INFLUENCE OF ABIOTIC ENVIRONMENTAL FACTORS ON PHYSIOLOGICAL RESPONSES AND MIXOTROPHY IN FRESHWATER AND MARINE CHRYSOPHYTES

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ABSTRACT

Global climate change represents one of the greatest threats to biodiversity. Phenomena such as rising surface water temperature, increased UV radiation, and ocean acidification have brought negative impacts to ecosystems and their inhabitants. Sensitive to various abiotic factors, microbial eukaryotic communities in aquatic systems are particularly being affected by these environmental changes. Specifically, warming temperature not only can directly affect plankton through limiting growth and inhibiting physiological processes, but can also indirectly impact these organisms by altering light and nutrient availability via loss of sea ice and changes in thermal stratification in various environments.

Mixotrophic chrysophytes are an important lineage of protists that often dominate phytoplanktonic blooms in both freshwater and marine systems. Studies have shown mixotrophic organisms' nutrient-acquiring strategies are influenced by abiotic environmental factors. Temperature in particular, is known to alter growth rate and bacterivory. In response to rising temperature, mixotrophs can either become more phototrophic or more heterotrophic, depending on species, resulting in changes of their role in aquatic food webs and potentially leading to shifts in overall community composition and structure.

The objective of this research is to investigate the influence of different environmental factors on primary production and heterotrophic ingestion in marine and freshwater chrysophytes, providing an understanding on how climate change may alter physiological response and survival, with indicative changes in community structures and food webs.

The influence of irradiance, nutrient concentrations, and temperature on mixotrophic responses of the Arctic marine chrysophyte *Dinobryon faculiferum* was investigated, where our results demonstrated an increase in heterotrophic ingestion in response to rising temperature. We also found bacterivory contributes a major proportion of *D. faculiferum*'s carbon budget in comparison to primary production, which is different from previous studies on *Dinobryon* species that appeared to be more reliant on phototrophy.

Conversely, the freshwater chrysophyte *Chrysolepidomonas dendrolepidota*, exhibited the opposite temperature effect. The freshwater species was more reliant on primary production and ingested less as temperature increased. Such varying responses showcased diverse nutrient strategies on the mixotrophic spectrum, suggesting generalization of mixotrophic mode in predictive models should be approached with caution. Additional work was done to gain insight on the biogeography of *C. dendrolepidota*, of which little is known about its distribution. The presence of *C. dendrolepidota* was not detected through metadata analysis, nor was it detected across several waterbodies sampled in this study. Our results suggested possible rare distribution and endemism of *C. dendrolepidota*.

To my parents, Jack Chang and Tracy Lee

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CHAPTER 1

INTRODUCTION

1.1 Mixotrophy and Mixotrophic Chrysophytes

Aquatic protists are ubiquitous and important components of freshwater and marine ecosystems (Hartmann et al., 2012; Jeong et al., 2012; Mitra et al., 2014). Playing various roles in food webs, they are crucial links in the microbial loop and trophic systems (Jeong et al., 2010; Lofton et al., 2020). Autotrophic protists are important primary producers, while heterotrophs are predators of bacteria and other plankton (Montero et al., 2017; Sanders et al., 2001). Many protists have been found to be mixotrophic, having the ability to utilize both photosynthesis and heterotrophic ingestion to gain energy and limiting elements (Mitra et al., 2016). When solar energy is limiting, mixotrophic protists utilize phagotrophy to capture food and obtain essential elements such as nitrate through the consumption of prey. When dissolved nutrients are limited, mixotrophs can combine photosynthetic and phagotrophic nutrition and use ingested organic nitrogen and phosphorus to enhance population growth (Mitra et al., 2016; Sanders et al., 2001). Mixotrophy is a spectrum of relative photosynthetic and consumptive abilities, and species can either be more autotrophic or heterotrophic (Flynn et al., 2013).

As both primary producers and predators, mixotrophic protists are known to play an important role in aquatic food webs and nutrient cycling (Adl et al., 2007; McKie-Krisberg & Sanders, 2014; Mitra et al., 2014). Mixotrophic flagellates have been found to be responsible for up to 40 - 95% of total bacterivory across a variety of aquatic

environments (Chan et al., 2019; Domaizon et al., 2003; Havskum & Hansen, 1997; Unrein et al., 2007). Studies in polar regions suggest mixotrophic flagellates have significant ecological impacts on polar food webs as well (Countway et al., 2010). Not only does bacterial grazing by mixotrophs introduce previously unavailable resources into higher trophic levels, in some cases, it can also facilitate remineralization of nutrients that are released in dissolved form during bacterivory (Princiotta et al., 2016).

The ability to utilize two nutritional modes enables mixotrophic protists to overcome abiotic limitations such as light and nutrients, as well as biotic constraints of competition and prey availability (Chan et al., 2019; Wilken et al., 2020). As anthropogenic-driven climate change continues to reform the environment, mixotrophic protists are projected to be more tolerant of environmental changes, and perhaps in some cases less dependent on phototrophy (Wilken et al., 2013).

Chrysophyta, also known as the golden algae, is a diverse group of flagellated protists (Lengyel et al., 2022). Chrysophytes are defined by the presence of two flagella, one long and one short (heterokont), along with chloroplasts containing chlorophyll a and c (Andersen, 2004). With several distinct lineages, chrysophytes exhibit a wide array of morphological traits and characters; although mainly unicellular, colonial species are also observed (Lengyel et al., 2022; Walter & Whiles, 2010). In some chrysophytes, proteinaceous shells or silica scales are known to cover the cell, offering a form of protection (Walter & Whiles, 2010). Silica-scaled chrysophytes have also been suggested to be important silicifiers in aquatic systems (Feng et al., 2009; Lengyel et al., 2022).

1.2 Effects of Temperature and Nutrients on the Freshwater Chrysophyte ***Chrysolepidomonas dendrolepidota***

Most chrysophytes have been found to be mixotrophic, of which some are considered as primarily phagotrophic phototrophs while others are more dependent on photosynthesis (Lim et al., 2019; Princiotta et al., 2016; Wilken et al., 2013). Mixotrophic chrysophytes play important roles in aquatic food webs as primary producers and predators of bacteria and phytoplankton, often dominating biomass in planktonic blooms in freshwater rivers and lakes (Adl et al., 2007; Charvet et al., 2014; Søgaard et al., 2021).

Chrysolepidomonas dendrolepidota was recently reported to be mixotrophic (Hamsher et al., 2020). Yet little is known of its distribution, ecology or mixotrophic responses to abiotic environmental factors. We hypothesized it would shift its nutritional mode to become either more phototrophic or heterotrophic along changing temperature and nutrient levels.

1.3 Temperature Dependent Phagotrophy and Phototrophy in the Arctic Marine Chrysophyte ***Dinobryon faculiferum***

Climate change has been projected to have a pronounced effect in Arctic waters (Lewis et al., 2019; Screen & Simmonds, 2010; Steele et al., 2008). Phytoplankton blooms typically happen in late spring to early summer in polar systems when there is more available sunlight and mixing of dissolved nutrients. The loss of sea ice has increased stratification and reduced thermocline depth in these waters (Teufel et al., 2017), limiting mixing of nutrients and inhibiting phytoplankton growth. As a result of

the shift in nutrient cycling, the growing season and size of planktonic blooms also changes (Ardyna et al., 2014).

Polar mixotrophs experience a narrow range of summer temperature and are thus expected to be sensitive to warming oceans (Charvet et al., 2014), yet summer temperatures in parts of the Arctic and Southern Oceans already exceed optimal temperature thresholds for several polar phytoplankton (Hop et al., 2020).

Species of the genus *Dinobryon* are known from both freshwater and marine phytoplankton communities. Although mainly phototrophic, freshwater *Dinobryon* species have been observed to have high rates of bacterivory, especially in environments where light is not optimal and nutrient levels are low (Bird & Kalff, 1987; Caron et al., 1993; Unrein et al., 2010). Less is known for marine species, with only one study of bacterivory for a strain of *Dinobryon* (Unrein et al., 2010). We examined mixotrophic responses of *Dinobryon faculiferum* under an assortment of light, temperature, and nutrient levels. Based on previous studies, we hypothesized that *D. faculiferum* would become more heterotrophic under the stress of rising temperature and limited nutrient availability (Wilken et al., 2013).

1.4 Exploring Biogeographical Distribution of a Poorly Identified Freshwater Chrysophyte

Even though protists play crucial roles in nutrient cycling and biogeochemical processes, there are surprisingly limited studies on the biogeography and distribution of these eukaryotic microorganisms (Dolan, 2005; Logares et al., 2014, 2015). The lack of attention on microbial biogeography stems from the long-standing assumption that

protists and other microbes are ubiquitous, i.e., that everything is everywhere (Fenchel & Finlay, 2004) , and are least of concern when discussing potential distributions and rarity (DeWit & Bouvier, 2006; Dolan, 2005; Logares et al., 2015).

The ubiquity model however has many flaws, with more and more studies coming out in support of restricted distribution and potential endemism of protists (Cotterill et al., 2008, 2013; Dolan, 2005; Gusev et al., 2018; Škaloud et al., 2014; Weinbauer & Rassoulzadegan, 2007).

In addition, advancements in molecular methods have led to discoveries of rare protist taxa and abundance patterns (Debroas et al., 2015, 2017). The term "rare biosphere" has been coined to describe the vast number of rare species found in protist communities (Debroas et al., 2015; Logares et al., 2014; Sogin et al., 2006).

C. dendrolepidota is a freshwater chrysophyte that was isolated and identified from Lake Medora, Michigan USA, in 1984. No subsequent field studies or research have reported its presence. To gain insight on potential distribution of this species, we used molecular methods to investigate for the presence of *C. dendrolepidota* in several freshwater systems.

CHAPTER 2

EFFECTS OF TEMPERATURE AND NUTRIENTS ON THE FRESHWATER CHRYSOPHYTE *CHRYSOLEPIDOMONAS DENDROLEPIDOTA*

2.1 Abstract

Mixotrophic chrysophytes are known to have significant contributions to primary productivity and grazing impacts within the microbial loop in aquatic systems. Understanding how projected changes in a warming environment might alter physiological responses in mixotrophic protists is crucial. In this study, we investigated mixotrophic responses of the freshwater chrysophyte, *Chrysolepidomonas dendrolepidota*, under an array of temperatures (14-20°C) and nutrient conditions (1-50% DY-IV nutrient media). Overall, photosynthetic rates of C. *dendrolepidota* were reduced in lower nutrient concentrations and increased with rising temperature. Bacterivory rates were not significantly different across nutrient regimes but decreased with rising temperature. A higher temperature promoted rapid initial growth in nutrient replete conditions, but did not sustain population size, presumably due to nutrient depletion. Our results indicate that phototrophy and phagotrophy by *C. dendrolepidota* are strongly affected by temperature, with a shift towards more phototrophic nutritional mode as temperature increased. While some mixotrophs have been shown to be more heterotrophic with increasing temperature, photosynthesis contributes more to *C. dendrolepidota*'s carbon budget with increasing temperature. These findings demonstrate environmental factors can induce different physiological responses along the gradient of mixotrophic abilities, which should be taken into consideration in future works involving models of climate-change impacts on mixotrophs.

2.2 Introduction

Chrysophytes are a diverse group of protists characterized by having chloroplast(s) containing chlorophyll a and c, and the presence of a heterokont flagella (Stramenopiles) (Lengyel et al., 2022; Kristiansen & Škaloud, 2017). This group has distinct morphological lineages and traits, existing in both freshwater and marine systems (M.Jeong et al., 2019; Lengyel et al., 2022). However, out of approximately 1200 species described, most are predominant in freshwater systems (Kristainsen & Preisig, 2001). Chrysophytes are important primary producers in freshwater systems, where they are often the major phytoplankton group and can contribute up to 85% of the phytoplankton community biomass (Kalinowska & Grabowska, 2016; Kristiansen & Škaloud, 2017). Among freshwater chrysophytes, mixotrophic species (as defined in Mitra et al 2016 as photo-osmo-phago-mixotrophs) are known to play a major role in aquatic food webs and microbial loops, often dominating in planktonic blooms (Lengyel et al., 2022; Sina et al., 2007).

C. dendrolepidota is a mixotrophic chrysophyte that was originally isolated and described from Lake Medora, Michigan, USA (Peters & Andersen, 1993). Little is known about this species; however, the genus is known to have a wide distribution. Members of this genus have been identified via microscopy and/or DNA sequencing from freshwaters in the USA, Canadian Arctic and Great Britain (Peters & Andersen, 1993; Charvet et al., 2014; Esteban et al., 2012) and *Chrysolepidomonas* (formerly *Sphaleromantis*) *marina* and *Chrysolepidomonas spp.* have also been identified along coastal marine waters of western USA and southern Australia (Boopathi et al., 2015; John et al., 2011; LeRoi & Hallegraeff, 2006).*C. dendrolepidota* is the only member of its genus thus far reported to

be mixotrophic. Some environmental variables that influence ingestion were investigated by Hamsher et al. (2020), who found mixotrophy in *C. dendrolepidota* was influenced by nutrient availability and light levels. The effects of temperature, a known factor limiting physiological processes and growth in protists, have not been documented previously for this species. Some mixotrophic species can become more reliant on heterotrophic feeding as temperature increases, suggesting changes in mixotrophic species' role from producers to consumers (Kang et al., 2020; Wilken et al., 2013;You et al., 2020). Other mixotrophs reduce ingestion at higher temperatures (González-Olalla et al., 2019; Princiotta et al., 2016). It is expected for global surface temperature to rise another 1.4 to 5.8ºC in the next century (Heino et al., 2009). With temperature having varying effects on phagotrophy in mixotrophic species, it is difficult to predict the role they will play in future aquatic systems. Yet understanding how different mixotrophs respond to temperature is necessary since dominance of mixotrophs can alter composition of food webs and efficiency of carbon transfer in microbial loops (Jeong et al., 2010; Kristiansen & Škaloud, 2017; Vad et al., 2020).

The aim of this study is to investigate the influence of temperature, nutrient availability, and their combined effects on phagotrophy and phototrophy in the mixotrophic *C. dendrolepidota*. The experimental ranges used in this study are currently observed in natural systems and provide a baseline to changes projected to occur in a warming environment. Along with growth rate assessed under different temperature conditions, we determined phagotrophic and phototrophic carbon acquisition in *C. dendrolepidota,* using the uptake of fluorescent microspheres as tracers of bacteria ingestion and ^{14}C carbon fixation, respectively.

