogen and phosphorus, but maintenance of the Redfield N:P ratio of DY-IV (DNPM, 16:1), resulted in an increased grazing rate that was similar to

the treatments with only N or P decreased (Fig. 2.6). There were no significant differences in grazing rates between any of the media preparations in which one or both macronutrients were decreased.



**Figure 2.6:** Rates of bacterivory by *Dinobryon sociale* incubated under different dissolved nutrient conditions and constant illumination ( $66 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at  $16^\circ\text{C}$  (DNM = decreased nitrogen media, DPM = decreased phosphorus media, DNPM = dilute-nutrient media with a concurrent decrease in nitrogen and phosphorus)

## 2.5 Discussion

The effect of temperature on photosynthetic and heterotrophic processes can vary and bacterivorous protists, in particular, may have much greater growth rates at higher temperatures than phototrophs (Rose & Caron, 2007). The effect of temperature on the balance of phototrophic and heterotrophic processes in mixotrophs is less clear.

Mixotrophic phytoflagellates from various taxa represent a range of nutritional strategies from primarily phototrophic to primarily heterotrophic (Jones, 1997). Consequently, the balance of these metabolisms is likely to vary depending on the importance of phagotrophy to the taxa, i.e., the position along the continuum of phototrophic to phagotrophic nutritional abilities. Studies of mixotrophy in several *Dinobryon* species suggest that it is an obligate phototroph with substantial phagotrophic abilities (Caron et al., 1993b, Rottberger et al., 2013). *Dinobryon sociale* examined in our study certainly fits the pattern of photosynthesis dominating its nutrition, and our results demonstrate that temperature and light influence both bacterivory and primary productivity, albeit in different ways.

The rates of  $^{14}\text{C}$  fixation were generally related to irradiance, as expected for a primarily photosynthetic organism, but temperature altered photosynthesis as well (Fig. 1, 2). The PE relationships indicated that temperature influenced not only the maximum photosynthetic rate ( $P_{\text{max}}$ ), but also the irradiance at which primary productivity reached that maximum. Although photosynthetic rates dropped substantially under higher light conditions in the 16 and 20°C treatments (Fig. 1), photoinhibition was only significant at 20°C (Table 1).

In the experiments examining concurrent bacterivory and photosynthesis, carbon fixation by *D. sociale* increased with irradiance up to a maximum at the highest light level ( $130 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) for all temperatures above  $8 \text{ }^{\circ}\text{C}$  (Fig. 2). Light had less effect on photosynthesis at  $8 \text{ }^{\circ}\text{C}$  where rates were low and less variable with changing irradiance (Fig. 2). Temperature dependence of photosynthesis is known to interact with light conditions (Falkowski and Raven, 1997), and this interaction is ecologically important in determining the distribution of planktonic organisms in nature (López-Urrutia *et al.*, 2006). Under light-saturated conditions, photosynthetic rates are generally positively related to temperature, however photosynthesis in light-limited conditions is less temperature sensitive (Davison, 1991). Our experiments with *D. sociale* demonstrate that these predictions can also hold true for a phagotrophic alga. Under “high” light conditions, photosynthesis by *D. sociale* increased with increasing temperature between  $8^{\circ}\text{C}$  and  $16 \text{ }^{\circ}\text{C}$ , where maximum photosynthetic rate occurred. A similar trend occurred under “medium” light conditions (Fig. 2), however, in the “low” light conditions there were not extensive differences in photosynthesis across the temperature treatments, supporting a reduced effect of temperature on light-limited photosynthesis (see Fig. 2,  $25 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ). The overall low photosynthetic rate at  $8 \text{ }^{\circ}\text{C}$ , irrespective of light conditions, may signify light saturation at a lower irradiance or a reduced ability to use light at reduced temperatures (Davison 1991).

As with photosynthetic rates, *D. sociale* ingestion rates were maximal at  $16 \text{ }^{\circ}\text{C}$  (Fig. 3). Greater rates of bacterivory with increasing temperature have been noted previously in both field and laboratory studies of heterotrophic flagellates (Marrasé *et al.*, 1992, Rose & Caron, 2007, Vaqué *et al.*, 1994, Vázquez-Domínguez *et al.*, 2012). There

are fewer data on the effect of temperature on feeding by mixotrophs, though several species of *Dinobryon* have been investigated. Bird and Kalff (1987) found that bacterivory rates averaged for four species of *Dinobryon* in three lakes in Quebec Province, Canada were positively related to temperature between approximately 6 and 20 °C, but were not correlated to light levels. Jones and Rees (1994b) found no relationship between light intensity and clearance rates for *D. divergens*, but there was a strong positive relationship between clearance rates and temperatures between 5 to 20 °C in *D. sertularia* adapted to temperature for only 10 minutes. In the current experiments, adaptation to different light levels did not influence ingestion rate (Fig. 3). However, Heinze et al. (2013) found that the same isolate of *D. sociale* ingested bacteria at a greater rate when adapted to 25  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  than at 67  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Given the interactive effects of temperature and light on photosynthesis in *D. sociale*, and the low photosynthetic rates at 25  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Fig. 2), the “medium” light treatment (66  $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ ) may be close to a threshold below which photosynthesis alone is inadequate for growth. The increased ingestion of bacteria may become a more important carbon source at lower light levels in *D. sociale*.

In contrast to the above findings, laboratory experiments by Caron *et al.* (1993b) found that increased light led to higher feeding rates on fluorescently-labeled bacteria by *D. cylindricum*. Those experiments were conducted at 20 °C, a temperature that led to reduced growth and feeding in the *D. sociale* strain utilized in this work (Fig. 3, Heinze et al., 2013). *Dinobryon* spp. are typically not abundant at temperatures <6°C and >20°C (Heinze et al. 2013), and it is at those extremes that the interaction with light appears to produce different phagotrophic responses in members of this genus. Interspecific

differences in phototrophy and phagotrophy have been noted for several ochromonads that belong to the same order as *Dinobryon* (class Chrysophyceae, order Chromulinales), though ochromonads are on the phagotrophic end of the mixotrophic continuum (Holen, 1999b, Sanders et al., 2001a, Wilken *et al.*, 2014a, Rothhaupt, 1996a) and *Dinobryon* is primarily photosynthetic.

Other factors besides temperature and light can modify ingestion rates. Food concentration generally has a strong effect on feeding rate in phagotrophic protists (Fenchel, 1987) and this was true for *D. sociale* in our experiments (Fig. 5). Additionally, there was an interaction between the effects of particle concentration and temperature in this mixotroph. Feeding rates at 6 °C increased with increasing microsphere concentration below  $1 \times 10^7$  particles  $\text{ml}^{-1}$  and then remained constant at the maximum rate, while at 10 °C and 14 °C the maximum ingestion rates occurred at the highest particle concentration tested ( $5 \times 10^7$  microspheres  $\text{ml}^{-1}$ ), and at a rate that was approximately 3X that observed at 6 °C (Fig. 5). The maximum ingestion rates at 10 °C and 14 °C were not significantly different from each other in this set of experiments. Previous work by Jones and Rees (1994a) found that particle ingestion rate by *Dinobryon divergens* increased linearly to a maximum at a microsphere concentration of  $10^9 \text{ ml}^{-1}$ . Although a type III functional response curve fit the data significantly for each temperature treatment for *D. sociale* (Fig. 5), a linear regression could also be appropriate for 10 °C and 14 °C, suggesting that prey satiation may not yet have occurred for these temperature treatments. However, abundances greater than  $5 \times 10^7$  bacteria  $\text{ml}^{-1}$  are unlikely to be encountered by members of this genus in nature and are thus not ecologically relevant. Our work with *D. sociale* also supports the lack of selectivity

between two common tracers used to measure bacterivory, microspheres and fluorescently-labeled bacteria, that Jones and Rees (1994a) noted for *D. divergens*. *D. sociale* fed on these particle types at indistinguishable rates in our experiments.

Feeding in *D. sociale* also was affected by the concentration of dissolved nutrients. In comparison to nutrient replete media (50% DY-IV), *D. sociale* exhibited comparable increases in ingestion rate when grown in media with decreases in nitrogen, phosphorus or both macronutrients maintained at the Redfield ratio (Fig. 6). This suggests that *D. sociale* increases bacterivory to substitute organic N and P from bacterial biomass for dissolved forms of the nutrients. Nutrient limitation has been reported to induce or increase the rate of phagotrophy in several other mixotrophs (Johnson, 2015, Nygaard & Tobiesen, 1993b, McKie-Krisberg *et al.*, 2015).

Assuming that *D. sociale* can combine carbon from phototrophy and feeding to produce biomass, the balance between phototrophy and heterotrophy in this species was influenced by both temperature and light conditions. In all cases, phototrophy was the main source of carbon for *D. sociale*, particularly under “high” and “medium” light conditions. This was not surprising given previous reports of the genus’ dependence on photosynthesis for growth (Caron *et al.*, 1993b, Rottberger *et al.*, 2013). We hypothesize that *D. sociale* supplements reduced photosynthetic efficiency with heterotrophic nutrition under low light conditions, as exhibited by the increase in percent carbon potentially acquired by bacterivory at  $25 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Table 2). Although we did not determine ingestion directly at  $25 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  during these experiments, instead using the feeding rates of Heinze *et al.* (2013) for this strain, the feeding rates between the two studies were consistent at other temperature and light levels. The

interaction between temperature and irradiance seem to trigger a shift in trophic mode between 8 °C and 12 °C, where carbon acquisition from photosynthesis declines and that from bacterivory increases. At lower temperatures, it may be that neither photosynthesis nor phagotrophic ingestion of bacteria alone are sufficient to maintain population growth.

If *Dinobryon* shifts to the more heterotrophic end of the nutritional spectrum when photosynthesis drops to low levels at cold temperatures as our data suggest, it is opposite to the response noted for *Ochromonas* sp. (Wilken et al., 2013a). Wilken et al. (2013a) proposed that mixotrophic organisms will become more heterotrophic at increased temperatures. However, the conclusions of Wilken et al. (2013a) were based on studying *Ochromonas* sp., and this genus of mixotrophs is primarily heterotrophic (Sanders et al., 2001a, Wilken et al., 2013a, Rothhaupt, 1996a), while *Dinobryon* species are obligate phototrophs. The increased heterotrophy noted by Wilken et al. (2013a) also occurred at temperatures between 21 and 33 °C, temperatures at which *Dinobryon* tend to be rare in natural systems (Heinze et al., 2013). In a later study, Wilken et al. (2014a) examined phototrophy in an axenic strain of another ochromonad, *O. danica*, and found there was a shift in the function of the photosynthetic apparatus from providing carbon and energy when axenic to providing mainly energy in the presence of bacterial food. The related *Poterioochromonas malhamensis*, also primarily phagotrophic, had a morphological response of reducing the size of its chloroplasts and incorporation of carbon from  $\text{NaH}^{14}\text{CO}_3$  additions when bacterial food was added to axenic cultures, and reverted back to larger chloroplasts when bacteria fell below a threshold feeding level (Sanders et al., 1990a). We did not observe a change in chloroplast size in *D. sociale* in our experiments, which suggests that ochromonads and *Dinobryon* may have very

different physiological and physical responses to utilization of phagotrophy versus photosynthesis.

With the exception of low light conditions, *D. sociale* gained a greater percentage of carbon from photosynthesis than from bacterivory. This raises the question of the importance of bacterivory in the nutrition of *D. sociale*. Since we saw an increase in ingestion when we reduced the concentration of N or P, without altering the availability of other micro- and macro-nutrients, we suggest that, under optimal light conditions, this mixotroph sustains phagotrophic ingestion of bacteria primarily to obtain nitrogen or phosphorus. This is supported by the work of Caron *et al.* (1993b) that indicated *Dinobryon cylindricum* utilized bacterivory to provide major nutrients, although those authors did estimate greater contributions (up to 25%) to the carbon budget from phagotrophy than we observed for *D. sociale*. *Dinobryon* species are a frequently common and abundant component of the plankton in oligotrophic environments where grazing on bacteria can provide additional nutrients. Bacteria act as a potential source of phosphorus and nitrogen because they are more efficient at sequestering nutrients than algae (Bratbak, 1985a, Doddema & van der Veer, 1983), and phagotrophy may confer a potential competitive advantage to mixotrophs that can allow them to dominate phytoplankton communities in oligotrophic waters (Arenovski *et al.*, 1995b, Hartmann *et al.*, 2012a, Kamjunke *et al.*, 2007a).

This study showed that both phototrophy and phagotrophy by *Dinobryon sociale* responded to changing conditions of light and temperature. Freshwater protists, whether phototrophic or heterotrophic, are likely to be influenced by climate change through both direct temperature effects and indirect effects related to changing influxes of dissolved

organic carbon that can lead to a shallower photic zone and nutrient limitation (Thrane *et al.*, 2014, Snucins & Gunn, 2000). For example, Wilken *et al.* (2013a) suggested that some mixotrophs become more heterotrophic at higher temperatures, though we do not observe this in *D. sociale*. Knowledge of the responses of various mixotrophic plankton, especially common species such as *Dinobryon*, to expected global climate change is imperative to building a framework for understanding its future effect on the planktonic food web overall (Mitra *et al.*, 2014).

**CHAPTER 3**  
**HETEROTROPHIC AND MIXOTROPHIC NANOFLAGELLATES IN A**  
**MESOTROPHIC LAKE: ABUNDANCE AND GRAZING IMPACTS ACROSS**  
**SEASON AND DEPTH**

**3.1 Abstract**

Nanoflagellates are recognized as fundamental members of the planktonic microbial food web due to their contribution to photosynthetic fixation of carbon and phagotrophic ingestion of bacteria. Although the presence, and often dominance, of mixotrophic nutrition among phytoflagellates has been well documented within a variety of aquatic ecosystems and in a range of taxa, few studies have assessed the abundance and grazing impact of mixotrophs in comparison to their photosynthetic and heterotrophic counterparts across seasons at multiple depths. Abundance and, as appropriate, bacterivory of phototrophic, heterotrophic and mixotrophic nanoflagellates were quantified at three depths over a 15-month period in Lake Lacawac (Pocono Mountains, Pennsylvania, USA) via microscopic analysis of ingested bacterial surrogates. The absolute and relative abundances of all nanoflagellate trophic groups varied with season and depth, likely as a result of differences in physiological tolerance. Whereas the abundance of phototrophic and heterotrophic nanoflagellates varied with depth in both the presence and absence of thermal stratification, mixotrophic protists were evenly distributed throughout the water column during mixis. The abundances of phototrophic and mixotrophic protists were significantly correlated, but only in surface waters during periods of thermal stratification. Grazing rate and consequent impact by bacterivorous nanoflagellates varied with depth across season, particularly during thermal stratification.