2.3 Materials and Methods

2.3.1 Strain Information and Experimental Setup

C. dendrolepidota (CCMP293) was originally isolated from Lake Medora, Keweenaw County, Michigan (47.4374° N, 87.9611° W) during spring 1990. Non-axenic cultures were obtained from National Center for Marine Algae and Microbiota (NCMA; Bigelow Laboratory for Ocean Sciences, Boothbay, ME). Stock cultures were maintained at 14°C in a 50% DY-IV algal medium originally designed for *Dinobryon* (Sanders et al., 2001) with a few grains of rice under fluorescent lamps with a light illumination of 100 $\mu mol \cdot sec^{-1} \cdot m^{-2}$. The light: dark photoperiod was set at 14h:10h. pH level of the cultures were maintained by the MES buffer included in the DY-IV algal medium (Sanders et al., 2001). Cultures were tested using a pH meter (YSI model No. pH100; YSI Inc., Yellow Springs, Ohio, USA), over the course of experimental work and the pH was maintained at 6.8 – 6.9. For experimental setups, cultures of *C. dendrolepidota* were incubated at three different temperatures, 14°C, 17°C, and 20°C with three replicate flasks (50 mL) maintained for each temperature. Temperature was controlled in an incubator with maintenance levels of irradiance. To simulate low and high nutrient conditions, a nutrient dilution series without autoclaved rice was prepared to obtain the three experimental levels of 1%, 5% and 50% DY-IV.

2.3.2 Growth Assessment

Growth of *C. dendrolepidota* was examined for all experimental temperatures under maintenance nutrient level (DY-IV 50%). Cultures were acclimated at each temperature for a total period of two weeks to. To determine growth of *C. dendrolepidota* after acclimation, the cultures were diluted with 50% DY-IV to a starting abundance of 1,000 cells mL^{-1}, and three 3 mL samples were collected and fixed with 10% Lugol's iodine approximately every 24 hours. Abundance was determined using a 1.35 mL settling chamber examined with the Zeiss Axiovert 10 inverted microscope at 400x magnification. A minimum of 20 random fields per sample were counted.

The growth rates for *C. dendrolepidota* at each temperature were calculated from the change of cell abundance over time (days). The maximum growth rates were determined between day 1 and day 3 for 20°C and 17°C, and between day 4 and day 7 for 14°C. Likewise, cell yields for each temperature were calculated as the maximum average abundance minus the average abundance at T = 1 day.

Time needed for the population to double in abundance (doubling time) was calculated from the exponential portion of the growth rate of each temperature. Carbon needed for growth doubling was estimated as a product of the number of cells, and the carbon content of a single cell estimated using cell volume calculated as a sphere. Pictures of cells were taken and analyzed using ImageJ, where no differences in cell volume and diameter at different temperatures were observed. Applying the carbon to volume relationship determined by Menden-Deuer and Lessard (2000), where pg C

cell^{-1}= 0.216×volume$^{0.939}$, the value of carbon content for a single cell was approximately 18.3 pg C cell^{-1}.

2.3.3 Grazing Experiments

Before assessment, triplicate culture flasks were acclimated to each desired experimental temperature and nutrient condition for two weeks. For each condition, polycarbonate microspheres (0.6 µm dia., Polysciences) were added to 3 mL cultures at a final concentration of 5 x 10^5 µspheres mL^{-1}. The microspheres were of similar size to the resident bacterial population and served as a tracer for ingestion. The sample vials were rotated and inverted to allow thorough mixing and dispersal of microspheres upon addition. Subsamples were taken and fixed with Lugol's solution immediately (T0) to account for background incidence. The replicates were fixed after 30 minutes of incubation (T30) following a protocol that prevents egestion (Sherr & Sherr, 1993) – i.e., fixation with Lugol's solution, followed by formalin, and clearing with sodium thiosulfate. Samples were collected onto 0.2 µm pore-size polycarbonate filters, mounted on a slide with Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA), and observed under 1000x magnification using epifluorescence on an Olympus BX41 (Olympus Corporation, Shinjuku, Tokyo, Japan) microscope.

An average number of ingested microspheres consumed at each temperature and nutrient level combination was determined by examining a minimum of 100 cells per replicate. Overlapping of non-ingested microspheres with cells was accounted for by subtracting T0 counts from the number of ingested microspheres in each T30 replicate

(Sherr & Sherr, 1993). Bacteria were enumerated by epifluorescence microscopy from T0 samples and average abundance was determined.

Under the assumption that microspheres were ingested at the same rate as bacteria, the rate of ingestion was determined using the following equation:

Ingestion=

$$\frac{\text{bacterial–sized particles}}{\text{protist} \times \text{hour}} = \frac{(\text{Microspheres ingested} \times \frac{\text{bacteria}}{\text{microsphere}}) + \text{microspheres ingested}}{\text{protist} \times \text{incubation time (hr)}}$$

Where microspheres ingested = average number of microspheres ingested, bacteria = bacteria concentration (cells mL^{-1}), microspheres = microsphere concentration (beads mL^{-1}), protist = number of protists with ingested microspheres (100 cells). This is the total ingestion rate of bacterial-sized particles. Carbon from bacterivory was estimated using the product of carbon content (20 fg) of a single bacterium (Lee and Fuhrman, 1987), the ingestion rate per protist cell, and the number of protist cells produced during doubling.

2.3.4 Primary Production

To assess the phototrophic ability of *C. dendrolepidota*, fixation of carbon by protists grown under different abiotic factors was measured via the uptake of ^{14}C using methods adapted from MacIntyre et al. (1996) with additional centrifugation techniques modified from Smith and Azam (1992). For each experimental condition, 2 mL samples were taken (4 replicates), [^{14}C] sodium bicarbonate was added to a final specific activity of 0.5 µCi (18.5 kBq) per sample. Samples were all incubated at the same acclimated photosynthetic active radiation level of 100 $\mu mol \cdot sec^{-1} \cdot m^{-2}$. Dark controls accounted for

possible ^{14}C uptake not due to photosynthesis. After 2 hours of incubation, samples were centrifuged at 16000 g for 15 minutes, followed by the aspiration of supernatant. The resulting pellets were resuspended in distilled water and fumed overnight with 6 M HCl to remove residual ^{14}C that had not integrated into the cells. During the following day the solution were neutralized using 6 M NaOH. Afterwards, scintillation fluid (ECONO-SAFE；RPI, Mt. Propsect, IL, USA) was added to each solution and the radioactivity of samples was measured using a scintillation counter (Beckman LS6500；Beckman Instruments Inc., Irvine, CA, USA).

Average counts per minute were converted into disintegrations per minute via quench correction. DIC per sample was calculated using the “phytotools” package in R statistical software program.

The rate of carbon fixation per hour was calculated as follow:

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

where C_{fixed} = total carbon fixed via photosynthesis; DIC_{sample}= amount of dissolved inorganic carbon available for fixation in media; $DPM^{14}C_{sample}$= disintegrations per minute of the algae pellet; $DPM^{14}C_{total}$ = disintegrations per minute of total available ^{14}C. The resulting net primary production was normalized to a per cell basis using protist abundance enumerated by light microscopy on samples fixed with Lugol’s iodine solution. Carbon acquisition from phototrophy was determined as a product of the rate of photosynthesis per protist cell and the number of protist cells produced during population doubling.

2.3.5 Statistical Analysis

All statistical analysis were run using R software program. For both primary production and grazing experiments, two-way ANOVA were used to analyze differences of ingestion and photosynthetic rate for all combinations of treatment variables. The two-way ANOVA were followed with pair-wise comparisons using Tukey HSD test.

2.4 Results

2.4.1 Effects of Temperature and Nutrients on Photosynthesis/Carbon Acquisition

Temperature had significant effects on the primary production rate of *C. dendrolepidota* (ANOVA, F=14.707, p=0.000164). Within each nutrient treatment, the highest rates of primary production occurred at 20°C. At 20°C, the highest experimental temperature, *C. dendrolepidota* had the highest carbon fixation rate at 50% nutrient level, with an acquisition rate of 0.47 pg carbon per cell $\cdot hr^{-1}$ (Figure 2.1). As temperature decreased to 17°C and 14°C, carbon fixation rates decreased significantly, with the fixation rates being 55% and 70% lower, respectively, compared to 20°C. The same trend was observed at lower nutrient levels of 5% and 1% DY-IV where carbon fixation rates decreased with decreasing temperatures. The highest carbon fixation rates for both nutrient conditions were found at 20°C, with an average rate of 0.40 and 0.17 pg carbon per cell $\cdot hr^{-1}$. However, the differences in carbon fixation were not significant between the two lower temperatures at 5% and 1% nutrient percent level using Tukey HSD ($p > 0.05$).

The concentration of nutrient medium also had significant effects on primary production of *C. dendrolepidota* (ANOVA, F = 6.53, p = 0.009). The carbon fixation rates

were significantly different for the three nutrient levels when comparing each experimental temperature individually (Figure 2.1), with the only exception at 14°C, 50% and 5% nutrient level. In summary, at all three temperatures, the highest carbon fixation rate was observed for cultures in 50% nutrient media. As the nutrient concentration was reduced, carbon fixation rates decreased, with the lowest fixation rate of only 0.05 pg carbon fixed per cell · hr^{-1} observed at 1% nutrient media level at 14°C. There was no significant interaction effect between nutrient concentration and temperature on rates of primary production (ANOVA, F= 1.173, $p > 0.05$).

2.4.2 Effects of Temperature on Bacterivory

Temperature had a significant effect on bacterivory by *C. dendrolepidota* (ANOVA, F= 49.228, $p = 4.99 \times 10^{-8}$). The highest ingestion rate was observed at 14°C, 1% nutrient media condition, with a grazing rate of 4.15 bacterial-sized particles (bsp) ingested per cell $\cdot hr^{-1}$ (Figure 2.1). In comparison, the ingestion rate for the higher temperatures, 17°C and 20°C, under same nutrient concentration were 61% and 85% lower, respectively (Figure 2.1). The same trend is observed for all nutrient concentration conditions, where ingestion rate decreased significantly as the temperature increased (Figure 2.1). At higher temperatures of 17°C and 20°C, ingestion rates are 50% and 75% lower compared to the 14°C culture in 5% nutrient media, and 60% and 81% lower to that of 14°C in 50% nutrient media (Figure 2.1). However, unlike the results for primary production, there was no significant effect of nutrient concentration on the ingestion rate of *C. dendrolepidota* across any temperature (ANOVA, $p > 0.05$).

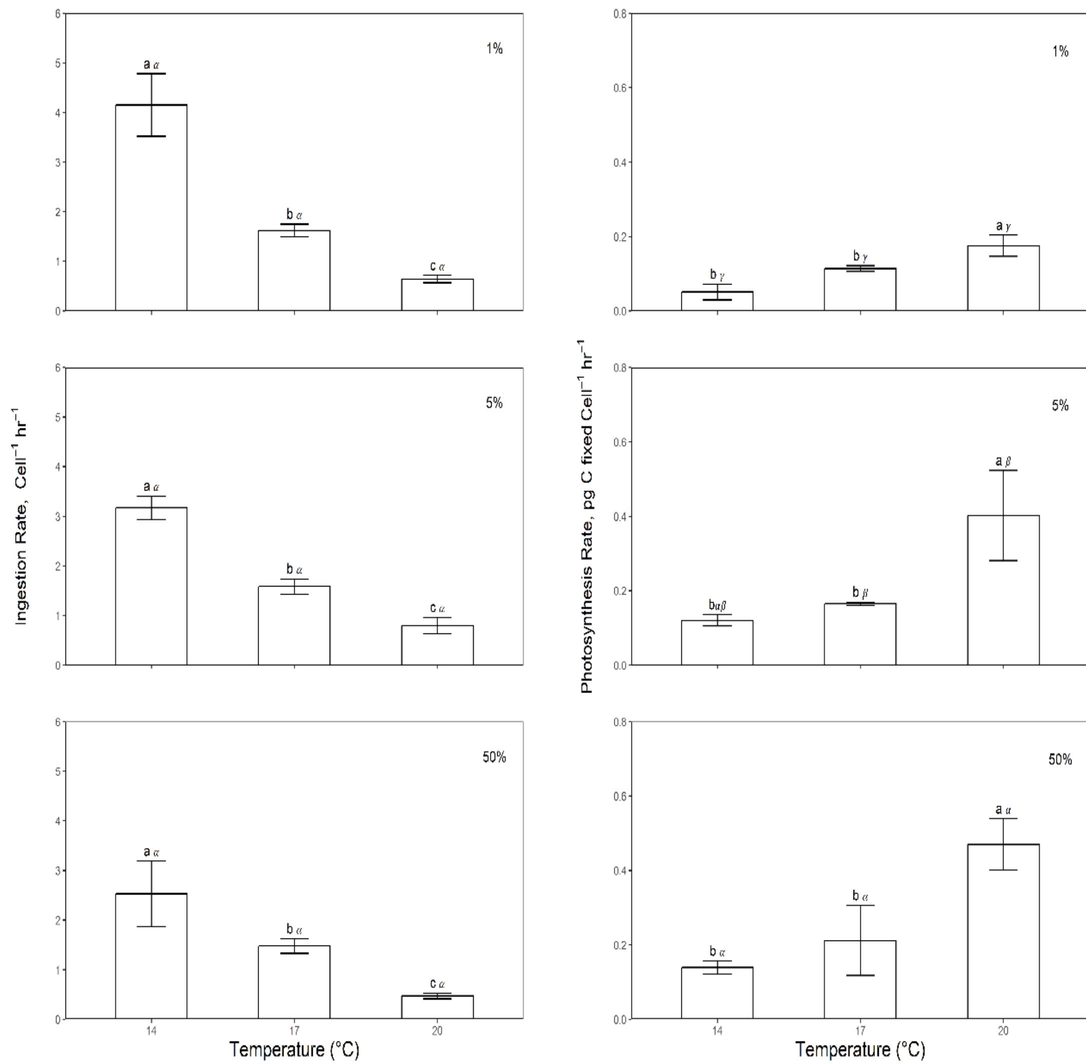

Figure 2.1 Photosynthetic and bacterivory rates of *C. dendrolepidota* under different temperature and nutrient conditions. Letters above bars indicate results of Tukey HSD comparisons for temperature (lower-cased, horizontal comparison) and nutrient conditions (Greek, vertical comparison), respectively, where no significant differences were found between treatments with same letters. The three nutrient media levels, 1% DY-IV, 5% DY-IV, and 50% DY-IV are indicated on each panel.

2.4.3 Temperature Effects on Growth

All temperatures treatments started with an initial rapid increase in cell abundance with similar increases at 14°C and 17°C, but greater in 20°C treatment (Figure 2.2). After day 1, growth was much slower at 14°C and 17°C, with cell yields of 1.9×10^4 cells mL^{-1} and 0.73×10^4 cells mL^{-1}, respectively (Table 2.1). At 17°C, the cells maintained a relatively steady population size after Day 1 and had the lowest specific growth rate ($0.07 \pm 0.005\ d^{-1}$) and cell yield ($0.73\ x10^4$ cells mL^{-1}). At 20°C, algal cells reached the highest abundance of approximately 6.5×10^4 cells mL^{-1} on the third day of incubation (Figure 2.2) resulting in the greatest measured growth rate and cell yield (Table 2.1). The cell abundance at 20°C also plateaued between Day 3 and 4, with a declining phase that was not observed at the other temperatures tested.

Table 2.1 Maximum growth rates and cell yields (n = 3, error bars ± SE) of *C. dendrolepidota* at three different temperatures at high (50% DY-IV) nutrient conditions over a period of 7 days. Light and nutrient concentrations were at maintenance levels.

Temperature	Growth rate μ (d^{-1})	Cell yield (cells·mL^{-1})
14°C	0.12 ± 0.018	1.9×10^4
17°C	0.07 ± 0.094	0.73×10^4
20°C	0.19 ± 0.089	2.8×10^4

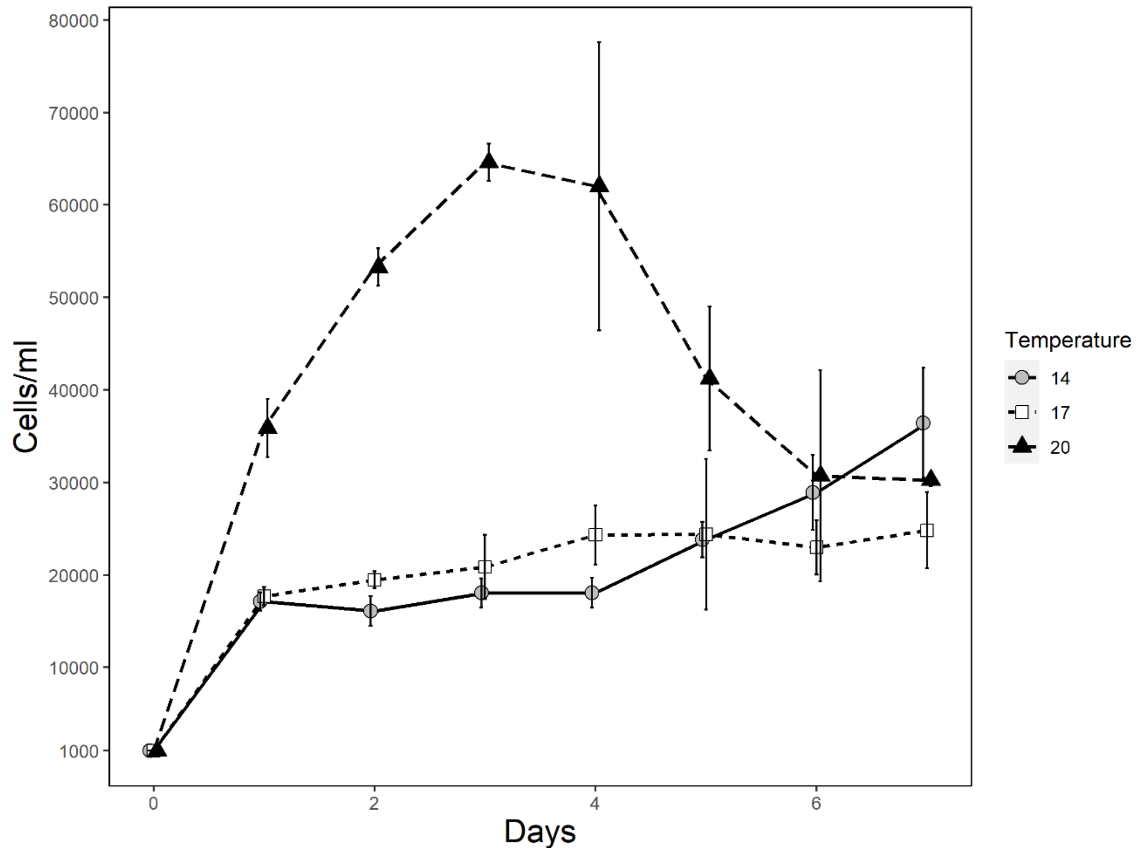

Figure 2.2 Change in population size of *C. dendrolepidota* under different temperatures across a seven-day time series. All replicates were maintained in 50% DY-IV nutrient media.

2.5 Discussion

2.5.1 Temperature Effects on Phototrophy

Our results suggest that *C. dendrolepidota* becomes more phototrophic and less of a heterotroph as the temperature increases regardless of nutrient levels. Rates of primary production increased more than twofold in each nutrient conditions as the temperature rose from 14°C to 20°C. Several mixotrophic species that are primarily phototrophic have been found to have a higher photosynthetic rate and lower ingestion rates at increased

temperatures. For instance, chrysophytes such as *Dinobryon sociale* and several strains of *Chromulina* showed decreases in phagotrophic ingestion with temperature elevation (González-Olalla et al., 2019; Princiotta et al., 2016; Sutton 1972). Prymnesiophytes, which include mixotrophic species such as *Isochrysis galbana* have also been shown to depend more heavily on phototrophy than phagotrophy as temperature increases (González-Olalla et al., 2019).

Multiple reasons may explain why temperature increase promotes phototrophy relative to bacterivory in phototrophic-mixotrophs, i.e., mixotrophs that are closer to the phototrophic end of the photo-phagotrophic spectrum. Studies such as Halac et al. (2010) found warmer temperature could lower rates of photoinhibition and enhance photoprotective mechanisms. In addition, they found when coupled with UVR exposure, higher temperature can also lead to higher metabolism and restoration of the D1 protein, which is responsible for photosynthetic efficiency via electron transport in PSII (Halac et al., 2010). A model proposed by Gonzalez et al. (2022) suggests a shift to phototrophy may be the result of the lack of sustainability via pure heterotrophy because bacterial prey will eventually be depleted and no longer sufficient to maintain a phagotrophic population. Carbon from primary production alone is sufficient for maintaining observed growth and population of *C. dendrolepidota* at 17°C and 20°C (Table 2.2), suggesting *C. dendrolepidota* is a phototrophic-mixotroph. The 68% photosynthetic efficiency at 20°C was higher than most reported values for species identified as mixotrophic, which range from 5 - 45%, although most of these studies focus on phagotrophic-mixotrophs (Islabão et al., 2016; Terrado et al., 2017; Wilken et al., 2013). The few studies done on

phototrophic-mixotrophs have measured photosynthetic efficiency values higher than 50%, in some cases up to 80% (Modenutti et al., 2004; Terrado et al., 2017).

Table 2.2 Carbon acquisition by phototrophy and phagotrophy per cell per day in *C. dendrolepidota* in experimental conditions as in Table 2.1. Photosynthetic efficiency estimates assume carbon from phototrophy accounts for the total amount of carbon needed for doubling and ingested carbon is respired. The unit for carbon is in pg.

Temperature	Growth Doubling Time (Days)	Carbon needed for growth	Carbon from Primary Production	Carbon from Bacterivory	Photosynthetic efficiency
14ºC	2.36	6.1	3.3	1.2	NA
17ºC	4.36	4.2	5.1	0.7	68%
20ºC	2.97	7.7	11.3	0.2	66%

2.5.2 Bacterivory in C. dendrolepidota

The phagotrophic ingestion rate of *C. dendrolepidota* decreased as temperature increased, which also supports *C. dendrolepidota* being more reliant on photosynthesis as temperature increase. Studies using the Metabolic Theory of Ecology (MTE) model have suggested that mixotrophs would respond to higher temperatures by becoming more phagotrophic (Cabrerizo et al., 2019; Wilken et al., 2013). However, this trend may be too generalized to be applicable to all mixotrophic species since its expression is along a gradient of phagotrophy versus phototrophy rather than being binary (Gonzalez et al 2022). In addition, MTE does not account for important factors such as the phylogeny of the mixotroph and environmental interactions. For example, Lepori-Bui et al. (2022) found evolutionary responses of mixotrophs are influenced by the presence of plastic

lineages and selection pressure, leading to different responses to temperature. Ecological feedback such as prey and light availability that are known to shift mixotrophic response are also not considered in MTE (Gonzalez et al., 2022), further limiting its applicability. It is also possible higher temperature simply increases metabolic activity that leads to quicker resource limitation (Wilken et al., 2013).

2.5.3 Effects of Nutrient Concentration on Mixotrophy Responses

Availability of dissolved nutrients had a significant effect on phototrophy of *C. dendrolepidota*. Under the 50% DY-IV nutrient condition, the photosynthetic rates were consistently higher than that of lower nutrient level conditions, regardless of temperature. Although differences between photosynthetic rates of 14°C and 17°C at lower nutrient levels were not significant (Figure 2.1, 1% and 5% DY-IV), we still observed the trend of increased photosynthetic rate with increasing temperature. This is consistent with *C. dendrolepidota* being a phototrophic-mixotroph and relying primarily on dissolved inorganic nutrients for photosynthesis as opposed to organic N and P supplied from ingested food (Anderson et al., 2018; González-Olalla et al., 2019). However, it is known that nutrient availability played a role on the rate of bacterivory in *C. dendrolepidota*, in earlier experiments (Hamsher et al., 2020), just as it did for some other mixotrophs, where limitation of nutrients increased phagotrophy in mixotrophs (McKie-Krisberg et al., 2015; Princiotta et al., 2016). The study by Hamsher et al. (2020) indicated that *C. dendrolepidota* had a higher ingestion rate under nutrient depleted conditions, i.e., 5% DY-IV, leading to the suggestion that nutrient acquisition was the major driver for ingestion of bacteria. The current experiments using 50%, 5% and 1% dilutions of DY-IV were performed with a higher light level (6.0 x 10^{15} quanta·sec^{-1}·m^{-1}) and did not result in

significant nutrient effects on ingestion at any temperature (Figure 2.1). However, we found nutrient availability to be a significant factor for photosynthetic rates (Figure 2.1). Together, these data suggest that a complicated interaction between temperature, light and nutrient concentration could alter the relative use of bacterivory and photosynthesis in the carbon and nutrient budget of the primarily phototrophic *C. dendrolepidota*.

2.5.4 Effects of Temperature on Growth and the Contributions of Phagotrophy and Phototrophy

Our results show *C. dendrolepidota* in nutrient replete media had the highest growth rate at 20°C (Table 2.1), which is consistent with previous studies showing increased mixotrophic growth rate at higher temperatures (Butterwick et al., 2005; Ferreira et al., 2022; You et al., 2020). The increased growth rate is explained by the higher metabolic activity in regards to temperature below a maximum temperature and threshold for metabolic processes when enzymes denature under excess heat (Halac et al., 2010; Lepori-Bui et al., 2022). Other than metabolic dysregulation, rapid initial growth may also lead to faster population saturation, which in turn depletes available nutrients and causes the population to crash (Gonzalez et al., 2022), as we observed for the 20°C culture (Figure 2.2).

The low growth rate and cell yield of *C. dendrolepidota* despite the steady population increase at 17°C was unexpected, since this was the temperature at which the culture was originally maintained. Recent study has shown mixotrophs going through rapid change or short-term acclimation to a new temperature may exhibit a sudden change in physiological behavior in response, but eventually adapt and recover (Lepori-Bui et al., 2022). In environments where temperature fluctuates, plastic responses are

often observed as a reaction to change (Schaum et al., 2013). It is possible that the higher growth rates observed at 14°C and 20°C were a response to stress during an acclimation period compared to growth at the longer-term adaptation at 17°C. Our results, suggest that growth rates in *C. dendrolepidota* were supported primarily by phototrophy and that an increased temperature led to an elevated photosynthetic efficiency and higher carbon fixation with reduced phagotrophy (Table 2.2). At a reduced temperature, *C. dendrolepidota* increased bacterivory, possibly to obtain essential nutrients for growth, including carbon.

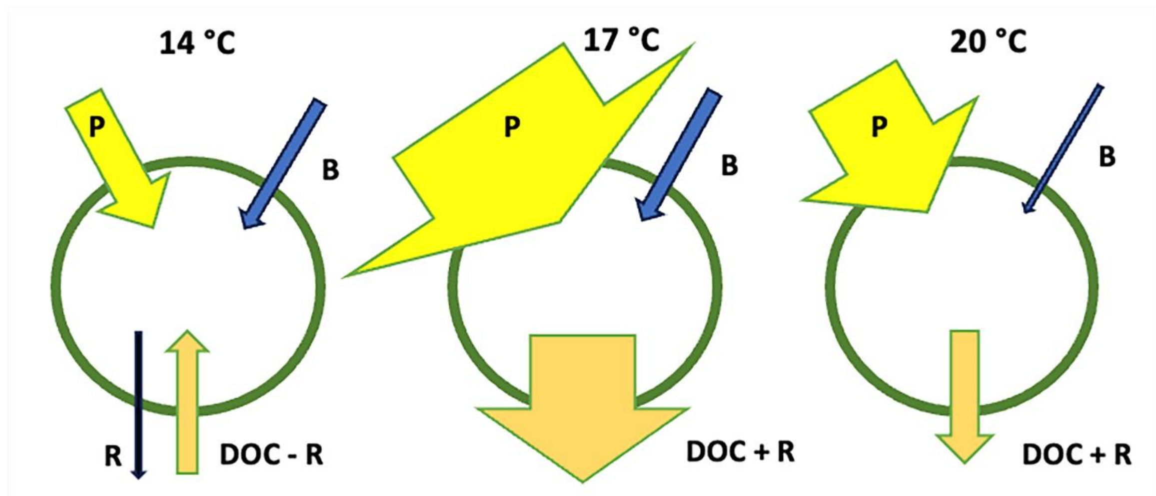

Figure 2.3 Carbon budgets relative to that required for observed population growth at the three experimental temperatures. Arrow size indicates the maximum proportion of growth attributed to photosynthesis (P) and bacterivory (B). At 14°C uptake of dissolved organic carbon (DOC) is proposed as a mechanism to balance carbon required for growth. At 17°C and 20°C, measured photosynthesis produces an excess of carbon that is respired or lost as DOC.