Mixotrophic bacterivory exceeded that of heterotrophs in the epi- and metalimnion during stratification, removing a maximum of 80% of the bacterial standing stock day<sup>-1</sup>. The relative contribution of heterotrophic nanoflagellates to community bacterivory increased with depth, even during mixis, and dominated the grazing impact in the hypolimnion.

### **3.2 Introduction**

One of the central goals of ecological study is to better understand patterns in distribution and abundance. In freshwater communities, predictable seasonal shifts in water column attributes can influence plankton dynamics through a series of “species replacements” (Sommer *et al.*, 1986b). Development of thermal stratification creates distinct vertical horizons in plankton community structure based partly on the association of temperature with physical and physiological processes that underlie protistan metabolism and ultimately distribution (Butterwick *et al.*, 2005, Raven & Geider, 1988, Reynolds *et al.*, 1983). The onset of thermal stratification in temperate lakes during summer acts as a major structuring force for planktonic communities because it affects light regimes, oxygen concentration and nutrient flux throughout the water column (Hampton *et al.*, 2014, Reynolds *et al.*, 1983, Winder & Sommer, 2012).

A vertically heterogeneous pattern in plankton distribution is also determined by the differential abilities of microbial organisms to maintain their position in the water column. Whereas some groups such as diatoms are susceptible to sinking, many nanoflagellates are highly motile and can maintain their position in the water column. The stratification of motile flagellates are often to a species-specific optimal environment

based on light, temperature and/or nutrient availability (Heinze & Sanders, 2009, Ptacnik *et al.*, 2003). For example, several flagellate species from a hyper-eutrophic lake were distributed into discrete vertical niches during thermal stratification by virtue of species-specific behavioral responses (Clegg *et al.*, 2007).

Flagellates in the nanoplankton size range (2-20  $\mu\text{m}$ ) play intermediate roles in the planktonic food web as prey for zooplankton and predators of bacteria (Bennett *et al.*, 1990a, Domaizon *et al.*, 2003a). Although grazing by heterotrophic nanoflagellates exhibits significant top-down control on bacterial communities, phagotrophic phytoplankton are increasingly recognized as important bacterivores in planktonic food webs (Mitra *et al.*, 2014, Sanders, 1991b). Field studies have revealed that mixotrophic protists are often numerically dominant in freshwater systems and can exhibit a greater grazing impact on the bacterial community than pure heterotrophs (Berninger *et al.*, 1992b, Bird & Kalff, 1989b, Domaizon *et al.*, 2003a). For example, pigmented flagellates accounted for 70% of bacterial consumption in the epilimnion of oligotrophic Lake Alatsee (Oikonomou *et al.*, 2014). Mixotrophs have the potential to survive or even thrive in a variety environmental conditions, relying on bacterivory during periods of reduced irradiance or dissolved nutrient concentration and on photosynthesis when prey concentration is low (Jones, 2000b, Sanders, 1991b). Despite the potential metabolic costs of maintaining both photosynthetic and heterotrophic machinery, the combination of trophic modes may provide a competitive advantage along the vertical gradient of resources (irradiance, nutrient concentration and prey densities) created as a result of thermal stratification (Holen, 1999b, Raven, 1997b, Tittel *et al.*, 2003). There may also be niche variation within mixotrophic species since exist along a nutritional gradient from

primarily autotrophic to primarily heterotrophic, with some demonstrating stricter resource requirements for optimal growth or feeding (Jones, 2000b). Further, species often move within this gradient, changing their relative reliance on heterotrophy or phototrophy based on abiotic conditions and unique physiology. Members of the genus *Dinobryon*, common freshwater mixotrophs, can exhibit restricted growth and feeding in continuous darkness despite the presence of supplemental bacteria (Caron et al., 1993b). At the heterotrophic end of the spectrum, some species of *Ochromonas* require high concentrations of bacteria to reach a maximal growth rate and can grow in the dark (Sanders *et al.*, 2001b). Mixotrophs such as these are more likely to be spatially or temporally restricted to a smaller niche.

Despite considerable work towards a better understanding of the role of heterotrophic and mixotrophic nanoflagellates in the planktonic food web, few studies have focused on the influence of vertical habitat heterogeneity in shaping patterns of nanoflagellate distribution and bacterivory across longer temporal scales. Many studies are limited to surface waters and few data are available that address how the combination of thermal stratification and seasonality affect the depth-distribution of metabolically diverse nanoflagellates. This work describes nanoflagellate dynamics over a 15-month period in a temperate lake with a protected watershed that is free from pollution and excessive nutrient runoff. The aims of this work were to investigate vertical distribution of phototrophic, heterotrophic and mixotrophic nanoflagellates, and associated changes in their bacterivory as water column structure changed with periods of stratification and mixis.

### 3.3 Methods

#### 3.3.1 Study site and sampling

Lake Lacawac (41°22.912'N, 75°17.543'W, 439 m altitude) is a 21 ha freshwater lake in the Pocono Mountains of northeastern Pennsylvania, USA. This 13,000 year old glacial kettle lake ( $z_{\max}=13\text{m}$ ) is in a protected watershed. Lake Lacawac is dimictic and exhibits seasonal patterns of thermal stratification, with a strong thermocline in late spring and summer. The lake typically remains ice-covered from December to March. Previous studies have shown that chrysophycean algae, including mixotrophic strains, dominate the annual phytoplankton community (Berninger et al., 1992b, Siver & Chock, 1986b). Physiochemical characteristics including temperature, dissolved oxygen, and photosynthetically active radiation (PAR) for each sampling date are summarized in Supplementary Table 1. Although dissolved nutrients were not measured during the current study, Siver and Chock (1986b) reported nitrate and total phosphorus levels in November, January, March and June to be less than their detection limit ( $10\ \mu\text{g NO}_3\ \text{N liter}^{-1}$  and  $10\ \mu\text{g PO}_4\ \text{P liter}^{-1}$ , respectively) throughout the water column.

Water samples were collected monthly from May 2013 through Sept 2014 from the central and deepest point in Lake Lacawac with the exception of December 2013 and February 2014 during which the lake was inaccessible due to unstable ice cover. On each date, water was collected with a vertical Van Dorn sampler from three depths corresponding to the summer epilimnion (1 m), metalimnion (3-5 m), and hypolimnion (7-8 m). Hypolimnetic water samples were always collected several meters above the sediment-water interface. Prior to sampling, light intensity, dissolved oxygen

concentration and temperature were measured at 0.5 to 1 m intervals from the surface to 10 m. PAR levels were determined with a LI-250A light meter and LI 193 spherical quantum sensor (LI-COR INC., Lincoln, Nebraska). A Secchi disk was also used for a comparative determination of the depth of 1% surface PAR. A YSI model 58 dissolved oxygen meter (Yellow Springs Instruments Co., Inc., Yellow Springs, Ohio) was used to determine vertical profiles of temperature and oxygen. Mean relative thermal resistance to mixing (RTR) was calculated to evaluate vertical stratification patterns and stability of the water column during the time of sampling, according to:

$$RTR = \frac{(\rho_{z2} - \rho_{z1})}{\rho_4 - \rho_5}.$$

RTR is a dimensionless value where  $\rho_4$  and  $\rho_5$  are the water densities ( $\text{kg m}^{-3}$ ) at 4°C and 5°C, respectively, and  $\rho_{z1}$  and  $\rho_{z2}$  are the water densities at the bottom and top of the stratum being considered (Wetzel, 2001a). For the purposes of this study,  $Z_1$  and  $Z_2$  represent depths 2 meters above and below the depth identified as the center of the metalimnion. A RTR value  $\geq 50$  was used to categorize a given date as thermally stratified (See Supp. Table 1, Chimney *et al.*, 2006, Song *et al.*, 2013).

### 3.3.2 Ingestion experiments

Grazing rates of heterotrophic and mixotrophic nanoflagellates were determined by short-term grazing experiments using fluorescently-labeled microspheres with subsamples from each depth. Incubations took place on shore in 5 replicate whirl pack bags that were filled with lake water immediately following collection. Fluorescent polycarbonate microspheres (0.6  $\mu\text{m}$  diameter, Polysciences) were added to each

replicate at tracer levels (<20% bacterial abundance). Subsamples were fixed immediately following tracer addition to account for background incidence ( $T_0$ ) and after 20 minutes of incubation. All samples were preserved using the Lugol's-formalin technique to prevent egestion of tracer particles (Sherr *et al.*, 1987a).

### 3.3.3 *Microscope enumeration and determination of bacterivory*

Concentrations of suspended bacteria and microspheres were determined by filtration of a 500  $\mu$ L subsample from each depth onto a 0.2-  $\mu$ m membrane (GE Water and Process Technologies, Trevose, PA). All filters were mounted onto slides with Vectashield mounting media (Vector Laboratories, Inc., Burlington, CA) containing DAPI stain.

In order to determine grazing rates and abundances of phototrophic, heterotrophic, and mixotrophic nanoflagellates (hereafter referred to as PNAN, HNAN and MNAN), 25 mL from each replicate was stained with primulin (final concentration 250  $\mu$ g/mL) and stored at 4°C overnight (as described by Sanders & Porter, 1986). The following day, 2 mL was filtered onto 0.8- $\mu$ m polycarbonate filters (GE Water and Process Technologies, Trevose, PA) and washed with 0.1M Tris-HCl (pH 4) to remove excess stain. All prepared slides were kept frozen until enumeration to preserve chlorophyll autofluorescence.

Cells were first visualized for primulin fluorescence with a Zeiss Axiovert microscope at 1000X. Flagellates were identified as phototrophic or heterotrophic based on the presence of chlorophyll autofluorescence, and mixotrophs were identified as the subset that contained both chlorophyll autofluorescence and one or more latex

microspheres within the cell. A minimum of 100 grids were counted on each filter to determine mean abundance of each group. Bacterial ingestion rates for both HNAN and MNAN were calculated by multiplying the rates of microsphere uptake and the ratio of natural bacteria to microspheres in the sample. Grazing impact ( $\text{bacteria h}^{-1} \text{ ml}^{-1}$ ) of each nanoflagellate group was estimated by multiplying ingestion rate by abundance. This value was then used to determine the percentage of the bacterial assemblage removed daily as a result of grazing by HNAN or MNAN.

#### *3.3.4 Statistical analyses*

Statistical analyses were performed using the JMP® Pro 12 software package (SAS Institute Inc., Cary, NC). Cell abundance, bacterial ingestion rate and bacterial grazing impact were  $\log_{10} + 1$  transformed prior to analysis. Non-parametric Mann-Whitney U-tests and subsequent Kruskal-Wallis tests were used to analyze differences in abundance, bacterial ingestion rate and grazing impact of nanoflagellate groups across depth and time (date). Spearman rank correlations were performed in order to analyze relationships between nanoflagellate abundance and feeding behaviors with physical parameters (temperature, dissolved oxygen, light) and abundance of other nanoflagellate groups.

### **3.4 Results**

#### *3.4.1 Environmental conditions*

Lake Lacawac was thermally stratified from May 2013 to October 2013. The thermocline initially spanned depths from 2 to 7 meters, but extended to 9 meters prior to

the onset of mixis. During this time, the temperature of the epilimnion increased from 16.5°C (May) to 26.6°C in June before decreasing to 13.1°C in October. The water column was isothermal from November 2013 to mid-April 2014, after which thermal stratification was established for the remainder of the study period. The lake was ice-covered from December 2013 through March 2014, with ice-out in early April. From November through March, average water temperature throughout the water column was 4°C. The temperature at all depths was slightly warmer by April, but RTR indicated that the water column was not yet stratified. Mean Secchi disc depth was 3.3 meters, with greatest light penetration in late summer during both years. Photosynthetically active radiation (PAR) was greatly attenuated in the meta- and hypolimnion and did not exceed  $9 \mu\text{mol m}^{-2} \text{s}^{-1}$  below 6 meters. Epilimnetic dissolved oxygen concentration ranged from 7.28 mg L<sup>-1</sup> in the summer to 15.65 mg L<sup>-1</sup> in the fall. The hypolimnion in Lake Lacawac was hypoxic (< 2 mg L<sup>-1</sup> DO) during July 2013 and from June 2014 through August 2014. When RTR < 50 (non-stratified conditions), the water column averaged  $9.9 \pm 2.0$  mg L<sup>-1</sup> dissolved oxygen throughout.

#### *3.4.2 Bacterial abundance*

There was a significant effect of depth ( $p < 0.0001$ ,  $df = 2$ ) and date ( $p < 0.0001$ ,  $df = 14$ ) on bacterial abundance. Bacterioplankton abundance ranged from  $3.5 \times 10^4$  cells ml<sup>-1</sup> to  $9.3 \times 10^5$  cells ml<sup>-1</sup>, reaching a peak in the August metalimnion. During periods of water column mixis (RTR < 50) bacteria were generally more abundant in surface waters and were positively correlated with oxygen (Spearman  $\rho = 0.75$ ,  $p = 0.0001$ ) and PAR (Spearman  $\rho = 0.49$ ,  $p = 0.001$ ) (Table 2). Conversely, bacteria were negatively correlated

with oxygen (Spearman  $\rho = -0.20$ ,  $p < 0.05$ ) and Secchi disc depth (Spearman  $\rho = -0.36$ ,  $p = 0.0001$ ) during periods of thermal stratification (Table 1). Bacteria were positively correlated with PNAN abundance during both stratification and mixis (Table 1, Spearman  $\rho = 0.20$ ,  $p < 0.05$ ; Table 2, Spearman  $\rho = 0.34$ ,  $p < 0.05$ ).