Our measurements of primary production and bacterivory at 14°C do not completely account for carbon needed for growth based on cell counts (Table 2.2). While this may be due to an underestimate of bacterivory, photosynthesis or both, we hypothesize that the carbon for growth at the lower temperature may come from osmotrophy (Figure 2.3). Uptake of dissolved organics is well documented for mixotrophs and other phytoplankton (Flynn et al., 2013; Granéli et al., 1999; Selosse et al., 2017), and carbon gain from this source is feasible at all experimental temperatures. However, at 17°C and 20°C, photosynthesis alone supplies carbon in excess of *C. dendrolepidota* population growth requirements (Table 2.2); loss of carbon from cells via excretion of dissolved organic carbon and increased respiration are more likely components of the carbon budget (Figure 2.3).

CHAPTER 3

TEMPERATURE- AND NUTRIENT-MEDIATED PHAGOTROPHIC AND PHOTOTROPHIC RESPONSES IN A MIXOTROPHIC ARCTIC CHRYSOPHYTE

3.1 Abstract

Temperature, variation of light levels, and changes in nutrient availability are among the top environmental factors known to have a profound effect on growth, grazing, and photosynthesis in mixotrophic species. Polar species, in particular, are facing rapid changes in their environment due to climate change. In the present study, mixotrophic responses of a chrysophyte isolated from Arctic waters, *Dinobryon faculiferum*, were investigated under different temperatures (2-6°C), light levels (0-200 µmol photons $m^{-2}s^{-1}$), and nutrient treatments (12.5 and 50% f/2+Si nutrient media in 32 PSU artificial seawater). We found phagotrophic ingestion by *D. faculiferum* increased with rising temperature, while photosynthetic rates were unexpectedly low under all experimental regimes; phagotrophy appeared to contribute more to the carbon budget than phototrophy. Furthermore, *D. faculiferum* survived long periods of darkness, implying it may not be an obligate phototroph as found for some other species in the genus. Our results suggested that nitrogen had a marked effect on both phototrophy and phagotrophy in *D. faculiferum*, impeding metabolic processes when strongly limited.

Our results demonstrated influences of abiotic environmental factors on mixotrophic responses, providing further understanding of how protists in the polar systems may respond to changing climate.

3.2 Introduction

In recent years, more and more studies have recognized mixotrophs as an important component of aquatic systems (Flynn et al., 2019; Leles et al., 2017; Millette et al., 2023; Sanders & Gast, 2012; Stoecker et al., 2009). Capable of combining both phototrophy and heterotrophy as trophic strategies, mixotrophs make significant contributions in nutrient and carbon cycling as both primary producers and consumers (Caron et al., 1993; Jeong et al., 2010; Sanders et al., 1990; Unrein et al., 2007). Mixotrophic protists have been reported in abundance in both Arctic and Southern Oceans, where substantial grazing impact on the bacterial standing stock has been observed (Gast et al., 2018; Sanders & Gast, 2012).

Polar systems, especially the Arctic, have experienced major changes in the past few decades due to the warming climate (Hop et al., 2020; Lewis et al., 2019). Loss of sea ice has increased stratification and reduced thermocline depth (Sallée et al., 2021; Teufel et al., 2017), limiting nutrient mixing and further resulting in changes of phytoplankton growth season and blooms (Fernández-Méndez et al., 2015; Paulsen et al., 2018; Teufel et al., 2017). Studies have shown several mixotrophic species to become more phagotrophic in response to rising temperature (Kang et al., 2020; Wilken et al., 2013; You et al., 2020), while higher ingestion by mixotrophs are frequently observed in nutrient limited scenarios (Christaki et al., 1999; McKie-Krisberg et al., 2015; Princiotta et al., 2016). This suggests environmental changes observed in the Arctic Ocean may shift mixotrophic metabolism, with potential to alter carbon and nutrient cycling in the microbial loop (Jeong et al., 2010; Paulsen et al., 2018). However, mixotrophic responses to environmental stressors are species-specific, complicating modeling of community

composition shifts and changes of the projected warming future (Gonzalez et al., 2022; Lepori-Bui et al., 2022; Millette et al., 2023). Hence, a more thorough and complete understanding of mixotrophic response to climate change is crucial.

Species of the genus *Dinobryon* are known from both freshwater and marine phytoplankton communities (Caron et al., 1993; Princiotta et al., 2016; Unrein et al., 2010). Although mainly phototrophic, *Dinobryon* species have been observed to have high rates of bacterivory (Bird & Kalff, 1986, Sanders & Porter, 1988, Kamjunke et al. 2007), especially in environments where light is not optimal and nutrient levels are low (Caron et al., 1993; Mckenrie et al., 1995). A temperate freshwater species*, D. sociale,* is known to have a temperature optimum for both photosynthesis and ingestion (Princiotta et al., 2016), but nothing is known on the temperature effects for marine *Dinobryon*. In the present study, we investigated light, temperature, nutrients, and their combined effects on phagotrophy and phototrophy in *Dinobryon faculiferum*, a marine chrysophyte common in the Arctic (Balzano et al., 2012).

The marine *D. faculiferum* has been found in both sea-ice and the open sea in the Arctic Ocean (Balzano et al., 2012; Kilias et al., 2014), and is known for having considerable rates of bacterivory. Unrein et al. (2010) reported a coastal strain of *D. faculiferum* contributing up to 4.5% of total bacterial grazing losses in NW Mediterranean waters leading to a significant impact on the bacterial community. High grazing rates by *D. faculiferum* raises an interesting question of whether this species is an obligate-photomixotroph, i.e., dependent on photosynthesis as reported for some other *Dinobryon* species (Caron et al., 1993; Princiotta et al., 2016).

Based on these previous reports, we hypothesized that *D. faculiferum* will thrive at warmer temperatures, with an increased ingestion rate, and that macronutrients (nitrogen or phosphate) are triggers for increased bacterivory. For all abiotic factors assessed for impact on phagotrophy and photosynthetic rate in this study, the ranges were chosen in regard to current conditions in the Arctic environment as well as forecasted changes due to climate change (Screen & Simmonds, 2010; Steele et al., 2008). Additional nutrient experiments were performed to identify if these macronutrients had different impacts on mixotrophic abilities.

3.3 Materials and Methods

3.3.1 Culture Origin and Maintenance

Non-axenic cultures of *Dinobryon faculiferum* (RCC2290) were obtained from the Roscoff Culture Collection (France) and were maintained in f/2 + Si media made with 32 PSU artificial sea water (ASW) in 120 mL culture flasks. Cell cultures were exposed to constant illumination (20 μmol photons $m^{-2}s^{-1}$) at 4°C in a monitored temperature-controlled cold room. Media were exchanged once a month to prevent development of highly dense populations that can lead to nutrient deprivation and limitation.

Three different temperature regimes were used for this experiment: 2°C, 4°C, and 6°C. Replicate 50 mL culture flasks were maintained for each temperature prior to experimentation. For each temperature level, the cultures were acclimated to two different nutrient media levels, which were 50% and 12.5% of the full-strength f/2 + Si media by dilution using 32 ASW. Light levels for each experimental culture were changed after acclimation to nutrient levels for a week. The four different light treatments

were: dark (0 μmol photons $m^{-2}s^{-1}$) maintenance (20 μmol photons $m^{-2}s^{-1}$), moderate (100 μmol photons $m^{-2}s^{-1}$), and high (200 μmol photons $m^{-2}s^{-1}$). The cultures were then allowed to acclimate for one more week before experimentation.

3.3.2 Grazing Experiment

Prior to grazing assessment, triplicate 50 mL culture flasks were acclimated to desired experimental regimes. For assessment, fluorescent polycarbonate microspheres (0.6 μm dia., Polysciences were added to a 3 mL subsample for each replicate at a final concentration of 5 x 10^5 μspheres mL^{-1}). The microspheres had similar sizes to the resident bacterial population, and served as a proxy for phagotrophic ingestion. Additional subsamples were fixed immediately upon addition of the microspheres with Lugol's solution to account for background incidence (T0). Subsamples were gently inverted and rotated briefly to ensure thorough microsphere mixing. After a grazing period of 30 minutes (T30), subsamples were fixed using a protocol known to prevent egestion; Lugol's iodine and formalin were added in succession; sodium thiosulfate was then added to clear the sample for microscopic enumeration (Sherr & Sherr, 1993). Each subsample was collected onto a 0.8 μm pore-size polycarbonate filter, and mounted onto a slide with Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). Enumerations were made using epifluorescence on an Olympus BX41 (Olympus Corporation, Shinjuku, Tokyo, Japan) microscope at 1000X magnification.

To determine the average number of microspheres ingested at each experimental condition, we examined a minimum of 20 fields of view, and at least 100 cells for each slide. Visual overlap of microspheres that potentially were not ingested by cells was

accounted by subtracting the T0 counts (background) from the number of ingested microspheres in T30 replicates (Sherr & Sherr, 1993).

Following the presumption of which ingestion rate of microspheres is the same as ingestion of bacteria, the ingestion rate of bacterial-sized particles were determined using the equation outlined below:

Ingestion=

$$\frac{\text{bacterial–sized particles}}{\text{protist}\times\text{hour}}=\frac{(\text{Microsphere ingested}\times\frac{\text{bacteria}}{\text{microsphere}}+\text{microsphere ingested})}{\text{protist}\times\text{incubation time (hr)}}$$

Where bacterial-sized particles = sum of bacteria and microsphere ingested, microsphere ingested = average microsphere ingested, bacteria = concentration of bacteria (cells mL^{-1}), microspheres = microsphere concentration (beads mL^{-1}), protist = number of protist cells). Carbon from ingestion was estimated using the product of carbon content (20 fg) of a single bacterium (Lee &Fuhrman, 1987) and the ingestion rate of bacterial-sized particle per protist cell. We estimated relative percentage of carbon acquisition via bacterivory by only accounting carbon acquired through bacterivory and photosynthesis.

3.3.3 Measurement of Photosynthetic Activity

Photosynthesis vs. irradiance (PI) curves were used to assess the phototrophic ability of the mixotrophic *D. faculiferum*. Fixation of carbon by *D. faculiferum* under each experimental condition was measured through the uptake of ^{14}C using a protocol adapted from Maclntyre et al (1996), modified with centrifugation technique from Smith and Azam (1992).

For each of the experimental condition, four replicate 2 mL aliquots were inoculated with [^{14}C] sodium bicarbonate to a final specific activity of 0.5 μCi (18.5 kBq) per sample. Samples were incubated at photosynthetically active radiation (PAR) levels corresponding to the experimental light conditions (0 μmol photons $m^{-2}s^{-1}$, 20 μmol photons $m^{-2}s^{-1}$,100 μmol photons $m^{-2}s^{-1}$,200 μmol photons $m^{-2}s^{-1}$). Control groups ran in total darkness accounted for any ^{14}C uptake that were not due to photosynthesis; consequently, the treatment without light had no recorded photosynthetic carbon fixation. The samples were incubated for 2 hours, after which samples were centrifuged for 15 minutes at 16,000g. After centrifugation supernatants were aspirated, followed by resuspension of the pellets in distilled water. 6 M HCl was then added to each sample and left to fume overnight, removing any residual ^{14}C not integrated into the cells. Sample solutions were neutralized via the addition of 6 M NaOH, scintillation fluid was then added and radioactivity determined using a scintillation counter (Beckman LS6500 with external standard; Beckman Instruments Inc., Irvine, CA, USA).

Average counts per minute were converted into disintegrations per minute using a quench correction curve. The “seacarb” R package was used to calculate dissolved inorganic carbon (DIC) in each sample.

The rates of carbon fixation were determined as follow:

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

where C_{fixed} = total carbon fixed by photosynthesis; DIC_{sample}= amount of dissolved inorganic carbon (DIC) available for fixation in sample; $DPM^{14}C_{sample}$= disintegrations

per minute of the algae pellet; $DPM^{14}C_{total}$ = disintegrations per minute of total available ^{14}C. Net primary productivity was normalized to a per cell basis using abundance determined by light microscopy on subsamples fixed prior to addition of ^{14}C.

3.3.4 Macronutrient Manipulation Experiment

To further understand the effects of major nutrients on ingestion in *D. faculiferum*, we performed a set of experiments limiting nitrogen, phosphorus or both. Four different combinations of nutrient media were made, including the macronutrient complete medium (MCM) of 50% strength f/2+si with 32 PSU ASW. Reduced-nitrogen medium (RNM), reduced-phosphorus medium (RPM), and the reduction of both nitrogen and phosphorus medium (RNPM) were made by modifying the MCM via exclusion of each respective macronutrient. Three 120 mL replicates of each nutrient treatment were incubated at 4°C for 7 days prior to experiments. Ingestion rates were then determined using the methods as described in the previous sections.

3.3.5 Statistical Analysis

All statistical analyses were run using R statistical software program. For grazing experiments, two-way ANOVA was used to analyze differences in ingestion and photosynthetic rate for all combinations of treatment variables. The two-way ANOVA was followed by pair-wise comparisons using Tukey HSD test.

PI curves were made by fitting to Eilers-Peters light limitation curve (Eilers & Peters, 1988), a model where photosynthesis is defined as the product of maximum photosynthetic rate and light limitation of photosynthesis at given irradiance. The "phytotools" R package was utilized to graph the PI curves (Silsbe & Malkin, 2015).

3.4 Results

3.4.1 Heterotrophic Ingestion by D. faculiferum

The grazing rates of *D. faculiferum* ranged from 0.8-12 bacteria-sized particle (BSP) per cell $\cdot hr^{-1}$ across treatments adapted to different temperature, nutrient, and light level regimes (Figure 3.1). Ingestion rates were affected significantly by temperature, nutrient and light levels, but also had significant interactions between the three factors (ANOVA, $p < 0.0005$ for temperature; $p < 0.0005$ for light; $p < 0.0005$ for nutrient $p < 0.05$ for interaction between temperature, light and nutrient levels). Ingestion rates were consistently significantly greater under the higher nutrient conditions (50% f/2+Si media) at all temperatures in the maintenance light conditions and at 6°C under all light conditions (Figure 3.1). In other combinations of light and temperature, ingestion rates tended to increase with rising temperature, but nutrient level had no significant effect on ingestion. The highest ingestion rates, ranging from 5.9 to 12 BSP per cell $\cdot hr^{-1}$, were found at 6°C, 50% nutrient level under all light treatments. The only exception was found under maintenance light level, where the ingestion rate at 4°C (7.7 per cell $\cdot hr^{-1}$) was slightly, but not significantly, higher than that of 6°C (7.1 per cell $\cdot hr^{-1}$) (Figure 3.1). In comparison, the ingestion rates at reduced nutrient conditions (12.5%) tended to be lower, with the highest average grazing rates observed at 4°C. However, across nutrient and light treatments, there were no significant difference in grazing rates at the lower nutrient concentration (Figure 3.1).

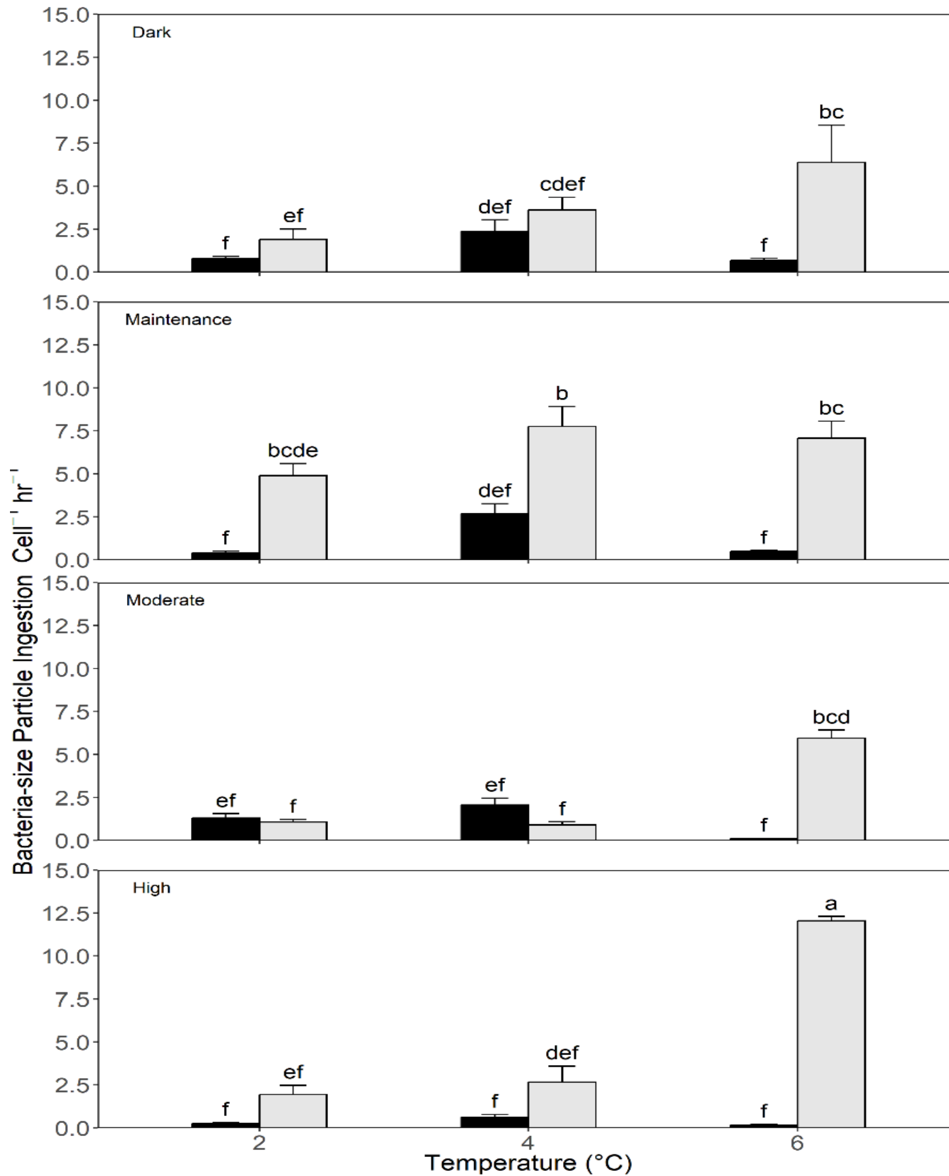

Figure 3.1 Bacterial-sized particle (BSP) ingestion rate of *Dinobryon faculifurm* under different temperatures, light levels, and nutrient regimes. The four different light levels are dark (0 μmol photons $m^{-2}s^{-1}$), maintenance (20 μmol photons $m^{-2}s^{-1}$), moderate (100 μmol photons $m^{-2}s^{-1}$), and high (200 μmol photons $m^{-2}s^{-1}$). Black and grey bars indicate the two different nutrient levels, 12.5% and 50% f/2 media). Letters above bars show results of Tukey HSD test, where the same letters indicate there are no significant difference found between treatments.

3.4.2 Phototrophy of D. faculiferum

The differences in rates of primary production and optimal irradiance level for *D. faculiferum* adapted to different temperature and nutrient treatments are depicted using PI curves (Figure 3.2), alongside estimates of photosynthetic activity parameters from the Eiler-Peeters model (Table 3.1). Temperature, nutrient, and the interactive effects of temperature and nutrients all had significant effects on the photosynthetic rates of *D. faculiferum* (ANOVA, $p < 0.001$ for temperature, nutrient and interactive effects.

The maximum photosynthesis rate was observed at 6°C under nutrient replete condition. The estimated maximum rate was 38.4 fg C per cell · hr^{-1}, and the optimal irradiance was around 57.7 μmol photons $m^{-2}s^{-1}$. Nutrient availability significantly affected photosynthetic rates ($p < 0.05$), with higher rates observed in nutrient replete conditions. When compared to the reduced nutrient treatments, estimated maximum photosynthetic rates in nutrient replete conditions were more than two-fold and five-fold higher at 4°C and 6°C, respectively (Table 3.1, Figure 3.2). Photoinhibition effects, on the other hand, were estimated to have a significant effect only at 6°C under nutrient replete conditions (Table 3.1). In regards to temperature effects on photosynthesis, 4°C and 6°C treatments followed the same trend where higher maximum photosynthetic rates were measured in the higher nutrient conditions. The peak rate of photosynthesis at 4°C and 6°C was in the irradiance range of 50 - 60 μmol photons $m^{-2}s^{-1}$, dropping off at higher irradiance, presumably due to photoinhibition (Figure 3.2). The highest optimal irradiance calculated was for treatments held at 2°C with high nutrients (Table 3.1), due to the minimal decrease in photosynthetic rates at higher light intensities within the range tested (Figure 3.2).

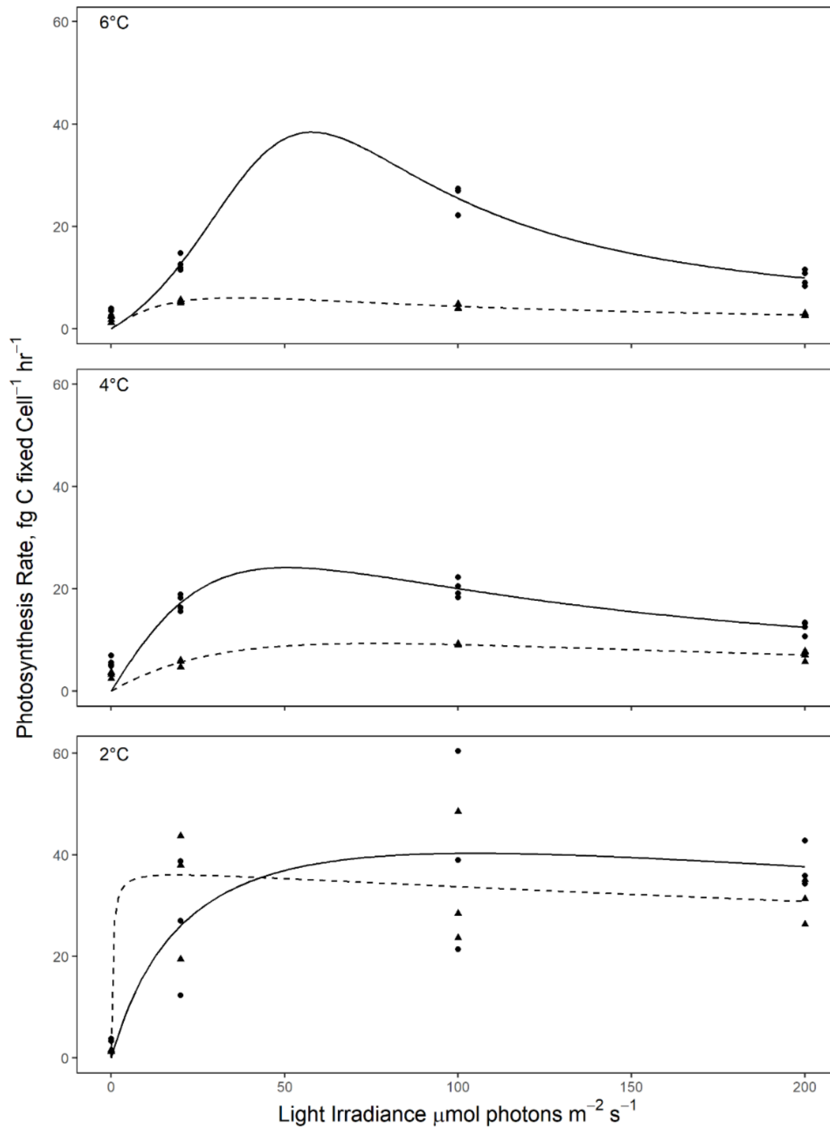

Figure 3.2 Photosynthesis vs. Irradiance Response (PI) curves of *D. faculifurum* adapted to different temperatures and nutrient regimes. Temperatures are: 2°C, 4°C, and 6°C (from bottom to top). Solid lines and circles indicate the higher nutrient media (50% f/2+ Si), where dashed line and diamonds indicate reduced nutrient media (12.5% f/2+Si).

Table 3.1 Photosynthetic parameters from the photosynthesis versus irradiance (PI) curves of *D. faculiferum* fitted to the Eilers-Peeters model.

	2℃			4℃			6℃		
	Estimate	SE	Pr(>|t|)	Estimate	SE	Pr(>|t|)	Estimate	SE	Pr(>|t|)
Low Nutrient									
P_{max}	33.94	78.76	**	9.29	1.19	***	6.02	1.43	**
I_{opt}	67.53	313.21	n.s.	75.77	15.17	***	36.46	11.53	**
β	5.64	76.33	n.s.	0.59	0.90	n.s.	0.46	1.63	n.s.
High Nutrient									
P_{max}	42.05	15.77	*	24.14	3.57	***	38.41	7.15	***
I_{opt}	130.27	75.25	n.s.	50.76	4.88	***	57.77	3.08	***
β	-0.19	1.92	n.s.	0.17	0.59	n.s.	-0.69	0.10	***

***P < 0.001, **P < 0.01, *P<0.05, n.s., not significant.
P_{max} is the maximum photosynthetic rate at the optimal irradiance level (I_{opt}). β is a non-dimensional parameter that estimates degree of photoinhibition

3.4.3 Nutrient Limitation and Bacterivory

When the roles of specific macronutrients on ingestion rates were examined, effects were significant (Figure 3.3). An ANOVA indicated that *D. faculiferum* grown with reduced nitrogen (RNM and RNPM) had ingestion rates that were significantly lower than when nitrogen was added to the media ($p < 0.05$). Tukey HSD tests indicated substantial differences between both the control (MCM) and the reduced phosphorus-media (RPM) relative treatments with to reduced nitrogen: (RNM) and nitrogen- and phosphorus- reduced (RNPM) media were significant. The ingestion rates at nitrogen reduced treatments were similar to that observed in 12.5% f/2+Si treatments.

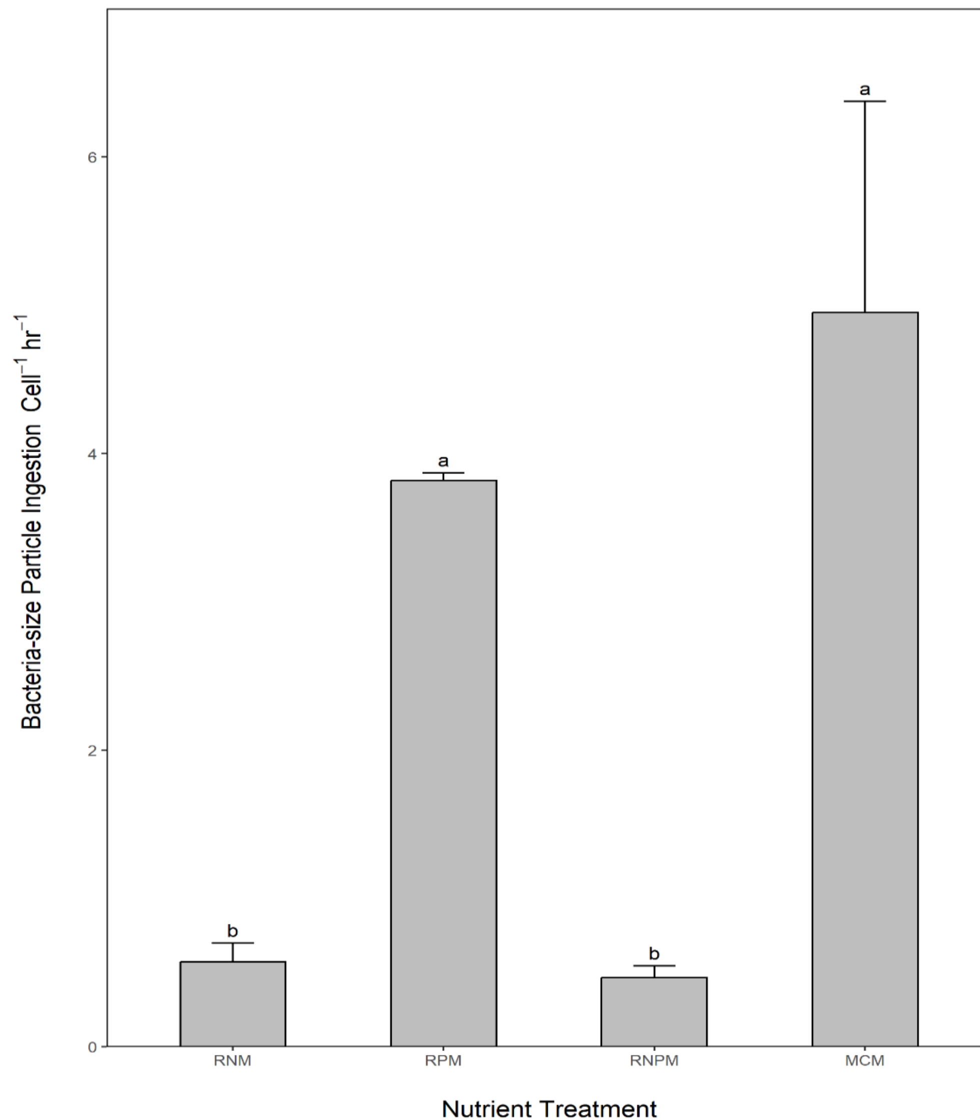

Figure 3.3 Bacterial-sized particle ingestion (mean ± SE) by *D. faculiferum* under different nitrogen and phosphorus supplements. Nutrient treatments are indicated as: RNM, reduced-nitrogen media; RPM, reduced- phosphorus media; RNPM, reduced-nitrogen and -phosphorus media; and macronutrient complete media (MCM, 50% f/2 + Si). Letters above bars show results of Tukey HSD test, where the same letters indicate there are no significant difference found between treatments.