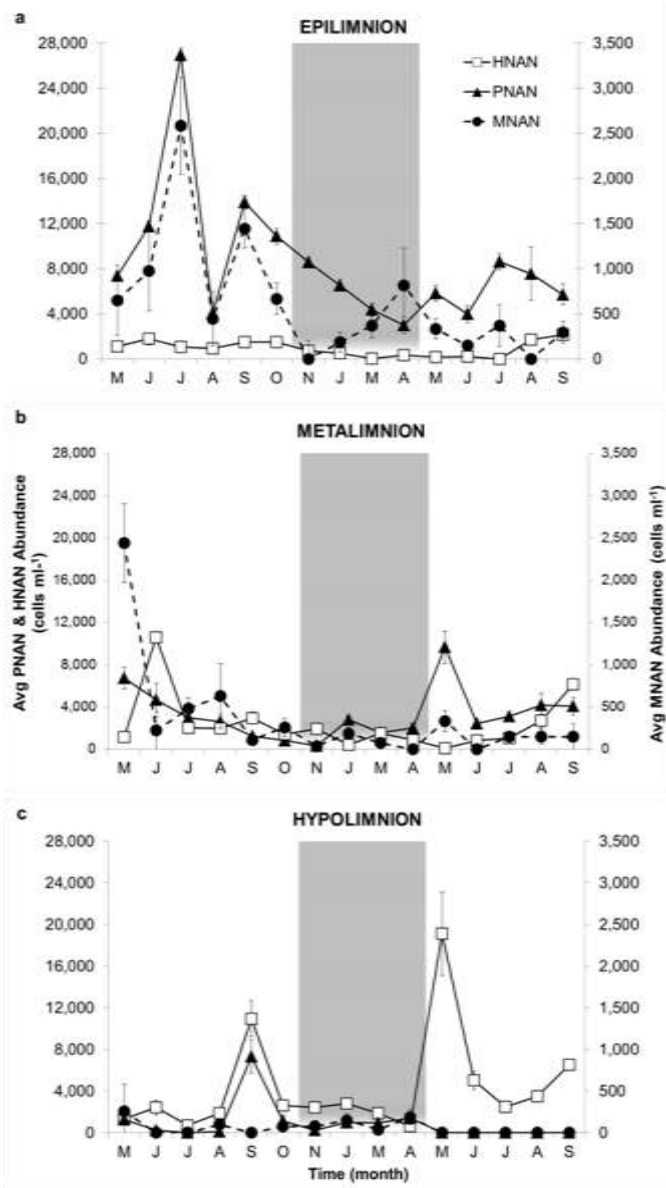
### 3.4.3 *Nanoflagellate abundances*

PNAN dominated the nanoplanktonic assemblage in the epilimnion during the entire study period, accounting for 70-92% of the nanoflagellate assemblage. MNAN accounted for a maximum of 20% of the nanoflagellate assemblage in surface waters (April 2014). PNAN and MNAN abundance in the epilimnion increased precipitously between May and July 2013, reaching epilimnetic peaks of  $> 26,900$  cells  $\text{ml}^{-1}$  and  $2,580$  cells  $\text{ml}^{-1}$ , respectively (Fig. 1a). Abundances of these pigmented nanoflagellate groups were highly correlated in the epilimnion when the water column was stratified from May 2013 through October 2013 (Spearman  $\rho = 0.75$ ,  $p = 0.0003$ ) and from May 2014 through September 2014 (Spearman  $\rho = 0.60$ ,  $p = 0.01$ ). Abundances of epilimnetic PNAN, HNAN and MNAN declined after September 2013, albeit at different rates. Whereas abundance of PNAN and HNAN declined throughout the breakdown of thermal stratification in the epilimnion, MNAN abundance declined initially and then increased during mixis (Fig. 1a). Epilimnetic PNAN and MNAN abundances during spring and summer in 2014 were considerably lower than in 2013. Abundance of these nanoflagellate groups reached 2014 maxima of  $8,624$  cells  $\text{ml}^{-1}$  (PNAN, July, Fig. 1a) and  $814$  cells  $\text{ml}^{-1}$  (MNAN, April, Fig. 1a). Although maximum PNAN abundance was detected in July during both years of study, the peak in 2014 was 68% less than that in 2013. In contrast, epilimnetic HNAN

abundance recovered following the fall 2013 decline and a peak for the study period of 2,147 cells ml<sup>-1</sup> in September 2014 (Fig. 1a). In comparison to PNAN and MNAN, HNAN abundance was relatively low in surface waters, ranging from not detected to 1.8 x 10<sup>3</sup> cells ml<sup>-1</sup> (Fig. 1a).

Two major metalimnetic peaks in nanoflagellate abundance were observed during the study period. HNAN reached 10,585 cells ml<sup>-1</sup> in June 2013 and PNAN peaked at 9,660 cells ml<sup>-1</sup> in May 2014 (Fig. 1b). The abundance of PNAN and HNAN in the metalimnion was generally higher than that of MNAN. MNAN abundance remained relatively low in the metalimnion over the entire study period with the exception of May 2013 (2,440 cells ml<sup>-1</sup>, Fig. 1b). PNAN abundance declined steadily in the metalimnion from May 2013 through November 2013, and remained low until the water column became stratified in May 2014 when the peak metalimnetic abundance was recorded (9,660 cells ml<sup>-1</sup>, Fig. 1b). As in the epilimnion, the metalimnetic trends in PNAN and MNAN abundance were highly correlated over the 15-month study period despite an order of magnitude difference in abundance between the population sizes (Spearman  $\rho=0.47$ ,  $p=0.0001$ ).

Nanoflagellates in the hypolimnion were generally dominated by HNAN, which displayed two peaks in September 2013 (10,955 cells ml<sup>-1</sup>, Fig. 1c) and May 2014 (19,098 cells ml<sup>-1</sup>, Fig. 1c). Abundance of all nanoflagellate groups at this depth was reduced during the November-April mixing period (Fig. 1c). MNAN were rare in the hypolimnion, never reaching greater than 300 cells ml<sup>-1</sup>, and PNAN abundance was also low throughout the entire study period, with a single hypolimnetic peak in September 2013 (7,000 cells ml<sup>-1</sup>, Fig. 1c).



**Figure 3.1:** Temporal fluctuations in abundance of phototrophic, heterotrophic and mixotrophic nanoflagellates in the (a) epilimnion, (b) metalimnion and (c) hypolimnion of Lake Lacawac from May 2013 through September 2014. Grey shaded area represents periods of water column mixing characterized by an RTR value  $<50$ . Secondary y-axis in all graphs corresponds to MNAN abundance.

#### *3.4.4 Water column structure and nanoflagellate dynamics*

There were significant differences in abundance within PNAN and HNAN across all depths regardless of thermal stratification pattern. However, MNAN were evenly distributed with depth during isothermal conditions, but not during stratification, during which their abundance was reduced in the hypolimnion ( $p < 0.0001$ ,  $df=2$ ). PNAN abundance was always positively correlated with PAR (Table 1; Spearman  $\rho=0.68$ ,  $p < 0.0001$  and Table 2; Spearman  $\rho=0.56$ ,  $p < 0.0001$ ). During thermal stratification, however, PNAN exhibited additional significant positive relationships with temperature (Table 1; Spearman  $\rho=0.57$ ,  $p < 0.0001$ ) and oxygen (Table 1; Spearman  $\rho=0.49$ ,  $p < 0.0001$ ). Like PNAN, MNAN abundance was positively correlated with temperature (Table 1; Spearman  $\rho=0.38$ ,  $p < 0.0001$ ), dissolved oxygen (Table 1; Spearman  $\rho=0.43$ ,  $p < 0.0001$ ) and PAR (Table 1; Spearman  $\rho=0.44$ ,  $p < 0.0001$ ) during stratification. During mixis, MNAN abundance was not significantly correlated with any of the measured abiotic variables, including PAR (Table 2). In contrast to PNAN and MNAN, HNAN were negatively correlated with measured abiotic variables during stratification (Table 1).

MNAN and HNAN abundances and grazing rates were not significantly correlated with bacterial abundance during the study period (Tables 1, 2). However, there were significant, positive correlations between MNAN bacterial ingestion rates and temperature (Spearman  $\rho=0.33$ ,  $p < 0.0001$ ), PAR (Spearman  $\rho=0.33$ ,  $p < 0.0001$ ) and oxygen (Spearman  $\rho=0.18$ ,  $p < 0.05$ ) during stratification that were not apparent for HNAN (Table 1). During mixis, there were no significant correlations between bacterivory rates and any abiotic factor for either MNAN or HNAN (Table 2).

**Table 3.1:** Spearman’s correlation analysis ( $\rho$ ) of abundance of bacteria, MNAN, HNAN and PNAN ( $\text{ml}^{-1}$ ), daily ingestion rate (bacteria  $\text{cell}^{-1} \text{hr}^{-1}$ ) of MNAN and HNAN, and abiotic variables during thermal stratification ( $\text{RTR} \geq 50$ ). Non-significant values are denoted n.s. ( $p > 0.05$ ) and asterisks are as follows: \*\*\* $p < 0.0001$ , \*\* $p < 0.001$ , \* $p < 0.05$ .

<b>Variable</b>	<b>Bacteria</b>	<b>MNAN</b>	<b>HNAN</b>	<b>PNAN</b>	<b>MNAN Ingestion rate</b>	<b>HNAN Ingestion rate</b>
<b>Temp.</b>	n.s.	0.38***	-0.40***	0.57***	0.33***	n.s.
<b>Oxygen</b>	-0.20*	0.43***	-0.27***	0.49***	0.18*	n.s.
<b>PAR</b>	n.s.	0.44***	-0.53***	0.68***	0.33***	n.s.
<b>Bacteria</b>	-	n.s.	n.s.	0.20*	n.s.	n.s.
<b>MNAN</b>	n.s.	-	-0.35***	0.63***	0.62***	n.s.
<b>HNAN</b>	n.s.	-0.35***	-	-0.35***	-0.28**	0.19*
<b>PNAN</b>	0.20*	0.63***	-0.35***	-	0.32**	-0.21*

**Table 3.2:** Spearman’s correlation analysis ( $\rho$ ) of abundance of bacteria, MNAN, HNAN and PNAN ( $\text{ml}^{-1}$ ), ingestion rate of MNAN and HNAN (bacteria  $\text{cell}^{-1} \text{hr}^{-1}$ ) and abiotic variables during periods of water column mixis ( $\text{RTR} < 50$ ). Non-significant values are denoted n.s. ( $p > 0.05$ ) and asterisks are as follows: \*\*\* $p < 0.0001$ , \*\* $p < 0.001$ , \* $p < 0.05$ .

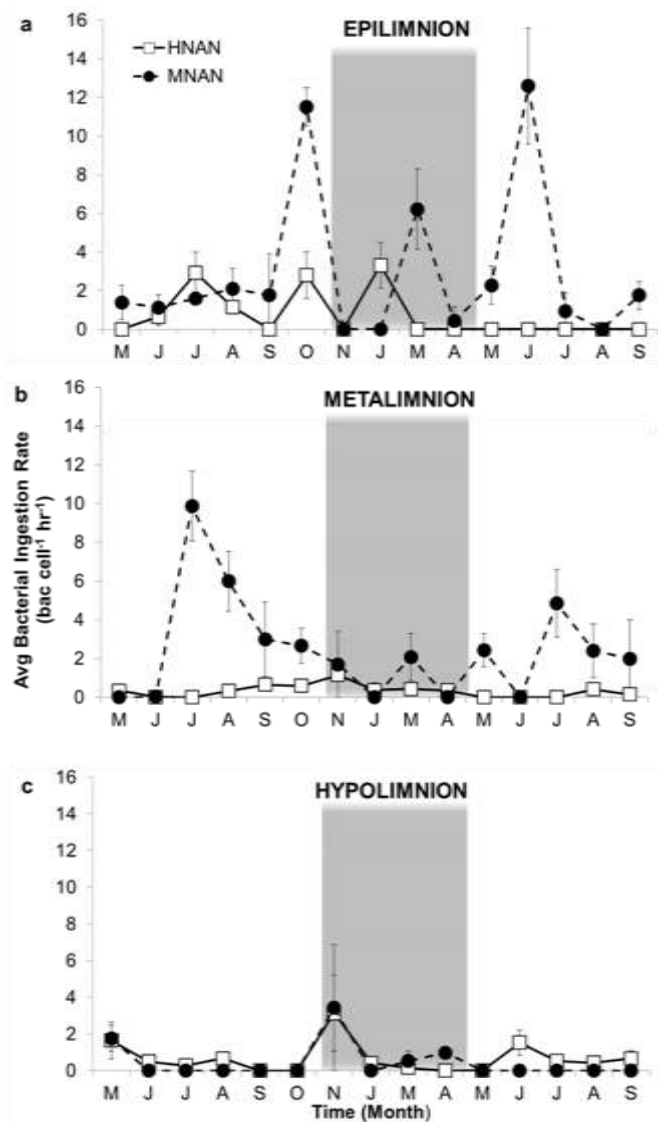
<b>Variable</b>	<b>Bacteria</b>	<b>MNAN</b>	<b>HNAN</b>	<b>PNAN</b>	<b>MNAN Ingestion rate</b>	<b>HNAN Ingestion rate</b>
<b>Temp.</b>	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
<b>Oxygen</b>	0.75***	n.s.	n.s.	n.s.	n.s.	n.s.
<b>PAR</b>	0.49**	n.s.	-0.57***	0.56***	n.s.	n.s.
<b>Bacteria</b>	-	n.s.	n.s.	0.34*	n.s.	n.s.
<b>MNAN</b>	n.s.	-	0.40**	-0.42**	0.37*	n.s.
<b>HNAN</b>	n.s.	0.40**	-	-0.67***	n.s.	0.47**
<b>PNAN</b>	0.34*	-0.42**	-0.67***	-	n.s.	-0.31*