3.5 Discussion

3.5.1 Temperature Effects on Phagotrophy

Temperature is known to have marked consequences on heterotrophic ingestion in mixotrophs, but the effects are often species-specific (Princiotta et al., 2016; Wilken et al., 2013). In our study, ingestion rate of *D. faculiferum* tended to increase with increasing temperature (Figure 3.1). Several other mixotrophic flagellates were also reported to become more phagotrophic as temperature increased. For example, Wilken et al. (2013) observed that the chrysophyte *Ochromonas* sp. had substantially greater bacterivory rates with rising temperature; other mixotrophic protists, including the dinoflagellates *Gymnodinium smadae* and *Alexandrium pohangense*, also had maximum ingestion rates at higher temperatures (Kang et al., 2020; Lim et al., 2019). Within the genus *Dinobryon*, studies on *D. divergens* and *D. sertularia* also showed positive correlations between temperature and ingestion rates (Bird & Kalff, 1987).

One rationale for mixotrophs to become primarily phagotrophic with rising temperature is based on the Metabolic Theory of Ecology (MTE), which states that metabolic rates increase as temperature increase (Cabrerizo et al., 2019; Wilken et al., 2013). A decoupling of phototrophy and heterotrophy is proposed to follow the increase in metabolic rates as investment in photosynthetic processes become inefficient compared to heterotrophy (Wilken et al., 2013). However, this model does not appear to hold true for all mixotrophic species. For instance, the freshwater *Dinobryon sociale*, an obligate-phototroph, exhibited a lower heterotrophic ingestion rate as temperature increased (Princiotta et al., 2016). Negative effects of rising temperature on ingestion were also found for another chrysophytes such as *Chromulina* sp. (González-Olalla et al., 2019).

These contrasting results demonstrate the complexity of mixotrophic responses, as species in the same genus can have totally different physiological behavior and traits in relation to the biotic and abiotic factors in their environment.

Previous studies, primarily from freshwater environments, have suggested species in the genus *Dinobryon* to be phototrophic-mixotrophs and several studies have shown them to be obligate phototrophs, incapable of living in even relatively short periods (days) of total darkness (Caron et al., 1993; Princiotta et al., 2016). However, as photo-mixotrophs *Dinobryon* still can have high grazing rates comparable to many purely heterotrophic flagellates (Sanders et al., 1989). Several strains of *Dinobryon* have been reported to have specific grazing rates as high as 40 - 60 bacteria cell $\cdot hr^{-1}$, and in one study ingestion rate of *Dinobryon cylindricum* went as high to approximately 138 bacteria cell $\cdot hr^{-1}$ (Bird & Kalff, 1987). Populations of *Dinobryon* in Lac Gilbert, Quebec that were primarily photosynthetic at the surface gained most of their daily carbon from bacterivory in deeper water, although the rate of ingestion was positively correlated to temperature and not light (Bird & Kalff, 1987).

Few studies have addressed grazing by marine *Dinobryon* species (Mckenrie et al., 1995; Unrein et al., 2010). Unrein et al. (2010) reported *D. faculiferum* in the Mediterranean to have a grazing rate of 7.8 bacteria cells per hour, similar to laboratory rates determined in the current study (Figure 3.1). Despite these relatively high grazing rates, *Dinobryon* from marine environments have long been assumed to be primarily phototrophic based on what is known about their freshwater counterparts described above. However, we present, to the best of our knowledge, the first study on primary production rates for a marine *Dinobryon* species. Contrary to laboratory measurements

for freshwater *Dinobryon* species (e.g., Caron et al., 1993; Princiotta et al., 2016), we found carbon acquisition from photosynthesis in *D. faculiferum* to contribute less than 10% of estimated total carbon uptake (Table 3.2). In addition, photosynthetic rates observed for *D. faculiferum* were considerably lower compared to the mainly phototrophic *Dinobryon* species in the freshwater studies noted above. These results suggest that, unlike previously studied *Dinobryon* species, *D. faculiferum* may be on the heterotrophic end of the mixotrophy spectrum regardless of light or temperature (Table 3.2).

Table 3.2 Percentage of carbon uptake from bacterivory relative to total carbon acquisition including photosynthesis.

	Dark 0 μmol $m^{-2}s^{-1}$	Maintenance 20 μmol $m^{-2}s^{-1}$	Moderate 100 μmol $m^{-2}s^{-1}$	High 200 μmol $m^{-2}s^{-1}$
High nutrient				
2°C	99	97	86	90
4°C	99	99	94	98
6°C	99	99	99	99
Low nutrient				
2°C	97	73	85	77
4°C	98	96	95	89
6°C	97	88	46	57

3.5.2 Impacts of Light on Bacterivory and Photosynthesis

With poor light conditions and ice cover, many protists in the polar system become adapted to low irradiance (Fernández-Méndez et al., 2015; Schünemann et al., 2007). Some studies suggest that species adapted to low irradiance are often phagotrophic mixotrophs, relying mostly on heterotrophic ingestion to sustain growth, where on the other hand phototrophic-mixotrophs tend to prefer environments where light is consistently available (Charvet et al., 2014; Schünemann et al., 2007). In the current study, *D. faculiferum* appeared to be adapted to lower light levels, with an optimal irradiance ranging from 36.5 to 75.8 μmol photons $m^{-2}s^{-1}$ in our nutrient replete conditions (Table 2.1).

Our results also showed that *D. faculiferum* can survive in darkness for a longer time period than freshwater *Dinobryon* species previously investigated. After one week in complete darkness for grazing experiments, we still observed active swimming and feeding cells. This is distinct from other cultured *Dinobryon* populations, such as *D. sociale* and *D. cylindricum,* of which were not able to survive more than a few days in total darkness or have positive population growth in continuous low light (Caron et al., 1993; Princiotta et al., 2016). The Arctic Ocean has a huge seasonal variability in irradiance, which is additionally limited by sea-ice formation throughout the year (Hop et al., 2020; Kilias et al., 2014; Schünemann et al., 2007). Cyst production, a viable strategy to survive the winter darkness, has also been observed for *D. faculiferum* (Kauko et al., 2018; Schünemann et al., 2007), but it also is possible that this polar strain of *D. faculiferum* gradually shifted its nutritional mode to primarily heterotrophic to enhance dark survival over winter.

3.5.3 Nutrient Effects on Bacterivory

In our experiments, both heterotrophic ingestion and phototrophic productivity decreased substantially when *D. faculiferum* was cultured in reduced nutrient media. This suggests that both bacterivory and phototrophy in *D. faculiferum* can be limited by available nutrients, which was confirmed in the macronutrient experiments that limited nitrogen, phosphorus or both. Nitrogen (N) was the strongest limiting nutrient for *D. faculiferum*, which is in accordance with several studies that suggest nitrogen to be the most important limiting nutrient in the Arctic Ocean for phototrophic protists (Lewis et al., 2019; Mills et al., 2018; Paulsen et al., 2018). Nitrogen supply is scarce in the Arctic, due to relatively low inorganic nitrogen input from runoff and stratification leading to poor nutrient mixing in the system (Paulsen et al., 2018). In addition, dissolved nitrogen availability also changes throughout the seasons, building up in the winter and becoming available later in spring. As light levels and daylength increase, phytoplankton blooms lead to a relatively rapid depletion of nutrients (Mills et al., 2018; Nöthig et al., 2020; Schünemann et al., 2007). Nitrogen limitation is detrimental to photosynthetic processes in phytoplankton, leading to decreased photosynthetic efficiencies (Kauko et al., 2018; Lewis et al., 2019).

In response to nitrogen limitation, it is known that phototrophic species can reduce their nitrogen-requiring cellular contents, lowering the amount of chlorophyll *a* and PSII light-harvesting antennae that are involved in the photosynthetic processes (Lewis et al., 2019; Mills et al., 2018). This leads to a substantial decrease in photosynthetic activity, which is what we observed for *D. faculiferum.* Conversely, it is not clear how nitrogen limitation would result in a reduction in grazing. It is, in fact,

contrary to expectations from most other studies in which nutrient limitation resulted in increased ingestion of bacteria (Liu et al., 2022; McKie-Krisberg et al., 2015; Pålsson & Granéli, 2004; Princiotta et al., 2016). One possible explanation for the low bacterivory rate by *D. faculifuerm* in nutrient-limited media is that prey ingestion was primarily for obtaining carbon. Under nitrogen limited conditions, mixotrophs have been shown to remobilize internal nitrogen by degrading pigments and proteins (Li et al., 2018), while obtaining carbon from means such as osmotrophy (Cecchin et al., 2018; Li et al., 2018). This may be the case for *D. faculiferum*, especially if active predation were more energy costly. Mixotrophs may cease activity or go into cell arrest in a nitrogen-stressed/nitrogen starvation environment, which is also a possible explanation of reduced ingestion rate observed for *D. faculiferum* (Dagenais-Bellefeuille & Morse, 2013). Further investigation on nutrient limitation, element stoichiometry, and heterotrophic metabolism in mixotrophic protists is needed to help elucidate this phenomenon.

CHAPTER 4

EXPLORING BIOGEOGRAPHICAL DISTRIBUTION OF A POORLY IDENTIFIED FRESHWATER CHRYSOPHYTE

4.1 Abstract

Being major primary producers and consumers in aquatic ecosystems, protists contribute greatly to carbon and nutrient cycling. However, biogeographical distributions of protist species in freshwater are understudied, presumably due to assumptions of ubiquitous distribution.

Here, we investigated freshwater lakes and rivers for the presence of *Chrysolepidomonas dendrolepidota*, a chrysophyte previously isolated from Lake Medora, Michigan, USA. First, a thorough metadata analysis for evidence that this species had been identified previously in freshwater systems located no references indicating its presence. We in addition, sampled from several lakes in geographically distinct areas, including Lake Medora, were screened for *C. dendrolepidota* and *Dinobryon*, a genus of chrysophyte known to be widely distributed, using High-Throughput Sequencing (HTS). Although *Dinobryon* was found in some water samples, *C. dendrolepidota* was not present in any of our samples and was not detected in any of the 31 HTS publications checked. Seasonality, species rarity, and low abundance are potential drivers that made *C. dendreolepidota* undetectable in water systems. Possible extinction of this species in its original habitat is also to be considered.

4.2 Introduction

Protists have been traditionally identified via microscopy. Morphological structures and characteristics such as cysts, scales, and color have proven to be successful

in identifying those with species-specific structures (Lengyel et al., 2022). However, many protists are nano-sized (<20 µm), often making accurate identification of species extremely difficult (Bachy et al., 2013; Gusev et al., 2018; Siver et al., 2018). In recent years new methodologies have been developed for detection and identification. From high quality scanning electron microscopy (SEM) to molecular methods that amplify ribosomal RNA and DNA genes, clone libraries and High-throughput sequencing have made better identification of small protist possible (Alteration et al., 2001; Charvet et al., 2014; Debroas et al., 2017; Gao et al., 2017; Penna et al., 2007; Stoeck et al., 2003).

Chrysophytes are important primary producers common in freshwater systems (Lengyel et al., 2022; Naselli-Flores & Padisák, 2023), with mixotrophic species known to play a role in carbon and nutrient cycling (Adl et al., 2007; Mitra et al., 2014). Although many chrysophytes have a cosmopolitan range, some species have been found to be endemic or having relatively restricted distribution (Boo et al., 2010; Charvet et al., 2012; Gusev & Martynenko, 2022).

Chrysolepidomas dendrolepidota (CCMP293) is a small freshwater chrysophyte with little known about its ecology and distribution. Currently, the only known distribution of this species is the lake from where it was originally isolated (Lake Medora, Michigan, USA). *C. dendrolepidota* is ovoid/ ellipsoid in shape with a maximum dimension <10 µm. Cells typically have one single parietal chloroplast, heterokont flagella (flagella of different length), with cylindrical and dendritic scales (Peters & Andersen, 1993).

The objective of this study is to investigate whether *C. dendrolepidota* is present in other freshwater systems using (1) metadata analysis and (2) molecular methods. For

the molecular analysis, a new set of primers that are specific to *C. dendrolepidota* was designed and used to search for its presence in water samples collected from thirteen different freshwater lakes and rivers in Michigan, USA and Northern Taiwan. In addition to the locations sampled here, a metadata analysis was conducted on high-throughput sequencing (HTS) databases searching freshwater systems mainly in North America. We also screened our community DNA samples for presence of *Dinobryon*, a genus of chrysophyte that have a wide distribution (Princiotta et al., 2016; Sanders et al., 1990; Unrein et al., 2010; Walter & Whiles, 2010).

4.3 Materials and Methods

4.3.1 Field Sample Collection and Preparation

Water samples of freshwater lakes and rivers were obtained from a number of locations in the United States and Taiwan (Figure 4.1; Table 4.1). For each of the samples, 100 mL of surface water were filtered, collected onto 5 - µm nitrocellulose filters and stored initially in 1 ml of DNA preparation buffer [RLT buffer; Qiagen, Texas, USA]. All samples were stored at -80 °C until further DNA extraction.

Figure 4.1 Photographs of sampled waterbodies in Taiwan and the United States. Waterbodies shown here are: A. YangMing Mountain Lovers Waterfall, Taiwan B. Spring Lake, USA C. Lake Medora, USA D. Lake Lory, USA.

Table 4.1 List of sampled waterbodies across Taiwan and United States.

Sample ID	Waterbody name	Collection Date	Latitude	Longitude	Temperature (°C)	pH
North America						
N1	Root beer Lake	May 2023	42.97	-85.82	11.7	4.61
N2	Muskegon Lake	May 2023	43.18	-86.22	15.587	8.04
N3	Mona Lake	May 2023	43.18	-86.22	12.3	8.22
N4	Spring Lake	May 2023	43.08	-86.17	17.8	8.64
N5	Lake Lory	June 2023	46.47	-87.88	21.8	5.24
N6	Lake Medora	June 2023	47.43	-87.96	16.3	NA
N7	Indian Lake	June 2023	45.94	-86.33	13.4	NA
N8	Lake Paradise	June 2023	45.69	-84.78	17.1	NA
Taiwan						
T1	Lengshuikeng - YangMing Mt. National Park	May 2023	25.16	121.57	18	6.18
T2	YangMing Mt. Lover waterfall	May 2023	25.14	121.52	24	7.8
T3	YangMing Mt. Lover waterfall	July 2023	25.11	121.46	25	7.6
T4	Guandu Pier	July 2023	25.14	121.52	27	7.65
T5	Rain water	August 2023	25.13	121.46	26	7.9

4.3.2 DNA Extraction

Water samples were either extracted using the CTAB method or Qiagen DNA/RNA kit (Texas, USA). Lab cultures of *C. dendrolepidota* and *D. faculiferum* were extracted following the CTAB method (Gast et al., 2004). Polymerase chain reactions (PCR) were performed using Q5® DNA Polymerase [NEB, MA, USA]. Each reaction tube contained a 20 µL mixture composed of 14 µL of H2O; 4 µL Q5 reaction buffer; 0.4

µL dNTP; 0.2 µL of Euk1A forward primer (5'- AAY CTG GTT GAT YCT GCC AG -3'), 0.2 µL of EukB2 reverse primer (5'- GAT CCT KCT GCA GGT TCA CCT A -3'); 0.2 µL of Q5 polymerase; and 1.0 µL of DNA. The following cycling condition were used: 98°C for 1 min, 34X (98°C for 30 sec, 58°C for 30 sec, 72°C for 30 sec), 72°C for 2 min. Amplified PCR products were verified on 1.5 % agarose gel and sent to Genewiz (South Plainfield, NJ) for cleaning and Sanger sequencing.

Sequence traces were assessed and cleaned using FinchTV. For *C. dendrolepidota*, resulted double peaked sequence indicated presence of another species in the culture. In order to obtain DNA of purely *C. dendrolepidota*, cloning was performed using TOPO™ TA Cloning™ Kit for Sequencing (Invitrogen), following the manufacturer's instruction.

PCRs of picked colonies were amplified in a 20 µL PCR mix containing 4 µL Green Buffer; 2 µL MgCl2; 0.4 µL dNTPs; 0.2 µL each of M13 forward and reverse primers; 0.2 µL of GoTaq polymerase; 0.1µL of DNA, and 12 µL of H2O. The cycling condition were as follow: 95°C for 2 min, 34 x (95°C for 30 sec, 55°C for 30 sec, 72°C for 2 min), 72°C for 5 min.

Verification and sequencing of picked colony PCR products were carried out the same as outlined above. Identified *C. dendrolepidota* colonies were kept inside a monitored temperature-controlled cold room for further use.

4.3.3 Primer Design

To design a species-specific PCR primer for *C. dendrolepidota* and genus-specific primer for *Dinobryon*, identification of a distinct region in the small-subunit ribosomal

RNA is needed. Sequences for both species as well as reference sequences were retrieved from NCBI GenBank. Reference sequences for *Dinobryon* were chosen from the genus, while references for *C. dendrolepidota* were closely related members in the class Chrysophycae. Sequences were aligned and curated using SeaView5 (http://doua.prabi.fr/software/seaview), with targeted fragments selected based on species-specific sites. A consensus sequence at 90% was also created for *Dinobryon* to identify genus-specific regions. The primer sets for both species were designed using BioEdit software and confirmed in silico using TestPrime on the silva database (https://www.arb-silva.de/search/testprime). Two sets of primers were identified for *C. dendrolepidota*, and four sets were identified for *Dinobryon* (Table 4.2). Gradient PCRs were used to evaluate the optimal annealing temperature for the primer sets, the temperatures ranged from 52 - 65°C for *Dinobryon*, and 65 - 72°C for *C. dendrolepidota*. The primers were then tested empirically for its specificity and amplification efficiency using both in-lab cultures and clones of *C. dendrolepidota* and *D. faculiferum*.

Polymerase chain reaction (PCR) on collected water samples using the designed primers were performed as outlined in a previous section for species isolation. All PCR products were then assessed on an 1.5% agarose gel.

4.3.4 Literature Search for C. dendrolepidota in Published Studies

To investigate whether *C. dendrolepidota* is present in other freshwater systems, literature searches were done to obtain 18S HTS files from various lakes and waters. Bioprojects and their associated SRA files were downloaded from NCBI (Table 4.2).

HTS files from each BioProject were used to check data accessibility and validity. For each of these files, the paired-end reads were blasted against the reference *C. dendrolepidota* sequence (GenBank Accession number: AF123297.1). OTUs with only one read, chimeric sequences, and OTUs that were too dissimilar (<65% similar to the most abundant OTU using Water Local Pairwise Alignment implemented in EMBOSS package) were removed to reduce noise produced by HTS. The OTUs were aligned within a curated full-length SSU rDNA alignment using Muscle (Edgar, 2004). Remaining files from the BioProject were downloaded and an OTU library was built following the HTS sequence guideline outlined in (https://github.com/jeandavidgrattepanche/Amplicon_MiSeq_pipeline/blob/master/Guide_MiSeqPipeline_2018.txt).

Taxonomy of each OTU was assigned by either using BLAST against the curated full-length SSU rDNA database or by assigning each OTU to their closest sister on the phylogenetic tree. MEGA7 was used to assign pairwise distance values and calculate similarity between the OTUs and their closest sister in our phylogeny.

Table 4.2 High-Throughput Sequencing BioProjects reviewed for *C. dendrolepidota* presence. Projects without associated publications are either yet published or data from public agencies.

	Geographic Location	Size Fraction (µm)	BioProject/ Accession number	Publications
North America and Arctic Ocean				
Milne Fiord	Nunavut, Canada	0.2-3	PRJNA326017	Thaler et al., 2017
Calvert island,	Canada	0.22	PRJEB24000	Okamoto et al., 2022
St. Charles River	Quebec, Canada	0.2-0.22	PRJNA541322	Cruaud et al., 2020
Georgia and Fraser River	British Columbia, Canada	0.2	PRJEB32009	
Calvert and Hecate Islands	Canada	0.22	PRJNA396681	Heger et al., 2018
Janus Arctic gateway	Arctic Ocean	0.2-3	PRJNA383398	Joli et al., 2018
Hudson river	NY, USA	0.2	PRJEB23919	
White lake	NJ, USA	0.2	PRJEB23923	
Bellingham Bay	WA, USA	0.2	PRJEB24157	
Riveria Maya	Mexico	0.2	PRJEB23922	
Natural Tunnel State Park	Virginia, USA	0.45	PRJNA434596	Cahoon et al., 2018
Lake ERA1-4;L05 Lake 1st and 2nd Lake Greiner Spawning Tahiryuaq	Virginia Island, Canada	0.2-3	PRJNA623385	Potvin et al., 2022
Nares Strait	Arctic	0.2-3	PRJEB24314	Kalenitchenko et al., 2019
Lake Clair, Clement Lake Saint-Charles Lake Saint-Augustin	Canada	0.2	PRJNA681583	Fournier et al., 2021

Table 4.2 (continued)

	Geographic Location	Size Fraction (µm)	BioProject/ Accession number	Publications
Amundsen Gulf	Arctic	0.2	PRJEB24025	
Chuckchi sea	Arctic Ocean	0.2	PRJEB24054	
Asia				
Lake Taihu Lake Chaohu	China	0.22 um	PRJNA330896	Li et al., 2017
Lake Chaohu	China	0.22	PRJNA534176	Shi et al., 2020
Xiamen Island	China	0.22	PRJNA255070	Yu et al., 2015
Lake Baikal	Russia	0.2-0.22	PRJNA657482	David et al., 2021
South America				
Parana' river	Argentina	2	PRJEB23471	Arroyo et al., 2018
Coral reef	Curacao	0.2	PRJNA482746	
Europe				
Drinking water network	Riga, Lativa	0.2	PRJEB31264	
LTER Helgoland research site	Germany	0.4	PRJEB37135	
Upper Bavarian lakes	Germany	0.2	PRJEB34585	
Lake Tovel	Italy	0.2	PRJEB32348	Obertegger et al., 2019
Oceania				
Chatham Chases	New Zealand	0.2	PRJNA670061	
Solomon Island lagoon	Solomon Islands	0.2	PRJEB24428	
Southern Ocean				
Filde Peninsula	Antarctica	0.2	PRJNA514205	Luo et al., 2020

4.4 Results

4.4.1 Primer Design

Novel sets of primers were designed and tested for the genus *Dinbryon* and *C. dendrolepidota*. Using gradient PCR, we tested the primers under an array of

temperatures, where successful amplification and clear bands on agarose gel were observed for primer set Chryso_F and Chryso_R2 at 68°C for *C. dendrolepidota*, and Dinobryon_F1, Dinobryon_R2 at 58°C.

We tested both primer sets on in-lab cultures and clones, as well as other in-lab chrysophyte cultures such as *Ochromonas*. Primer set Chryso_F and Chryso_R2 was species-specific and consistently amplified *C. dendrolepidota*; while primer set Dinobryon_F1 and Dinobryon_R2 was proven to successfully amplify *Dinobryon* genus.

Table 4.3 Novel Primers designed to test for *C. dendrolepidota* and *Dinobryon* presence.

Primer Name	Direction	Sequence
Chrysolepidomonas dendrolepidota		
Chryso_F	Forward	5'-TCA TTA ATT TGA TTT GTA CCT TTT GTC CGA -3'
Chryso_R1	Reverse	5' - CTA ACG TCA ATG CTA AGC ATT CAC -3'
Chryso_R2	Reverse	5' - ACT GCT AAG TCG CCA AAG GCG TCT CAG CTG -3'
Dinobryon		
Dinobryon_F1	Forward	5' - CAT CAA GCC CCG ACT TT -3'
Dinobryon_F2	Forward	5' - CCC GTA ACT TGG TG-3'
Dinobryon_R1	Reverse	5' -TGA TGG AGT CAT TAC A -3'
Dinobryon_R2	Reverse	5'- AAA GTC CCT CTA AGA AGC CAM MA -3'

4.4.2 Presence of C. dendrolepidota and Dinobryon in Freshwater Systems

For our freshwater samples, we first ran a PCR using eukaryotic primers EUK1A and EUKB2 to check whether microbial eukaryotes were present in the samples. Eukaryotic presence was detected in YangMing Mt. waterfall and Guandu pier for samples taken in Taiwan, while microbial eukaryotic DNA was present in all samples from lake samples taken in North America, with the exception of Lake Medora and

Indian Lake (Figure 4.2).

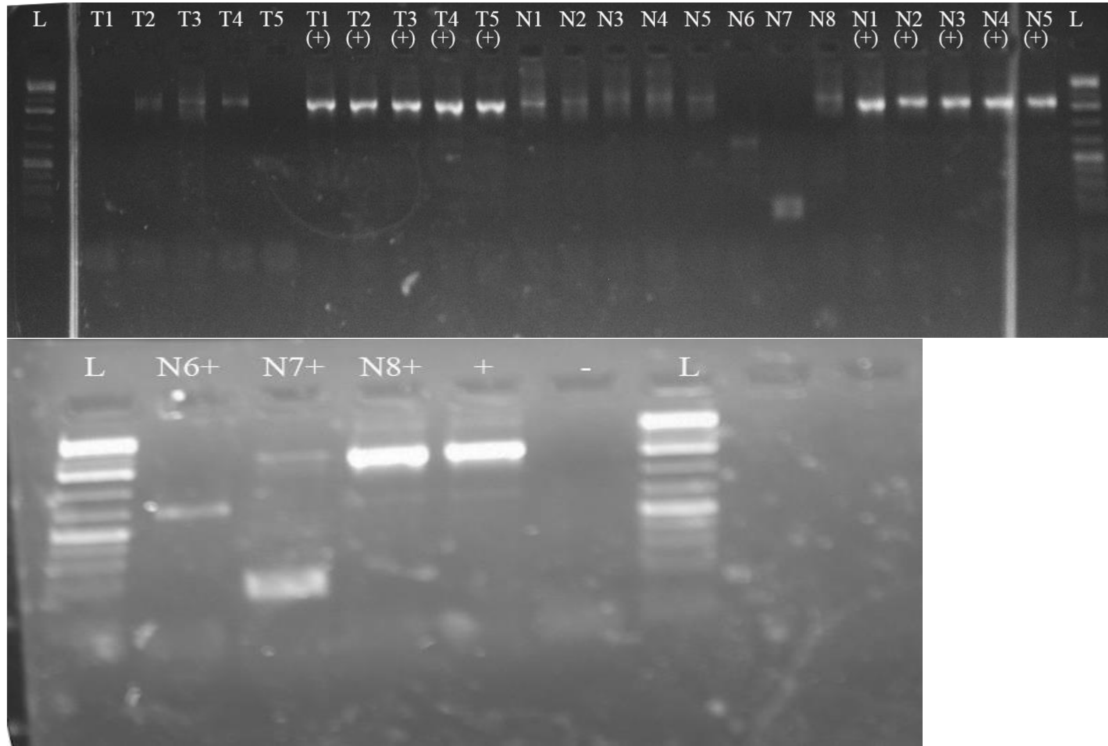

Figure 4.2 Agarose gel electrophoresis of PCR amplified products using Euk1A and EukB2 primers. Lanes marked T1 - T5 are water sampled from Taiwan, lanes marked N1 - N8 are samples from United States (Table 4.1). Lanes marked T1 - T5 (+), N1 - N8 (+), are positive controls where the sample was mixed with in-lab culture. Lane marked with "+" is positive control. Lane marked with "-" is negative control. Lane marked as "L" are 1kb DNA ladder.