### 3.4.5 Bacterivory by heterotrophic and mixotrophic flagellates

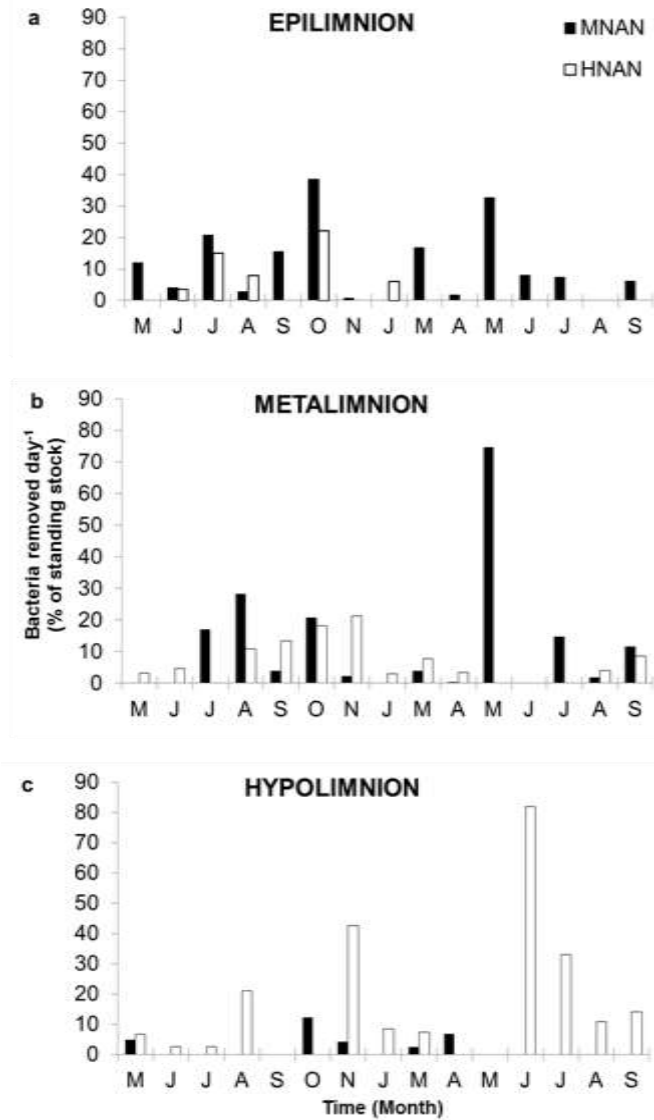
Whereas a majority of the phototrophic population was made up of pure phototrophs, MNAN accounted for a significant proportion of the bacterivorous flagellate population in the epilimnion, exceeding HNAN abundances in July 2013, and March, April, May and July 2014. Bacterial ingestion rates by HNAN and MNAN were highly variable during the study period (Fig. 2). Mann-Whitney U tests indicated significant effects of date ( $p=0.045$ ,  $df=14$ ) and depth ( $p<0.0001$ ,  $df=2$ ) on MNAN ingestion rate, but not for HNAN ingestion rates (date,  $p=0.06$ ,  $df=14$ ; depth,  $p=0.08$ ,  $df=2$ ). MNAN exhibited a maximum bacterial ingestion rate of  $12.6 \text{ bacteria cell}^{-1} \text{ hr}^{-1}$  in the June 2013 epilimnion (Fig.2a). HNAN bacterial ingestion reached a maximum of  $3.3 \text{ bacteria cell}^{-1} \text{ hr}^{-1}$  at the same depth in January (Fig.2a). Incidence of ingestion by both nanoflagellate groups was consistently low in the hypolimnion with the exception of a single date when HNAN and MNAN ingestion rates were approximately  $4 \text{ bacteria cell}^{-1} \text{ hr}^{-1}$  (November 2013, Fig.2c). Bacterial ingestion by HNAN was also relatively low in the metalimnion, never exceeding  $1 \text{ bacteria cell}^{-1} \text{ hr}^{-1}$ , while MNAN bacterivory was as high as  $10 \text{ bacteria cell}^{-1} \text{ hr}^{-1}$  in that water column layer (Fig.2b). Ingestion rates by MNAN were significantly greater than those of HNAN in the epilimnion and metalimnion during thermal stratification (epi-  $p=0.0003$ ,  $df=1$ ; meta-  $p=0.0179$ ,  $df=1$ ). The only period that HNAN feeding rates were significantly greater than those of MNAN was in the hypolimnion during stratification ( $p=0.0013$ ,  $df=1$ ).

Combined grazing impact by HNAN and MNAN ranged from undetected to  $3.09 \times 10^5$  bacteria  $\text{ml}^{-1} \text{ day}^{-1}$ ,  $1.21 \times 10^5$  bacteria  $\text{ml}^{-1} \text{ day}^{-1}$ , and  $2.71 \times 10^5$  bacteria  $\text{ml}^{-1} \text{ day}^{-1}$  in the

epilimnion, metalimnion, and hypolimnion, respectively. The largest impacts of bacterivory by the combined nanoflagellate assemblage removed 81% of the bacterial population daily in June (hypolimnion) and 75% in May (metalimnion) (Fig.3a, b). Depth had a significant effect on grazing impact on bacteria by HNAN ( $p=0.0036$ ) and MNAN ( $p<0.0001$ ), but the two groups exhibited opposing patterns. HNAN had the greatest impact on bacterial populations in the hypolimnion, whether the water column was stratified ( $p<0.0001$ ) or mixed ( $p=0.0038$ ). The greatest number of bacteria removed by HNAN feeding was  $2.47 \times 10^5$  bacteria  $\text{ml}^{-1} \text{day}^{-1}$  in the June hypolimnion (43% of the bacterial population  $\text{day}^{-1}$ , Fig.3b). MNAN alone had the most impact in the epilimnion and metalimnion (Fig. 3), with the greatest number of bacteria ingested in the October epilimnion ( $1.96 \times 10^5$  bacteria  $\text{ml}^{-1} \text{hr}^{-1}$ , 39% of the bacterial population  $\text{day}^{-1}$ , Fig.3a).



**Figure 3.2:** Average bacterial ingestion rate by heterotrophic and mixotrophic nanoflagellates in the (a) epilimnion, (b) metalimnion and (c) hypolimnion of Lake Lacawac from May 2013 through September 2014. Grey shaded area represents periods of water column mixing characterized by an RTR value <50.



**Figure 3.3:** Average percent of bacterial standing stock removed ( $\text{day}^{-1}$ ) by MNAN (solid columns) and HNAN (open columns) from the (a) epilimnion, (b) metalimnion and (c) hypolimnion over a 15-month period in Lake Lacawac.

### 3.5 Discussion

Nanoflagellates play key roles in aquatic biogeochemical cycling as primary producers and links to upper trophic levels via ingestion of other microorganisms (Worden *et al.*, 2015). They are considered the dominant bacterivores in most aquatic ecosystems, though ciliates, rotifers and cladocerans can contribute to top-down control of bacterioplankton (Oikonomou *et al.*, 2014, Pace *et al.*, 1990, Sanders *et al.*, 1989b). Mixotrophs are now known to be a major component of nanoflagellate grazing impact in a variety of planktonic ecosystems (Sanders *et al.*, 1989b, Bird & Kalff, 1986a, Hartmann *et al.*, 2012a). Although there has been increasing effort to differentiate the grazing impacts of heterotrophic and mixotrophic protists on bacterial communities, most studies have focused on a single depth (Domaizon *et al.*, 2003a, Isaksson *et al.*, 1999b), season (Jones, 1988a) or even day (Oikonomou *et al.*, 2014). However, seasonal alterations between isothermal mixing and the onset of stable thermal gradations within the water column drive patterns in succession seen in plankton communities, and are highly likely to influence the vertical distribution of metabolically diverse protists (Reynolds *et al.* (1983). The goal of the current study was to characterize the seasonal and spatial (depth) dynamics for phototrophic, heterotrophic and mixotrophic protists with focus on the role of thermal stratification in shaping nanoflagellate abundances and feeding rates.

#### *Bacterioplankton dynamics*

Bacterial abundance in Lake Lacawac was consistently on the low end of the range typical for oligo- and mesotrophic lakes (Carrias *et al.*, 1996a, Domaizon *et al.*,

2003a, Pålsson & Granéli, 2003). However, these data are consistent with the only previous study to examine bacterial abundance in Lake Lacawac (Berninger et al., 1992b). Influx of considerable recalcitrant colored dissolved organic matter (CDOM) known for this lake (Osburn *et al.*, 2001) may have led to lower bacterial production and consequently relatively low abundances. During both mixis and stratification, bacterial abundance in the present study was positively correlated with that of PNAN, indicating a potential reliance on uptake of dissolved organics released by PNAN (Simek & Straskrabová, 1992). Grazing pressure by heterotrophic and mixotrophic protists can also contribute to low bacterial abundance (e.g., Hadas & Berman, 1998), and feeding by a combined nanoflagellates did impose significant losses at times (up to 61%, 75% and 82% of the bacterial standing stock per day in the epilimnion, metalimnion and hypolimnion, respectively, Fig.3)

#### *Temporal and vertical zonation in nanoflagellate abundance*

The seasonal patterns for phototrophic, heterotrophic and mixotrophic nanoflagellates in Lake Lacawac had similarities to those observed over shorter time scales in other freshwater systems (Bennett et al., 1990a, Carrias et al., 1996a, Domaizon et al., 2003a). For example, heterotrophic protists in temperate, mesotrophic lakes typically exhibit a bimodal seasonal distribution with peaks in the spring and late summer/autumn (Sonntag *et al.*, 2006). HNAN in Lake Lacawac displayed two major abundance peaks in September 2013 and May 2014, however, these were apparent only in the hypolimnion (Fig.1c). Conversely, peak abundances of PNAN and MNAN occurred in the epilimnion or metalimnion (Fig. 1a, b). Interannual differences in

nanoflagellate abundances noted here may partially result from rapid growth rates of protists and bacteria and/or their short predator-prey cycles relative to the period of time between sampling dates (4-6 weeks). Bennett et al. (1990a) also observed interannual differences in the relative abundance of heterotrophic and phototrophic flagellates during a seasonal study in an eutrophic lake, and Berninger et al. (1992b) even noted considerable variability in MNAN abundance over a 3-day period in Lake Lacawac.

In a previous study of phytoplankton dynamics in Lake Lacawac, Siver and Chock (1986b) reported ranges in total phytoplankton abundance much lower than were noted in 2013/2014, ranging from  $2.3 \times 10^2$  cells  $\text{ml}^{-1}$  to  $9.0 \times 10^3$  cells  $\text{ml}^{-1}$ , though our use of epifluorescence microscopy may have enhanced our detection of very small flagellates. Siver and Chock (1986b) noted that phototrophs were evenly distributed throughout the water column during the fall and spring mixis, which also contrasted with 2013/2014 when PNAN abundance was greatest in the surface waters independent of stratification or mixis.

Abundances of nanoflagellate groups in Lake Lacawac were differently affected by water column structure. Whereas surface waters always were dominated by PNAN, likely as a positive response to availability of PAR (Table 1), HNAN were always numerically dominant in the hypolimnion (Fig. 1). MNAN reached peak abundances of approximately  $2,500 \text{ ml}^{-1}$  in spring and mid-summer in metalimnetic and epilimnetic waters, respectively. Domaizon et al. (2003a) noted a coupling between phototrophic and mixotrophic population sizes in the epilimnion of Lake Annecy that was also observed in Lake Lacawac (Fig.1a). This may reflect a shared reliance on PAR between PNAN and

MNAN, and speaks to the composition of the mixotrophic community in these lakes in that it may be dominated by those on the phototrophic end of the nutritional gradient.

MNAN abundances were very low in the hypolimnion (Fig 1c), which is reflected by the significant positive relationships between MNAN abundance and temperature, light and oxygen that were present only during stratification (Table 1). Thermal stratification may have prevented a significant increase of MNAN in the hypolimnion due to declines in average water temperature and PAR. Protistan metabolism can decline in cold and anoxic waters (Fenchel & Finlay, 1990), and MNAN may be particularly sensitive to decreases in either. Further, because MNAN were identified only by the combination of active ingestion and chlorophyll content, any negative effect on feeding by colder, low oxygen waters would have led to MNAN being identified as PNAN.

#### *Protistan bacterivory and grazing impact in Lake Lacawac*

Water column mixing patterns influenced depth-differences in feeding rates and grazing impacts by HNAN and MNAN. While there were no significant depth differences in bacterivory between HNAN and MNAN during isothermal conditions, HNAN ingestion rate exceeded that of MNAN only in the hypolimnion during stratification. This is reflected by the significant positive correlation of MNAN bacterivory with temperature and light during stratification that did not exist for HNAN (Table 1). Oikonomou et al. (2014) also found that pigmented flagellates were responsible for the majority of prokaryotic grazing losses in the epilimnion and heterotrophic nanoflagellates were the main grazers in the anoxic hypolimnion of a meromictic (rarely mixed) lake during autumn. While some mixotrophs can survive

continuous darkness, ingestion comes to a halt in others after a period in continuous darkness (Princiotta *et al.*, 2016). Consequently, lack of significant PAR may have constrained MNAN ingestion to the surface or midwater during stratification in these lakes. Combined lower abundances and reduced feeding rates for MNAN in the hypolimnion led to negligible grazing impact on bacterial standing stock with depth in Lake Lacawac (Fig.3).

As was evident in the photic zone of Lake Lacawac, ingestion rates by MNAN were greater than HNAN (54.8 bacteria cell<sup>-1</sup> hr<sup>-1</sup> versus 10.7 bacteria cell<sup>-1</sup> hr<sup>-1</sup>) in the epilimnion of Lake Annecy with the onset of thermal stratification (Domaizon *et al.*, 2003b). The highest ingestion rates for MNAN in Lake Lacawac were also in the epilimnion, but never exceeded 12 bacteria cell<sup>-1</sup> hr<sup>-1</sup> (Fig.2a). MNAN may compete better with PNAN when dissolved nutrients concentrations are low, which is often the case in the epilimnion during stratification (Isaksson *et al.*, 1999b). Bacterivory has the potential to increase access to nutrients by mixotrophs compared to non-phagotrophic algae that can only access dissolved nutrients. Nutrient limitation has been reported to increase the rate of bacterivory in phagotrophic phytoplankton (Nygaard & Tobiesen, 1993b, Pålsson & Granéli, 2003, Winder & Sommer, 2012). In oligotrophic Lake Annecy, mixotrophic taxa such as *Dinobryon* and *Ochromonas* were identified among the photosynthetic community, supporting the idea that mixotrophy may be competitively advantageous in a nutrient-depleted epilimnion (Domaizon *et al.*, 2003a). Although we did not measure dissolved nutrient concentrations in Lake Lacawac, Longhi and Beisner (2009) observed lower levels of total phosphorus in epilimnetic waters within 45 northern

temperate lakes, and in a previous seasonal study of phytoplankton dynamics in Lake Lacawac, nitrogen and phosphorus were below detectable limits (Siver & Chock, 1986b).

Lake Lacawac was ice-covered during two sampling periods in 2014. HNAN and MNAN abundances were relatively low, but MNAN ingestion rates were relatively high in March, when the MNAN constituted a maximum of 91% of the bacterivorous assemblage. Pålsson and Granéli (2003) also reported increased ingestion rates by *Cryptomonas* spp. under ice in Lake Skärlen. As in the current study, the under-ice nanoflagellate assemblage of Lake Lacawac was dominated by MNAN and PNAN in February 1991 (Berninger et al., 1992b). Compared to 1991, abundances of PNAN were 3 times greater in March 2014 and bacterivorous nanoflagellates relatively fewer. Despite the differences in abundance, grazing activity by MNAN and HNAN removed 17% of the bacterial standing stock daily in the March 2014 epilimnion compared to 8% reported by Berninger et al. (1992b). Siver and Chock (1986b) indicated highest phytoplankton concentration was in the epilimnion of Lake Lacawac through the duration of ice-cover despite low overall winter abundances. Under-ice sampling efforts in March 2014 also revealed greater abundances (Fig. 1) of PNAN and MNAN, as well as higher MNAN ingestion rates in the epilimnion (Fig.2a). Thus, mixotrophy may contribute to the maintenance of winter nanoflagellate populations that often face reduced irradiance for prolonged periods. Few studies of the microbial community within temperate lakes include under-ice sampling efforts (Hampton *et al.*, 2015, Pålsson & Granéli, 2003), highlighting the necessity for inclusion of winter months in studies of plankton dynamics, especially considering the importance that mixotrophic protists may play in the winter protistan community.