Using the primer sets we designed for *C. dendrolepidota* and *Dinobryon*, we then tested for the presence of both species in the water samples. No presence of *C. dendrolepidota* was detected in any of our samples. Presence of the genus *Dinobryon* on the other hand, was detected in YangMing Mt. waterfall from Taiwan, and Rootbeer Lake, Muskegon Lake, and Lake Paradise in the United States.

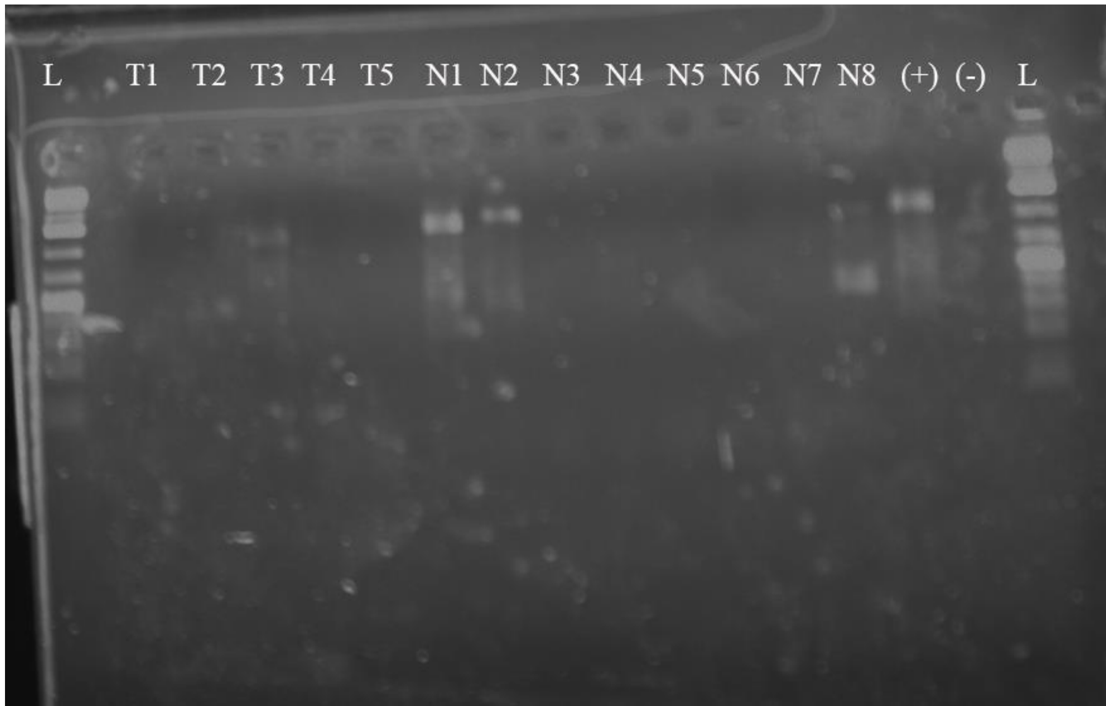

Figure 4.3 Agarose gel electrophoresis of amplified PCR products using primers Dinobryon_F1 and Dinobryon_R2. Lanes labeled T1 – T5 are samples from Taiwan, N1 – N8 are samples from United States (Table 4.1). Lane labeled (+) and (-) are positive and negative controls, respectively. Lanes marked as L are 1kb DNA ladder.

4.4.3 Literature Search of C. dendrolepidota

At first, the review of literature was planned only for freshwater systems in North America. However, during the initial reviewing process, a limited number of freshwater studies that implemented HTS to examine microbial eukaryotic biodiversity were identified. Thus, we expanded our search and included samples from other continents such as Asia, Europe, and Oceania (Table 4.2). Most samples from these areas were taken from freshwater systems, with a few exceptions of environments that include mixing of fresh and salt water. After excluding BioProjects with broken links and unreadable

datafiles, a total of 31 BioProjects were investigated to look for the presence of *C. dendrolepidota*. In all 31 projects, *C. dendrolepidota* had not been detected.

4.5 Discussion

In this study novel species-specific primers were designed for *C. dendrolepidota*. But the presence of *C. dendrolepidota* was not detected in any of our thirteen freshwater samples from the USA or Taiwan. *C. dendrolepidota* was also not identified in previously published studies. The presence of *C. dendrolepidota* in water samples collected from lake Medora, where it was originally isolated, was also undetectable.

Although this outcome was unexpected, there are several possible reasons for the absence of *C. dendrolepidota*.

4.5.1 Seasonal Variability and Taxon Turnovers

Perhaps the most reasonable explanation to consider for the absence of *C. dendrolepidota* from the samples is seasonal variability and possible community shifts on a temporal scale. Protist communities often exhibit seasonal patterns (Berge et al., 2017; Simon et al., 2015; Unrein et al., 2007). On an yearly scale, different community compositions are often observed for summer and winter, with seasonal taxonomical turnovers (Lewis et al., 2019; Millette et al., 2021). Strong seasonal succession and abundance patterns have been reported for chrysophyte species (Boopathi et al., 2015; Charvet et al., 2012). In addition, Nolte et al (2010) found lineages of the chrysophyte *Spumella* exhibits seasonal niche differentiation, where even with a broad distribution, specific lineages are more adapted to certain microclimates or seasons.

During seasons where conditions are not favorable, protists can undergo encystment in order to survive the harsh environment (Gao et al., 2017; Lengyel et al.,

2022; Schünemann et al., 2007), and most chrysophytes are assumed to encyst or go under similar resting stages (Foissner, 2007; Lengyel et al., 2022; Stoecker et al., 1997). During these periods of time, species exist in low frequencies, especially in the water column, making them harder to detect (Nolte et al., 2010). The lack of seasonal sampling hence could result in mis-labeling of species as rare.

4.5.2 Influences and Changes in Abiotic Factors

Dominant species and community compositions in aquatic systems are influenced by a number of abiotic parameters, such as temperature, light intensity, nutrient availability, and pH (Caracciolo et al., 2022; Charvet et al., 2014; Lewis et al., 2019). Nutrient input and pH, in particular, are known to play a role in chrysophyte abundance in freshwater systems (Kalinowska & Grabowska, 2016; Mushet et al., 2017; Rottberger et al., 2013)

C. dendrolepidota was first sampled from Lake Medora in the summer of 1984. The water temperature at that time was 22°C, with a pH of 7 (Peters & Andersen, 1993). Subsequent investigation of this species was done using in-culture strains (Hamsher et al., 2020), no further field samples have been collected. We collected our field samples from Lake Medora in June 2023, the water temperature recorded was 16°C. The difference between sampling month and temperature may have contributed to the inability to detect *C. dendrolepidota* in the lake. It is also highly possible that abiotic factors such as pH, dissolved oxygen, or water temperature changed in the time period. With the lack of field surveys for Lake Medora, we also unfortunately are not able to infer community compositional and structural changes in the past 40 years.

4.5.3 Abundance and Possible Rarity of C. dendrolepidota

The renown hypothesis on microbial cosmopolitanism states "everything is everywhere, but the environment selects" (Becking, 1934). This has led some to the assumption that protists and other microbial organisms are widely distributed (DeWit & Bouvier, 2006; Fenchel, 2005), with little attention on species rarity or possible local extinctions (Cotterill et al., 2008; Weinbauer & Rassoulzadegan, 2007; Weisse, 2008). However, it is reported that around one third of known protist species have restricted range (Cotterill et al., 2008). In recent years, more studies have been conducted on the biogeography of the microbial eukaryotes, revealing the presence of rare protist species, some of which had restricted distributions (Dolan, 2005; Logares et al., 2015; Pan et al., 2020; Weisse, 2008). For example, the chrysophyte *Synura hibernica*, was originally found only in a small area of western Ireland (Škaloud et al., 2014). Cotterill et al (2013) also found ciliate species that are endemic to the Krauthugel pond in Austria.

There is no known information on past abundance or even presence of *C. dendrolepidot*a in lakes, so it cannot be deduced whether *C. dendrolepidota* decreased in abundance in Lake Medora over the past 40 years, if it is seasonally more abundant, or if it was always rare in the system.

Several studies suggested protists, especially those in rarity, should increase in abundance when the environment is favorable (Logares et al., 2015; Weinbauer & Rassoulzadegan, 2007). Hence, if *C. dendrolepidota* existed in high abundance in the past, changes in abiotic and biotic variables may have led to a less desirable environment, limiting species population. On the other hand, *C. dendrolepidota* may have always been present in low abundance in aquatic systems, with the possibility of being a rare species.

There are many factors that may contribute to low abundance for protistan species. Biotic factors including competition and predation as well as abiotic factors such as resource limitation are well known to influence species abundance and community composition (Logares et al., 2014; Weinbauer & Rassoulzadegan, 2007). Being in low abundance however, are not necessary a disadvantage. Low abundance may lead to successful avoidance of predation and parasitism, as well as less competition pressure (Logares et al., 2014). If C. dendrolepidota is indeed a rare species, it may experience fluctuations of abundance on a temporal scale (Caracciolo et al., 2022; Nolte et al., 2010), making detection of the species even more difficult without long-term sampling.

More studies have been recognizing the presence and importance of protist rare biospheres, with species showing unique phylogenetic composition and biogeographical distributions (Boo et al., 2010; Cahoon et al., 2018; Debroas et al., 2017; Logares et al., 2014, 2015). Rare microbial species are proposed to host keystone species that have a disproportional effect on bio-geo-chemical processes relative to their abundance, playing crucial roles in nutrient cycling (Jousset et al., 2017). In addition, rare biospheres contribute to ecosystem resilience and promote recovery to disturbance events (Jousset et al., 2017; Lynch & Neufeld, 2015).

4.5.4 Sampling Issues

Under-sampling and sampling bias are known to skew our understanding of protist diversity and distribution (Coterill et al., 2008, Dougherty et al., 2016, Foissner et al., 2008, Leles et al., 2019). Protist distributions are not always homogenous, and patchiness frequently has been observed (Bulit et al., 2014; Grattepanche et al., 2016).

Given that the sampling for this study filtered a maximum of 0.5 L of water from

each freshwater system, *C. dendrolepidota* could have been excluded if it indeed exists in low abundance. Any future surveys in Lake Medora should sample larger volumes to further test whether the species is rare in general or potentially seasonally abundant.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 Temperature and Nutrient Influences on the Balance of Phototrophy and Phagotrophy in a Mixotrophic Freshwater Chrysophyte

Our findings showed that increased temperature influences both phototrophic, phagotrophic and potentially osmotrophic responses in *C. dendrolepidota*. Although the species is primarily phototropic, bacterivory is reduced as temperature increased, which is also the case for some other phototrophic-mixotrophs (Ferreira et al., 2022; González-Olalla et al., 2019; Princiotta et al., 2016). However, other studies have shown mixotrophic chrysophytes becoming more heterotrophic with increasing temperature (Kang et al., 2020; Lim et al., 2019; Wilken et al., 2013), complicating our ability to predict responses in a warming environment. Furthermore, the proposed uptake of dissolved organic carbon to balance the carbon budget at 14°C requires additional research as it adds additional uncertainty to how warming temperatures may affect mixotrophs. In addition, theoretical models on impacts of warming on protists often exclude interacting variables that may complicate predictions. To gain better clarity on the ecology of mixotrophs under warming scenarios, future studies should take into account that the interaction of abiotic and biotic factors are likely to affect mixotrophic species differently. Future work should also incorporate temporal aspects to better understand long-term mixotroph response to climate change.

5.2 Temperature- and Nutrient-Mediated Phagotrophic and Phototrophic Responses in a Mixotrophic Arctic Chrysophyte

D. faculiferum had varying mixotrophic responses under different levels of light, nutrients, and temperature; both heterotrophic ingestion and photosynthesis increased in response to rising temperature and decreased in response to limiting inorganic nutrients, especially nitrogen. In addition, *D. faculiferum* was shown to be more reliant on phagotrophy than freshwater species in the same genus. With projected warming sea temperatures and further loss of sea ice, it has been suggested that mixotrophic species may shift their nutritional mode, leading to changes in food web structures and carbon availability in the system (Hop et al., 2020; Jeong et al., 2010; Millette et al., 2023; Stoecker & Lavrentyev, 2018). However, the complex nature of interactions between abiotic and biotic factors and the variability between mixotrophic species hinder our ability to project or model how mixotrophic species will respond in a changing environment. The fact that *D. faculiferum* had a different mixotrophic mode compared to freshwater members of the genus is a reminder that responses can be species-specific, and that generalities and/or assumptions about how mixotrophic species fit into aquatic food webs should be approached with caution.

5.3 Exploring Biogeographical Distribution of a Poorly Identified Freshwater Chrysophyte

Novel primer sets were designed for *C. dendrolepidota* and *D. faculiferum* on a species-specific and genus-specific level, respectively. *C. dendrolepidota* was not identified in any of the freshwater samples collected for this study nor in our metadata analysis. Possibilities for the absence of the species include seasonal variability, low

abundance, species rarity, and patchy distribution. We also address the possibility for endemism and local extinction.

As demonstrated by the case of Lake Medora, where *C. dendrolepidota* was originally isolated, lack of continuous sampling and monitoring lead to difficulties understanding species abundance as well as community dynamics and successions. Future endeavors should focus on periodic sampling on a longer time-scale, with continuous tracking of physical and biogeochemical properties of freshwater systems.

Freshwater systems, especially smaller lakes and ephemeral water bodies can host high protist diversity (Debroas et al., 2017; Simon et al., 2015). More attention should be directed to these understudied systems for potential rare and new species.

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