This work in a mesotrophic, dimictic lake suggests that alterations between thermal stratification and isothermal water column conditions differently affect abundances, ingestion rates and consequently grazing impacts of mixotrophic and heterotrophic nanoflagellates on bacterioplankton. Indeed, plankton dynamics are intimately linked to fluctuations in vertical mixing processes, which will likely be influenced by climate-driven fluctuations in meteorological variables (Winder & Sommer, 2012). Lakes that experience ice-cover exhibited the highest surface warming rates globally (O'Reilly *et al.*, 2015), which is highly likely to influence protistan metabolism and phenology by way of decreased duration of ice cover or premature and increasing periods of summer stratification (Gerten & Adrian, 2000). Knowledge about the effect of water column structure on planktonic processes throughout the year is crucial to predicting how global climate change will impact lake ecosystems.

**CHAPTER 4**  
**SEASONAL DYNAMICS OF PHYTOPLANKTON AND BACTERIVORY BY**  
**LACUSTRINE PLANKTONIC PROTISTS**

**4.1 Abstract**

The concept of a microbial loop in which protists reintroduce dissolved carbon into the traditional food web via ingestion of bacteria was a major conceptual advance in understanding planktonic ecosystems. Bacterivorous protists are vital to efficient nutrient remineralization. Likewise, the identification of the important roles of phagotrophy by a variety of phytoplankton groups introduced a conceptual restructuring of the traditional view of the planktonic food web. This is because mixotrophic protists play dual roles as producers and consumers, thereby contributing to both primary and secondary production. Mixotrophic behavior is also highly variable and taxa differ largely in the relative contributions of photosynthesis and phagotrophy to their nutrition. Phagotrophic phytoflagellates have proven to be a major component of freshwater communities. A continuum of strategies often characterizes mixotrophic protists – an understanding of which is critical to that of carbon and energy flow through pelagic systems. Despite the ecological significance of mixotrophic nutrition, few studies have compared the *in situ* dynamics among various bacterivores, including mixotrophs. In this study, abundance and bacterivory of members of the planktonic community were quantified by ingestion of bacterial surrogates. Overall, our results support that mixotrophic protists have the ability to seasonally dominate planktonic communities and maintain a significant impact on bacterioplankton, particularly under ice. The community was largely dominated by

Chlorophytes and Chrysophytes; however, Cryptophytes were important in the hypolimnion.

#### **4.2 Introduction**

Phagotrophic protists, including heterotrophic and mixotrophic flagellates, are ubiquitous members of the microbial assemblage (Sherr & Sherr, 1994). The microbial loop concept has emerged as a dominant component of aquatic ecosystems in which dissolved organic carbon that would otherwise be lost to the system is taken up by bacteria and reintroduced to the classic pelagic food web by bacterivorous protists (Azam *et al.*, 1983). Bacteria are often superior competitors for nutrients in comparison to phytoplankton due to their high surface area to volume ratios (Bratbak, 1985b). Nitrogen and phosphorus excretion by phagotrophic protists is a major source of dissolved nutrient regeneration that stimulates growth of bacteria and phytoplankton, thereby closing the “loop” and providing a vital connection between primary producers and higher trophic levels (Tranvik, 1994).

Protistan grazing also has far reaching impacts on the bacterial community in terms of phylogenetic composition and morphology (van Hannen *et al.*, 1999). Although grazing by bacterivores is significant in shaping the bacterial community, differential grazing impacts may, in turn, have an impact on the composition of the planktonic community itself. Therefore, efficient bacterivores will be competitively superior and dominate the planktonic assemblage, potentially by increased regeneration of substrates or reduction of competitors.

In conjunction with heterotrophic forms, phagotrophic phytoplankton have been demonstrated to be important constituents and substantial bacterivores in freshwater food webs (Sanders, 1991a). Mixotrophy is a widespread phenomenon and has been identified in several taxonomic groups of aquatic organisms including nanoflagellates and ciliates (Jones, 2000a). Field and laboratory studies have demonstrated that bacterivory by mixotrophic protists is increasingly prevalent under low light and nutrient conditions, suggesting that this nutritional flexibility may provide a competitive advantage (Nygaard & Tobiesen, 1993a, Caron et al., 1993a). Therefore, the combination of phototrophy and phagotrophy may allow mixotrophic taxa to persist during periods that are otherwise unsuitable for efficient growth. Although mixotrophic organisms are increasingly prevalent in oligotrophic environments (Isaksson et al., 1999a), they are also common in eutrophic and humic waters (Jones, 1988b, Sanders, 1991a). The distribution of mixotrophic organisms in nature is often difficult to predict based on the spectrum of nutritional strategies that range from primary phototrophy to primary heterotrophy (Lones, 1997). Mixotrophs often respond differently to environmental conditions based on their own unique physiology that is predicted to dictate the relative balance between nutritional modes. Although the definition of mixotrophy extends to the osmotic uptake of dissolved organic compounds, this work will focus on the combination of phototrophy and phagotrophic uptake of bacteria (bacterivory).

Protistan community composition is largely a consequence of physiological responses to changes in water column attributes (Sommer et al., 1986a). Seasonal variation also plays a vital role in the structure of pelagic microbial food webs, which itself is governed by a complex suite of biotic interactions. Population dynamics of

individual taxonomic groups are highly dynamic, and spatial distribution of plankton groups varies over temporal scales in response to fairly predictable seasonal changes in light and nutrient availability. The quantitative importance of individual members of the planktonic assemblage varies considerably throughout the seasons (Sanders et al., 1989a). Although numerous studies have documented the seasonal dynamics of planktonic protists, many are limited in spatial (vertical) scale (Koiv & Kangro, 2005). Further, most research has been dedicated solely to either heterotrophic (non-pigmented) flagellates or ciliates. The present work describes the composition of the planktonic protistan assemblage, including mixotrophic taxa, in relation to season and depth in a freshwater lake.

### **4.3 Methods**

#### *4.3.1 Study site and sampling*

Lake Lacawac is a mesotrophic lake of glacial origin located in the Pocono Mountains of Northeastern Pennsylvania (USA) with a protected watershed that is undisturbed by human impact. A stable thermocline typically develops during late spring and summer, and the 13-m water column circulates in early autumn. During the study period, Lake Lacawac experienced ice-cover from December through March. Phytoplankton in Lake Lacawac tend to be most abundant during winter and spring, with dominance of flagellated chrysophytes (Moeller & Williamson, 1994). Procedures for collection of water samples and measurement of physiochemical characteristics were as previously reported (See Chapter 3.3.1 and Appendices A and B). In brief, whole water samples were collected monthly from a fixed, central location over the deepest point in

Lake Lacawac between May 2013 and September 2014. On each date, samples were taken with a vertical Van Dorn sampler from 3 depths corresponding to the summer epilimnion, metalimnion and hypolimnion. These depths were dictated by prior measurement of temperature and oxygen during thermal stratification in Lake Lacawac.

#### *4.3.2 Sample processing and grazing experiments*

Water samples were filtered through a mesh screen prior to experimentation in order to remove zooplankton and crustaceans. Short-term grazing experiments were conducted in order to determine grazing rates of planktonic protists and aid in detection of mixotrophic taxa. Subsamples from each depth were incubated in (5) replicate whirl-pack bags directly following collection. Fluorescent polycarbonate microspheres (0.6  $\mu\text{m}$  diameter) were then added to each subsample at tracer levels (approximately 20% of natural bacterial abundance). Fixation and preservation by the Lugol's-formalin technique was conducted immediately after addition of bacterial surrogates to account for background ingestion ( $T_0$ ) and after 20 minutes of incubation (Sherr *et al.*, 1987b). Preserved samples were stored in the dark at 4°C for a maximum of 24 hours until further processing.

#### *4.3.3 Microscopic enumeration, identification and assessment of bacterivory in planktonic protists*

Bacterial abundance and concentration of microspheres was determined by filtration of a 500  $\mu\text{L}$  subsample from each depth onto a 25 mm, 0.2  $\mu\text{m}$  membrane. Filters were mounted onto slides with Vectashield mounting media containing DAPI

stain and visualized by oil immersion at 1000X. All slides were kept frozen prior to analysis.

In order to analyze protistan community structure and bacterivory, 75-mL aliquots of preserved samples from each depth and sampling date were settled in darkness overnight. Samples were concentrated to approximately 10 mL by aspiration of the remaining volume with a sterile J-shaped tube, and stored at 4°C to preserve chlorophyll autofluorescence. Subsequent enumeration and identification of planktonic protists was performed on a Zeiss Axiovert inverted microscope at 40X. Up to 200 cells in the nano- to microplanktonic size range were identified and enumerated in a PhycoTech settling chamber. Identifications were made to species where possible, but ultimately grouped according to the classifications described in Table 4.1. Incidence of bacterivory was enumerated for both heterotrophic and mixotrophic plankton, the latter identified by a combination of chlorophyll autofluorescence and the presence of one or more latex microspheres within the cell. Bacterial ingestion rate was calculated by multiplying rates of microsphere uptake and the ratio of background bacteria to microspheres. Grazing impact was subsequently calculated by multiplying cell abundance ( $\text{cell ml}^{-1}$ ) by individual ingestion rates ( $\text{ingestion cell}^{-1} \text{ day}^{-1}$ ), and used to determine the daily percentage of bacterial standing stock removed by ingestion. Rates for all heterotrophic and mixotrophic flagellates were calculated by the identification of all individuals, including those that did not ingest bacterial surrogates.

#### 4.3.4 Data analyses

Multivariate statistical analyses were conducted using the PRIMER v6.1.13 package with PERMANOVA+ add-on to explore spatial and temporal patterns in planktonic community structure in Lake Lacawac. Samples were coded for depth, year and month. Abundance and grazing impact data were log transformed prior ( $\log x + 1$ ) to analysis. Bray-Curtis similarity matrices between samples for abundance were explored through ordination by non-metric multidimensional scaling (MDS) with cluster overlays based on group-average hierarchical cluster analysis. The same procedure was used for grazing impact, but with Euclidean distance matrices. MDS is an iterative method that plots samples in non-dimensional space in such a way that rank order of the distances between the samples agrees with the rank order of the samples from the Bray-Curtis similarity matrix. The congruency of this relationship was represented by a stress value. 2-dimensional MDS plots were constructed for abundance and grazing impact at each depth individually (across month and year) and for all 3 depths (across sampling month).

Type III permutational multivariate analysis of variance (PERMANOVA) with unrestricted permutations of transformed data was used to test for significant spatial (depth) and temporal (month) differences in planktonic community structure and grazing impact. Significant differences in community structure were verified by subsequent pairwise tests. PERMANOVA is a nonparametric permutation test that provides F-ratios analogous to Fisher's F-ratio in multivariate analysis of variance. Average similarity percentages (SIMPER) were calculated to identify the taxa or groups that contribute most to discrimination between observed depth clusters in abundance and grazing impact over month and year.

## 4.4 Results

### 4.4.1 Limnological conditions of Lake Lacawac during the study period

A complete description of measured environmental conditions during this study can be found in Chapter 3.4.1. In brief, sampling began in mid-May 2013 when water column stratification was present and increasing in stability. The thermocline depth deepened during the subsequent sampling months until October 2013, after which the water column was isothermal. The average water column temperature during mixis decreased from 6°C in November 2013 to 3°C in January 2014. By May 2014 thermal stratification was established and continued until the end of the study in September 2014. Lake Lacawac was ice-covered from December through March, but was inaccessible in December and February 2014 due to unstable ice conditions. Ice-out occurred prior to sampling in April 2014 when the water column maintained an average temperature of 7°C. Light attenuated considerably in Lake Lacawac, with PAR levels < 9  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  below 6 meters. Epilimnetic dissolved oxygen (DO) ranged from 6  $\text{ml l}^{-1}$  in July 2014 to 19  $\text{ml l}^{-1}$  in October 2013. The hypolimnion was hypoxic (< 2  $\text{mg l}^{-1}$  DO) in July 2013 and from June 2014 through August 2014.

### 4.4.2 General characteristics of the planktonic assemblage

In total, 9 taxonomic groups were informed by microscopic analysis; ciliates, coccoid and colonial green algae, chrysophytes, cryptophytes, cyanobacteria, desmids, diatoms, dinoflagellates and “unknown” nanoflagellates (Table 4.1). Within the Chrysophyceae, major taxa including *Dinobryon*, *Ochromonas*, and *Synura* were

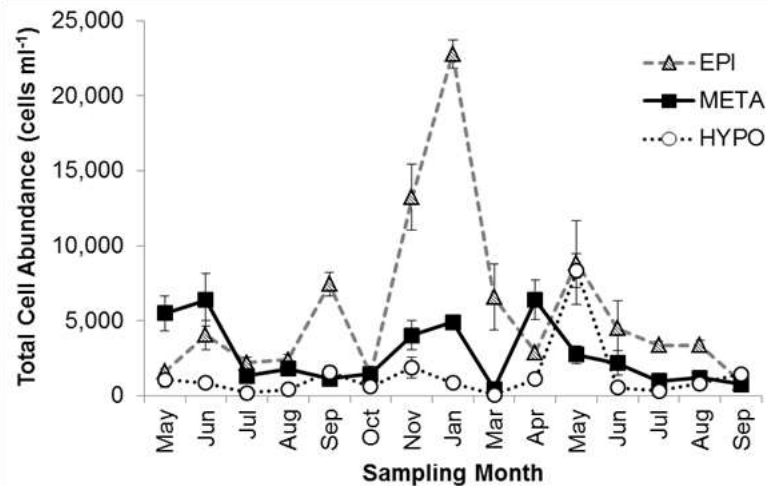
identified and analyzed as independent units in the multivariate analyses of community grazing impact. The same trend was used for members of Cryptophyta: *Cryptomonas* and *Rhodomonas*. “Unknown nanoflagellates” was used to describe non-pigmented, uni- or biflagellated cells (2-10  $\mu\text{m}$ ) that could not be identified to genus level due to lack of distinguishing morphological features. Plankton were considered rare if they occurred in <5% of the total sampling occasions (n=192), and were excluded from analyses.

Species similarity decreased with increasing depth on all sampling occasions. Whereas similarity observed in the epi- and metalimnion was due to the presence of coccoid and colonial green algae that at depth was due to *Cryptomonas*. Total cell abundance was generally greatest in the epilimnion, where a maximum was reached in January (22,800 cells  $\text{ml}^{-1}$ , Fig. 4.1). This was driven by a bloom in *Ochromonas* and *Dinobryon*. Similar patterns in total cell abundance were observed between the meta- and hypolimnion. There were two metalimnetic peaks in cell abundance in June 2013 and April 2014 (both 6,340 cells  $\text{ml}^{-1}$ , Fig. 1). Total cell abundance in the hypolimnion was generally <2,000 cells  $\text{ml}^{-1}$  (Fig. 4.1) with the exception of a peak in May 2014 (8,340 cells  $\text{ml}^{-1}$ , Fig. 4.1).

**Table 4.1:** Species of plankton in Lake Lacawac identified by light microscopy.

<b>Ciliates</b>	<b>Cryptomonads</b>	<b>Cyanobacteria</b>	<b>Desmids</b>
<i>Strombidium</i>	<i>Cryptomonas</i>	<i>Anabaena</i>	<i>Arthrodesmus</i>
<i>Strombilidium</i>	<i>Rhodomonas</i>	<i>Aphanothece</i>	<i>Cosmarium</i>
		<i>Merismopedia</i>	<i>Staurastrum</i>
		<i>Oscillatoria limnetica</i>	
<b>Diatoms</b>	<b>Dinoflagellates</b>	<b>Chrysophytes</b>	
<i>Cyclotella</i>	<i>Gymnodinium</i>	<i>Chrysosphaerella longispina</i>	<i>Epiphyxis</i>
<i>Navicula</i>	<i>Peridinium</i>	<i>Dinobryon bavaricum</i>	<i>Kephyrion</i>
<i>Nitzschia</i>		<i>D. cylindricum</i>	<i>Mallomonas caudata</i>
<i>Tabellaria</i>		<i>D. sociale</i>	<i>M. akrokomos</i>
		<i>D. sertularia</i>	<i>Synura</i>
			<i>Ochromonas</i>
			<i>Uroglena</i>
<b>Green Algae</b>			
<i>Ankistrodesmus falcatus</i>		<i>Oocystis</i>	
<i>Botryococcus braunii</i>		<i>Quadrigula</i>	
<i>Crucigenia rectangularis</i>		<i>Scenedesmus</i>	
<i>C. tetrapedia</i>		<i>Schroederia</i>	
<i>Dictyosphaerium</i>		<i>Sphaerocystis</i>	
<i>Elakatothrix</i>		<i>Stichoglea</i>	
<i>Kirchneriella</i>		<i>Tetrastum</i>	
<i>Monomastix</i>		<i>Unidentified pigmented colony</i>	
<i>Nephrocytium lunatum</i>		<i>Unidentified pigmented nanoflagellates</i>	

2



**Figure 4.1:** Total abundance of planktonic cells in the epilimnion (shaded triangles), metalimnion (solid squares) and hypolimnion (open circles) of Lake Lacawac recorded from May 2013 through September 2014.

There was a significant effect of month ( $F_{9, 50}=5.7$ ,  $p=0.0001$ ) on planktonic community composition in the epilimnion (Table 4.2). Coccoid and colonial green algae composed  $>50\%$  of the protistan community in late spring and summer. All epilimnetic sampling months contained chrysophycean algae, which were numerically dominant in the surface waters of Lake Lacawac in September, January and March (Fig 4.2a). Members of the mixotrophic genus *Dinobryon* made up a significant component of the epilimnetic community in September (77%, Fig. 4.2a), but reached peak abundance in January of the same depth (8,500 cells ml<sup>-1</sup>, data not shown). *Ochromonas* dominated the epilimnetic planktonic community in January and March (60% and 82%, respectively, Fig. 4.2a), and exhibited a peak of 13,670 cells ml<sup>-1</sup> (data not shown) in March. In fact,

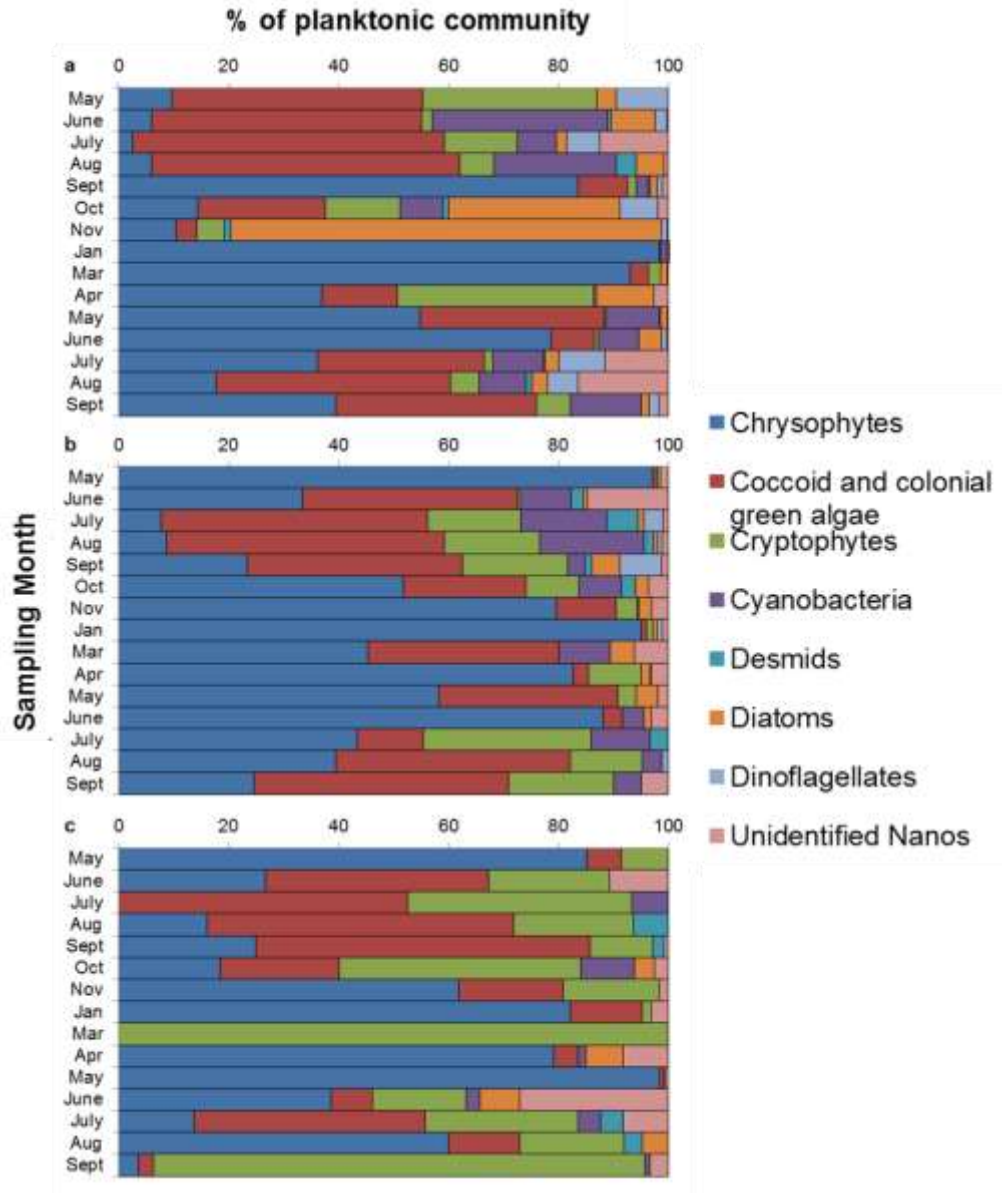
the epilimnetic community in January was largely composed of *Dinobryon* and *Ochromonas* (Fig. 4.2a).

There was also a significant effect of month ( $F_{9, 50}=4.8$ ,  $p=0.0001$ ) on the metalimnetic planktonic community (Table 4.2). Similarly to the epilimnion, coccoid and colonial green algae dominated the community in late spring and early summer (Fig. 4.2b). When green algae were not numerically dominant, chrysophycean algae were very abundant, comprising a maximum of 95% of the community in January (Fig 4.2b). Mixotrophic *Ochromonas* dominated the planktonic community composition in November (2,890 cells  $\text{ml}^{-1}$ , data not shown) and January (3,500 cells  $\text{ml}^{-1}$ , data not shown), contributing to 70% of the assemblage during both months (Fig. 4.2b). Whereas *Dinobryon* was common in the September epilimnion, this mixotroph was most abundant in the May and June metalimnion in 2014. *Dinobryon* was not numerically dominant in the May metalimnion during the first sampling year (2013), during which *Synura*, a non-bacterivorous chrysophyte, composed 97% of the planktonic community. *Synura* exhibited the greatest abundance of all planktonic cells within the metalimnion in May 2013 with a peak of 5,000 cells  $\text{ml}^{-1}$  (data not shown).

Total cell abundance was low in the hypolimnion relative to other depths (generally  $<1,000$  cells  $\text{ml}^{-1}$ , Fig. 4.1). A significant *Synura* peak in May 2013 (6,700 cells  $\text{ml}^{-1}$ , data not shown) drove the hypolimnetic maximum during the sampling regime. A PERMANOVA revealed a significant effect of month ( $F_{8, 42}=3.0$ ,  $p=0.0004$ ) on planktonic community composition in the hypolimnion (Table 4.2). Coccoid and colonial green algae were less important at depth except during September 2013 when they contributed to 69% of the community (Fig. 4.2c). Similarly to other depths, *Ochromonas*

dominated the planktonic assemblage in January (62%, data not shown). As opposed to other depths, the presence of members of the mixotrophic genus *Cryptomonas* were increasingly abundant in the hypolimnion. This taxon contributed to a maximum of 89% of the planktonic community in September 2014.

**Figure 4.2:** Relative abundance of protistan cells in the (a) epilimnion, (b) metalimnion and (c) hypolimnion of Lake Lacawac recorded from May 2013 through September 2014.



**Table 4.2:** Summary of independent one-way PERMANOVA analyses examining temporal differences in planktonic community composition within 3 depths in Lake Lacawac. Statistical analyses based on Bray-Curtis similarity matrix of log transformed abundance data.

	<i>Source</i>	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>Pseudo-F</i>	<i>P(perm)</i>
<b>Epilimnion</b>	Month	9	26246	2916.2	5.73	0.0001
	Res	50	25462	509.23		
	Total	59	51708			
<b>Metalimnion</b>	Month	9	39285	4365	4.83	0.0001
	Res	50	45144	902.88		
	Total	59	84429			
<b>Hypolimnion</b>	Month	8	30670	3833.8	3.05	0.0004
	Res	42	52845	1258.2		
	Total	50	83515			

MDS plots of abundance within each depth across month (data not shown) depict a great deal of variability in species composition both within and between months. The relatively high stress values (0.2) of the MDS plots indicate that the unconstrained ordination also provides a weak representation of the temporal relationship between depth and species composition in 2-dimensional space (data not shown). However, a two-way crossed PERMANOVA showed significant differences in community composition with depth ( $F_{2, 150}=11.2, p=0.0001$ ) and month ( $F_{9, 150}=5.0, p=0.0001$ ) despite the scatter

of points in the MDS plot (Table 4.3). Whereas temperature was a significant covariate in dynamics of planktonic community composition within all depths of Lake Lacawac, the effect of dissolved oxygen concentration was only significant in the surface and deep waters.

A PERMANOVA based on Bray-Curtis resemblance data revealed patterns in taxonomic composition across depth that varied with month. In June, August and March the taxonomic composition throughout all depths was significantly different from one another. During all other months only the epi- or hypolimnetic planktonic assemblage was distinct from other depths. September was the only sampling period in which the hypolimnion was different from the surface and mid waters. During May, July, October, November, January and April the meta- and hypolimnion were not different, but the epilimnetic community was distinct. An ANOSIM indicated greater overlap in community composition between the epi- and metalimnion ( $R=0.38$ ,  $p=0.0001$ ) and meta- and hypolimnion ( $R=0.41$ ,  $p=0.0001$ ). In contrast, there was a moderate degree of distinction between the epi- and hypolimnion ( $R=0.61$ ,  $p=0.0001$ ) between the surface and bottom waters.

**Table 4.3:** Summary of two-way crossed PERMANOVA examining differences in planktonic species composition across 16 months and 3 depths in Lake Lacawac.

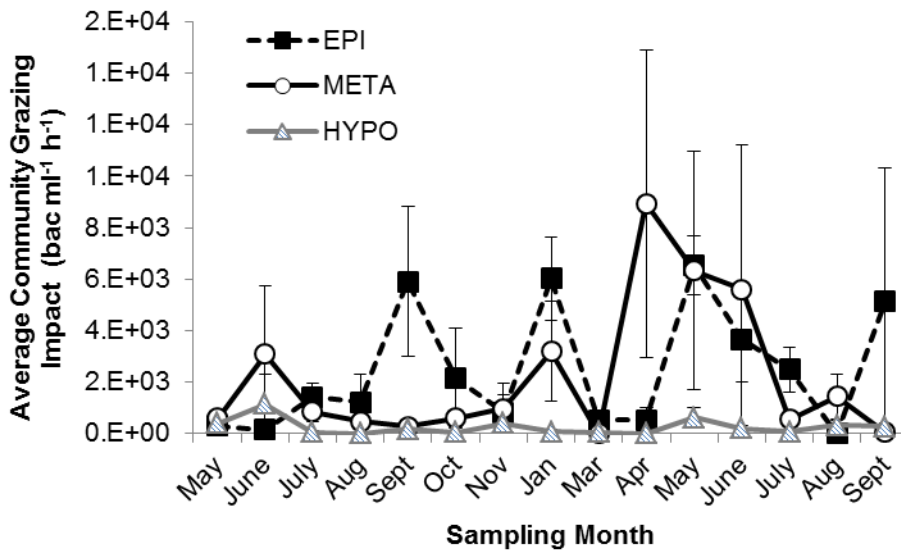
<i>Source</i>	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>Pseudo-F</i>	<i>P(perm)</i>
<b>Depth</b>	2	24602	12301	11.18	0.0001
<b>Month</b>	9	49717	5524	5.02	0.0001
<b>Depth x Month</b>	18	49054	2725.2	2.48	0.0001
<b>Res</b>	150	1.6 x 10 <sup>5</sup>	1100		
<b>Total</b>	179	2.9 x 10 <sup>5</sup>			

#### *4.4.3 Bacterioplankton and bacterivory by the planktonic community*

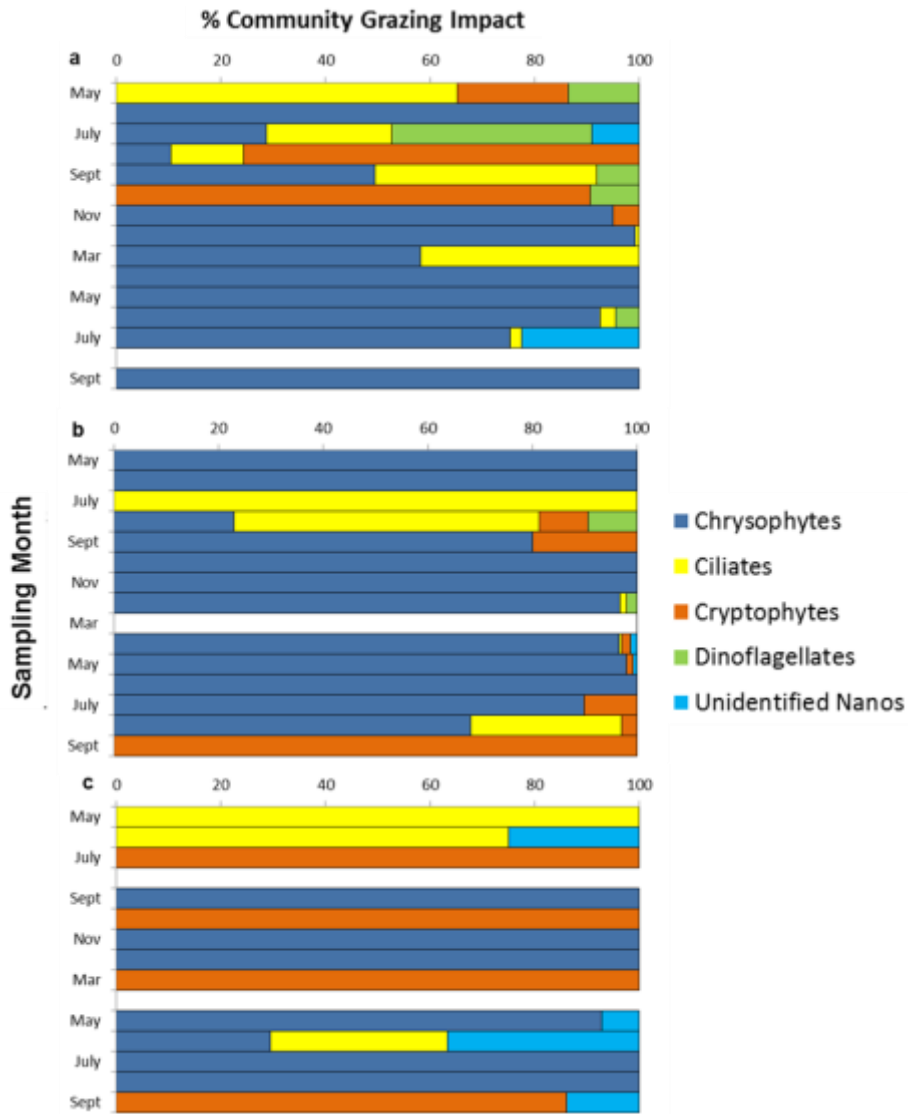
Bacterial abundance ranged from a spring minimum of  $3.5 \times 10^4$  bacteria  $\text{ml}^{-1}$  (recorded in May 2014 metalimnion) to a maximum of  $9.3 \times 10^5$  bacteria  $\text{ml}^{-1}$  in August 2014. Abundance of bacteria fluctuated widely during the entire study period. Protistan community grazing was highly variable, ranging from undetectable to  $8.9 \times 10^3$  bacteria  $\text{ml}^{-1} \text{hr}^{-1}$  (April 2014 metalimnion, Fig. 4.3). This was driven by grazing action of chrysophycean algae which contributed to 95% of the community grazing impact (Fig 4.4). Grazing impact by chrysophytes contributed substantially to community grazing in all depths of Lake Lacawac during the study period, removing a maximum  $8.6 \times 10^3$  bacteria  $\text{ml}^{-1} \text{hr}^{-1}$  in the April 2014 metalimnion. Bacterivory by ciliates rarely exceeded that of other protistan groups and reached a maximum of  $2.5 \times 10^3$  bacteria  $\text{ml}^{-1} \text{hr}^{-1}$  in the September 2013 surface waters. Community grazing impact in the hypolimnion was generally reduced (Fig 4.3), and was seasonally dominated by cryptophytes such as

*Cryptomonas* and *Rhodomonas* (Fig 4.4). Despite an increased dominance of cryptophytes in the depths of Lake Lacawac, their community grazing impact was relatively low compared to the chrysophycean population.

A two-way crossed PERMANOVA revealed a significant effect of depth ( $F_{2, 150}=6, p=0.001$ ), month ( $F_{9, 150}=1.6, p=0.039$ ) and interactive effect ( $F_{18, 150}=1.6, p=0.004$ ) on grazing impact by members of the planktonic community (Table 4.4). Observed similarity in taxa-specific grazing impact was driven by that of *Dinobryon* in the upper depths and *Cryptomonas* in the hypolimnion. Within month similarity in January and March across all depths was driven by *Dinobryon* and *Ochromonas* grazing impact, respectively.



**Figure 4.3:** Total community grazing impact of planktonic cells across 3 depths in Lake Lacawac.



**Figure 4.4:** Relative grazing impact by key protistan groups in the (a) epilimnion, (b) metalimnion and (c) hypolimnion of Lake Lacawac recorded from May 2013 through September 2014.

**Table 4.4:** Summary of two-way crossed PERMANOVA examining differences in planktonic grazing impact across 16 months and 3 depths in Lake Lacawac.

<i>Source</i>	<i>df</i>	<i>SS</i>	<i>MS</i>	<i>Pseudo-F</i>	<i>P(perm)</i>
<b>Depth</b>	2	261.61	130.8	6.03	0.001
<b>Month</b>	9	311.8	34.64	1.59	0.039
<b>Depth x Month</b>	18	637.81	35.43	1.63	0.004
<b>Res</b>	150	3254	21.69		
<b>Total</b>	179	4480			

#### **4.5 Discussion**

The planktonic community in Lake Lacawac showed seasonal patterns that were distinguished by depth. Such patterns in plankton abundance within Lake Lacawac were characterized by a series of “species replacements” in which each depth was often characterized by the dominance of a taxonomic group that varied over seasonal time. This is likely due to differential responses to changes in environmental variables or mixing patterns. Surface waters were characterized by dominance of coccoid and colonial green algae in late spring and early summer, likely as a response to ample availability of PAR and upward mixing of nutrients that precedes the spring bloom. Mid-depths of Lake Lacawac exhibited a similar pattern, but dominance of photosynthetic forms shifted in the late summer and early fall. There was a notable increase in the prevalence of

*Cryptomonas* with depth, particularly in Fall. This was previously observed in Lake Lacawac by Siver and Chock (1986), who documented seasonal population peaks in deeper water. In oligotrophic Lago Maggiore (Italy) *Cryptomonas erosa* was identified throughout the water column despite attenuation of PAR (Callieri *et al.*, 2006). The utilization of mixotrophic nutrition may allow this cryptophyte to survive in the hypolimnion where PAR is reduced (Tranvik *et al.*, 1989). However, Gasol *et al.* (1993) demonstrated sustained growth by *Cryptomonas* at low light levels without substantial bacterivory, which is reflected by the low grazing impact of *Cryptomonas* in Lake Lacawac.

Chrysophycean algae were an important feature throughout the water column in Lake Lacawac during this study. This supports previous findings in which the system was aptly described as a “Chrysophycean lake,” with members of the Chrysophyceae accounting for over 78% of the phytoplankton assemblage (Siver & Chock, 1986a). Dominance of Chrysophycean algae in freshwater lakes is not uncommon due to the range of trophic versatility exhibited by this group (Holen & Boraas, 1995, Koiv & Kangro, 2005, De Hoyos *et al.*, 1998). For example, a large proportion of the plankton biomass in Faroese lakes was composed of mixotrophic flagellates, including *Dinobryon* and *Ochromonas*, that exerted high grazing pressure on the bacterial community (Palsson *et al.*, 2005). Similarly to Siver & Chock (1986), we observed seasonal and vertical shifts in relative dominance amongst chrysophyte taxa. Similar successional changes were also observed in the epilimnion of the chrysophyte-dominated Lake Verevi (Koiv & Kangro, 2005) where succession was largely attributed to outcomes of competition for available phosphorus.

A majority of the planktonic community across all depths in Lake Lacawac was composed of *Ochromonas* in January. This phagotrophic phytoflagellate has been reported to consume bacteria under light and/or inorganic nutrient limitation, and produce antipredatory chemicals that may enhance its competitive abilities (Schmidtke *et al.*, 2006, Katechakis & Stibor, 2006, Hiltunen *et al.*, 2012). In contrast, *Dinobryon* was dominant in the September surface waters and spring metalimnion. Similar results were observed in a seasonal study of chrysophyte community composition in oligotrophic Lake Sanabria in which a substantial *Dinobryon* peak occurred in the autumn epilimnion (De Hoyos *et al.*, 1998). *Dinobryon* has been documented as a major bacterial grazer, particularly in nutrient-depleted environments (Bird & Kalff, 1986b). An example is provided in the epilimnion of Lake Constance, in which *Dinobryon* biomass increased during a period of phosphorus depletion and bacterivory was estimated to provide up to 77% of the phosphorus budget (Kamjunke *et al.*, 2007b). Comte *et al.* (2006) observed a spring bloom of *Dinobryon* throughout the water column of Lake Bourget, where the mixotroph accounted for up to 75% of the phototrophic population. *Dinobryon* may be able to reach numerical dominance during certain times in Lake Lacawac due in part to its ability reach a maximum photosynthetic rate under moderate light conditions and to increase ingestion rate in response to nutrient limitation (Princiotta *et al.*, 2016). It is not surprising that *Dinobryon* was less abundant in the hypolimnion of Lake Lacawac because although it is an efficient bacterivore, *Dinobryon* ceases grazing after extended exposure to darkness (Caron *et al.*, 1993a)

Although total cell abundance was generally reduced in the hypolimnion, a single peak in May 2014 was driven by the presence of *Synura*. This alga numerically

dominated the community in May during both years of the study, but exhibited an otherwise reduced abundance in Lake Lacawac. *Synura* is an exception to the pervasive incidence of mixotrophy within Chrysophyceae and has not been documented to ingest particles. The production of resting cysts (stomatocysts) in *Synura* increases cell survival and provides a seed bank for subsequent populations (Holen, 2014). This may explain the peak in abundance that occurred following ice-out in Lake Lacawac. In a review of Reynold's classification of plankton into distinct functional groups, (Padisak *et al.*, 2003) indicated that *Synura* is sensitive to high irradiances and tolerates humic substances in the water column, which provides support to the distribution patterns observed in Lake Lacawac.

During periods of ice-cover in lakes, aquatic organisms are subject to reduced light availability and low temperatures (Roberts & Laybourn-Parry, 1999, Phillips & Fawley, 2002). Despite these apparent abiotic pressures, the planktonic community in Lake Lacawac reached an overall peak in total cell abundance in the ice-covered surface waters during January. This was driven by a dominance of phytoflagellate members of the Chrysophyceae. Blooms of low-light and cold-adapted phytoplankton have been previously reported under ice (Phillips & Fawley, 2002) Seasonal variation in phagotrophic phytoplankton was also observed in Lake Oglethorpe with highest proportion of mixotrophs in winter (Bennett *et al.*, 1990b). In a study of phytoplankton species composition over 18 years in the winter community of Lake Muggelsee, Oezkunkakci *et al.* (2016) indicated the importance of nutritional mode and ability to form resting stages. Members of the Chrysophyceae are often low-light adapted and form cysts to survive inhospitable surroundings, including arctic lakes. (Sandgren, 1981,

Charvet *et al.*, 2012). The ability to ingest bacteria in primary phototrophs serves as an additional nutrient source, particularly under light-limited conditions.

With the exception of the hypolimnion, bacterivory by heterotrophic and mixotrophic taxa exerted pronounced grazing pressure on the bacterial standing stock. Taxon-specific bacterivory varied seasonally, with a community maximum in the surface waters during September. Total community grazing impact by protists reported here is similar to that for other freshwater systems (Comte *et al.*, 2006). A previous study in Lake Lacawac demonstrated an overall greater grazing impact by mixotrophic nanoflagellates under ice than that of heterotrophic bacterivores that was likely driven by a dominance of *Dinobryon*, which exhibited the greatest contribution to total bacterivory in this study (Berninger *et al.*, 1992c). Although *Dinobryon* is a primary phototroph that derives a majority of its carbon by photosynthesis, ingestion rates up to 95 bacteria cell<sup>-1</sup> h<sup>-1</sup> have been reported in natural plankton communities (Caron *et al.*, 1993a). Impacts on the bacterial community can be great. For example, spring *Dinobryon* blooms in a shallow humic lake coincided with a precipitous reduction in bacterial abundance and shift to grazing-resistant filamentous forms (Kent *et al.*, 2004). Several *Dinobryon* taxa were especially abundant in the surface waters of Lake Oglethorpe, accounting for up to 89% of total mixotroph grazing (Sanders *et al.*, 1989a).

Ciliates are also considered key bacterivores along with heterotrophic and mixotrophic nanoflagellates in many other freshwater systems, but they are typically less abundant with higher feeding rates than flagellates (Sonntag *et al.*, 2006, Carrias *et al.*, 1996b, Sanders *et al.*, 1989a). Ciliates generally contributed to <10% of the planktonic protistan abundance across all depths, but their concentration in Lake Lacawac was

comparable to that observed in other freshwater systems (Comte et al., 2006). The low relative abundance of ciliates resulted in reduced grazing impact on bacteria compared to flagellates. Ciliate bacterivory reached a peak in the June hypolimnion when grazing by other members of the community was relatively reduced. Several of the ciliates identified in Lake Lacawac are known to preferentially ingest algal cells, including autotrophic picoplankton and dinoflagellates (Esteban *et al.*, 2010, Pestova *et al.*, 2008).

This study provides a comprehensive perspective on planktonic community dynamics in a protected ecosystem. These results are generally consistent other analyses of the microbial assemblage, however, direct comparisons between studies can be difficult due to differences in taxonomic resolution. Further, studies of temporal changes in community composition are confounded by trophic state of the water body, which plays an ultimate role in the baseline “species inventory” (Berninger et al., 1992c). Most of the taxonomic groups described here displayed interannual patterns in abundance. Year-to-year variation in community composition is not uncommon and may have been due to the sampling frequency utilized during the study period. For example, (Soininen *et al.*, 2005) observed large variability in phytoplankton community assembly among years in a boreal lake. Transitions in the microbial assemblage can occur on the order of hours to days due to short generation times (Weisse *et al.*, 1990). Further, lakes with a small water volume are increasingly subject to climactic fluctuations (Padisak et al., 2003). Regardless, comparisons of patterns in planktonic community composition are essential to predicting how assemblages will change under global change scenarios. As the role of mixotrophic protists in ecosystem processes becomes increasingly evident, a better understanding of spatio-temporal changes in community composition and grazing impact

is vital. In addition, the eminence of mixotrophy as a popular strategy amongst phytoflagellates adds a further complication to studies of microbial food web dynamics.

## CHAPTER 5

### SUMMARY AND CONCLUSIONS

One of the fundamental goals of ecological study is to connect results from controlled culture experiments with *in situ* field observations. It can be argued that knowledge about individual plankton species is a prerequisite to understanding their relative contribution and importance in the plankton assemblage. The overarching goal of this body of work was to investigate mixotrophic behavior in freshwater plankton, from physiological work in culture to spatio-temporal dynamics in a freshwater lake. The culture work conducted in *Dinobryon sociale* (as described in chapter 2) provides a comprehensive investigation of the response of phototrophy and phagotrophy as they occur simultaneously under various abiotic conditions. Although considerable work has resulted in well-characterized responses of various metabolic processes in pure phototrophs and heterotrophs, less has been done in mixotrophic protists that span a gradient of nutritional modes. Mixotrophic protists have a range of metabolisms and likely exhibit physiological tolerances unlike those of pure phototrophs or heterotrophs. It is expected that this will be reflected in their distribution patterns in nature. The notion that mixotrophs can adapt to variable abiotic conditions that may push the physiological boundaries of nutritional generalists was examined in a field study that compared the abundance, distribution and grazing impact of heterotrophic and mixotrophic nanoflagellates in a freshwater lake (as described in chapter 3). Whereas a majority of previous field studies of seasonal dynamics of planktonic protists focus on a single depth, this work extended into vertical structure as a response to habitat heterogeneity provided

by thermal stratification. Incidence and importance of mixotrophic behavior was also studied on multiple taxonomic scales, first within a subset of the planktonic population (nanoflagellates), and next as a functional group within the greater planktonic community (as described in chapter 4). The field system utilized in this work provided an ideal baseline for the study of planktonic dynamics because anthropogenic impacts are reduced and mixotrophic taxa are seasonally dominant.

### *5.1 Dinobryon sociale: a model for mixotrophy*

Mixotrophic behavior was characterized in a Chrysophycean alga belonging to a genus with a near cosmopolitan distribution in lakes. Members of this taxonomic group are among the most common and abundant phytoplankton in temperate lakes of the northern hemisphere (Tolotti et al 2003). Although this work was conducted in a freshwater species, members of the genus *Dinobryon* can also be common in coastal oceans. *Dinobryon sociale* is an effective model for the study of bacterivory in phagotrophic phytoflagellates because although phototrophy represents the primary nutritional mode, these chrysophytes are capable of substantial impacts on the bacterial population at certain times. This suggests that there may be a physiological trigger that influences the relative rate of phagotrophy. The major objective of chapter 2 was to examine the potentially changing contributions of both nutritional modes utilized by *D. sociale* and identify factors that regulate bacterivory. The results of this chapter add to a growing body of work dedicated to the physiology of phagotrophic phytoflagellates.

Maximal rates of carbon fixation and bacterial ingestion rate in *D. sociale* occurred at 16°C, but the relationships were differentially influenced by temperature and

light conditions. Whereas the light levels utilized in these experiments did not influence ingestion rate, primary productivity generally increased with temperature and light with the exception of 8°C, the lowest temperature tested. PE curves revealed that at 16°C, a greater maximum photosynthetic rate ( $P_{\max}$ ) was reached at a lower optimal irradiance ( $I_{\text{opt}}$ ) than at 12°C and significant photoinhibition at 20°C. This supports the peak performance observed at the moderate temperature of 16°C. Calculations of the percent carbon acquired by either nutritional mode revealed that although a majority of the carbon budget was supplied by photosynthesis, there was an increase on reliance on bacterivory at 8°C under both light treatments. This can be attributed to the differential effects of temperature on heterotrophic vs. phototrophic metabolism. In comparison to other temperature treatments, there was a moderate reduction in grazing and significant reduction in primary production at 8°C that may have precipitated an increased nutritional contribution supplemented by bacterivory.

Field reports from a variety of aquatic ecosystems have indicated the importance of *Dinobryon* as a bacterivore. This is difficult to rectify considering the dominance of photosynthesis in its nutrition. This work has shown that ingestion rate by *Dinobryon sociale* increases in response to reductions in the concentration of nitrogen and phosphorus. Therefore, phagotrophy may be sustained in order to obtain limiting macronutrients. This is supported by previous field studies that have identified *Dinobryon* as an abundant component of the plankton in many oligotrophic environments.

The results of chapter 2 provide insight into the balance between phototrophy and bacterivory, and how this balance is moderated by abiotic conditions such as temperature, light and nutrient concentration, as well as prey availability. Future work should focus on

direct comparisons of photosynthetic abilities and phagotrophic efficiency between mixotrophs and pure phototrophs or heterotrophs, respectively. This can provide answers to a growing debate surrounding the possible competitive advantage of mixotrophic nutrition, where some argue that maintaining multiple metabolic apparatuses is unfavorable. Although such contrasts were not made in this dissertation work, it paves the way for experiments designed to compare competitive abilities of nutritionally diverse protists that may ultimately inform predictions for dynamics in nature.

### *5.2 Nanoflagellate mixotrophy in Lake Lacawac*

Summer thermal stratification is an important natural phenomenon in temperate aquatic systems that acts as a structuring force for planktonic communities by creating physical and chemical gradients in the water column. This leads to periods of increased habitat heterogeneity that are interrupted on a predictable, seasonal basis with the breakdown of thermal stratification in the fall. Planktonic organisms are influenced by fluctuations between mixing and thermal stratification in freshwater lakes and their spatio-temporal distribution are directly affected by the various abiotic factors that follow water column patterning. These include temperature, irradiance, and dissolved nutrients. The objective of chapter 3 was to explore dynamics of the nanoplanktonic assemblage, with a focus on variability between metabolically diverse flagellated forms.

Vertical distribution and bacterivory of nanoflagellates was related to water column structure in Lake Lacawac, but different patterns emerged based on trophic mode. Whereas phototrophic and heterotrophic nanoflagellates were unevenly distributed throughout the water column regardless of physical structuring, mixotrophic

nanoflagellates exhibited a change in vertical distribution depending on water column structure. The phagotrophic phytoflagellates in the nanoplanktonic size range were evenly distributed throughout the water column during mixis, suggesting a potentially opportunistic approach to niche discrimination. This is supported by the lack of significant correlations between mixotroph abundance and measured abiotic variables during isothermal conditions. In a water column marked by nearly isothermal conditions, some mixotrophic nanoflagellates may be able to take advantage of the warm, well-light epilimnion or the darker hypolimnion that may be light-limiting (with an exception during mixis) with ample bacterial food. During thermal stratification however, mixotroph abundance was significantly reduced in the hypolimnion. It is expected that this group was constrained to the surface and midwaters during stratification as reflected by significant, positive correlations between mixotroph abundance and temperature, oxygen and PAR. Whereas mixotrophs may have adapted a seemingly opportunistic approach to niche partitioning due to their ability to survive within a variety of conditions, pure phototrophs and heterotrophs may be bound by stricter resource requirements that dictate their distribution in the water column.

Mixotrophic nanoflagellates were a significant component of the bacterivorous population in Lake Lacawac. Water column structure altered the relative importance of grazing impact on bacteria by heterotrophic and mixotrophic nanoflagellates. Whereas heterotrophic nanoflagellates always had the greatest grazing impact in the hypolimnion, mixotrophic grazing exceeded that of pure heterotrophs in the epi- and metalimnion during thermal stratification. This may reflect the ability of mixotrophic organisms to utilize phagotrophic ingestion of bacteria when nutrients or PAR is limiting. Some

mixotrophic organisms, such as *Dinobryon*, require photosynthetic fixation of carbon and will not ingest bacteria in the absence of suitable levels of PAR. However, others such as *Poteroichroomonas* can survive in darkness in the presence of sufficient bacterial food. Ingestion by heterotrophs, on the other hand, is not likely moderated by light availability, allowing them to thrive even at depths of complete darkness. The results of this chapter speak to the advantages afforded by nutritional flexibility in mixotrophic nanoflagellates and elucidates the instances in spatio-temporal time when they may dominate the flagellate assemblage.

### *5.3 Mixotrophy in a community context*

Due to their pivotal roles in biogeochemical cycling, investigations of microbial diversity and community composition are important to understanding their part in the ecological functioning of the microbial loop and lake functioning as a whole. The prevalence of phagotrophy in members of the phytoplankton elicits a need to include mixotrophy in studies of the protistan assemblage. Because mixotrophic organisms possess a unique underlying metabolism, they may violate the seasonal patterns exhibited by other members of the plankton assemblage. Studies of planktonic community composition over vertical space and time that include the influence of mixotrophs are rare. This is likely due in part to the limitations of identifying organisms that have few distinguishable morphologies discernable by light microscopy. Further, staining and fixation protocols can often distort protistan morphology. Finally, documentation of mixotrophy by ingestion of bacterial surrogates only allows identification of actively-feeding members of the community.

In Lake Lacawac, community composition changed significantly with depth and season. Mixotrophs occurred within all depths, but the dominant species varied. This provides evidence that phagotrophy phytoplankton can occupy a number of different ecological niches with interspecific variability. The most remarkable changes in relative dominance occurred between the epi- and metalimnion and hypolimnion. Whereas Chrysophycean algae such as *Dinobryon* and *Ochromonas* were most abundant in surface and mid-waters, *Cryptomonas* dominated the depths. The cryptomonad may have a reduced requirement for PAR that could place spatial limitations on the described chrysophytes. A majority of the Chrysophyceae are obligate phototrophs that have shown global distributions related to nutrient concentration with a preference for oligotrophic lakes. Previous studies have suggested that cryptophytes are tolerant to anaerobic environments, as well as low light and temperature. Further, they have been described as an opportunistic group that increases in abundance when other algal groups are at a minimum. The dynamics between members of the Chrysophyceae and Cryptophyceae provides an excellent example of niche partitioning within mixotrophic species that reflects differences in physiological tolerance and competitive abilities.

Mixotrophic flagellates were significant members of the community in winter, supporting the idea that they are of major importance for the survival of the planktonic community under ice. This may be due to their ability to supplement photosynthesis with phagotrophy in conditions of reduced light availability. The results of chapter 4 suggest that mixotrophy plays a significant, year-round role in the dominant taxa of freshwater phytoplankton, thereby contributing significantly to the community composition of plankton communities.

**APPENDIX A**

**PHYSIOCHEMICAL CHARACTERISTICS OF LAKE LACAWAC (EPI- AND METALIMNION)**

	<b>Date</b>	<b>Temp (°C)</b>	<b>O<sub>2</sub> (mg L<sup>-1</sup>)</b>	<b>PAR (μmol m<sup>-2</sup> s<sup>-1</sup>)</b>	<b>Bacteria (x 10<sup>5</sup> ml<sup>-1</sup>)</b>	<b>Secchi (m)</b>	<b>RTR</b>
<b>Epilimnion</b>	2013 May 19	16.5	11.7	73.37	3.28	3.5	115.1
	June 26	26.6	7.6	331.4	6.80	3.5	249.7
	July 26	26	6.8	315.4	5.02	3.5	326.7
	Aug 24	24.5	9.5	348.8	3.23	4.5	241.8
	Sept 22	18.5	12.3	90.82	6.35	3.5	167.4
	Oct 24	13.1	19	109.87	5.09	2	49.9
	Nov 23	6.1	13.5	118.88	5.16	3.25	0.32
	2014 Jan 23	2.5	n.d.	61.54	7.29	1	1.72
	Mar 01	1.87	8.5	71.48	4.40	n.d.	4.43
	Apr 13	9.4	10.9	247.3	4.61	2.75	20.6
	May 14	18.5	8.8	361.4	0.745	4	136.4
	June 12	20.5	6.5	47.28	8.40	2.75	186.7
	July 08	25.6	6	334.5	3.17	5	295.2
	Aug 07	23.8	7.3	110.79	5.81	3.5	264.4
	Sept 26	18.4	8.0	227.6	2.51	3.75	115.4
<b>Metalimnion</b>	2013 May 19	10	12.5	3.52	3.33		
	June 26	15	10.5	14.23	1.95		
	July 26	20.1	4.5	18.63	7.14		
	Aug 24	21.5	9.7	37.72	2.64		
	Sept 22	14.5	5.8	2.87	3.71		
	Oct 24	12.9	7.5	5.67	1.09		
	Nov 23	5.6	12.3	8.12	2.56		
	2014 Jan 23	3.1	n.d.	1.95	1.82		
	Mar 01	3.6	12.2	5.72	1.92		
	Apr 13	7.5	10.7	59.12	2.77		
	May 14	13.2	10.4	103.31	0.346		
	June 12	15	7.85	9.34	2.59		
	July 08	19.7	4.7	41.01	1.68		
	Aug 07	19	2.6	38.94	9.33		
	Sept 26	17.1	7.55	13.25	2.45		

**APPENDIX B**

**PHYSIOCHEMICAL CHARACTERISTICS OF LAKE LACAWAC  
(HYPOLIMION)**

	<b>Date</b>	<b>Temp (°C)</b>	<b>O<sub>2</sub> (mg L<sup>-1</sup>)</b>	<b>PAR (μmol m<sup>-2</sup> s<sup>-1</sup>)</b>	<b>Bacteria (x 10<sup>5</sup> ml<sup>-1</sup>)</b>
<b>Hypolimnion</b>	2013 May 19	6	14.5	0.11	5.40
	June 26	8	4.3	2.1	7.75
	July 26	11.3	4.9	5.71	2.10
	Aug 24	13	8	5.25	1.44
	Sept 22	8.2	5.1	1.18	8.26
	Oct 24	9.1	3.1	1.33	2.79
	Nov 23	5.6	13.2	2.21	5.82
	2014 Jan 23	3.3	n.d.	0.45	3.04
	Mar 01	3.8	10	1.08	0.774
	Apr 13	6	10.3	3.71	0.933
	May 14	9.9	9.3	3.89	2.61
	June 12	10.7	4.77	2.31	2.0
	July 08	12.5	3.3	8.88	0.933
	Aug 07	11.6	0.5	1.59	3.79
	Sept 26	10.5	0.25	2.17	7.10

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