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High abundances and negligible grazing during winter by the mixotrophic chrysophyte *Dinobryon*

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Abstract: Grazing on bacteria by planktonic organisms is a channel for the biological transfer of organic matter through the aquatic food web. Much attention has been given to the importance of heterotrophic nanoflagellates and ciliates as primary grazers of bacteria. However, the prevalence of phagotrophy by phytoplankton across many environments emphasizes a need to include mixotrophy in studies of the protistan food web. Few studies have addressed the seasonal dynamics nor grazing activity of mixotrophs in freshwater environments, especially those that extend below surface waters. The goal of this work was to examine temporal patterns in mixotroph abundance and taxon–specific bacterivory, with a focus on *Dinobryon*. Results shown here support our general predictions of increased bacterivory by *Dinobryon* where a low light and nutrient environment may promote grazing. *Dinobryon* spp. were numerically dominant members of the community under–ice in winter, possibly as a result of their ability to supplement photosynthesis with phagotrophy of bacteria in conditions of reduced irradiance and day length. However, daily grazing rate and associated impact on the bacterial community during winter were not substantial. These results highlight the importance of including winter sampling when an active community of phagotrophic phytoflagellates may play a major role in ecosystem functioning. Results underscore the importance of including measurements of grazing activity with mixotroph occurrences, as high abundance of *Dinobryon* did not align with high rates of bacterivory.

Key words: bacterivory, *Dinobryon*, flagellate, mixotrophy, phagotrophy, seasonal succession

Introduction

The microbial loop has emerged as a dominant component of planktonic ecosystem dynamics that reintroduces dissolved organic carbon that would otherwise be lost from the system via protistan ingestion of heterotrophic bacteria biomass (Azam et al. 1983). Bacterivorous flagellates, including heterotrophic and mixotrophic species, are ubiquitous members of the microbial loop (Sherr & Sherr 1994). Protistan grazing in lacustrine ecosystems plays a significant role in bacterial mortality (Sanders et al. 1989; Ram et al. 2013) and has far–reaching impacts on the bacterial community in terms of phylogenetic composition and morphology (van Hannen et al. 1999). In addition to effects on the bacterial community, differential feeding by bacterivores may also impact protistan community composition via regeneration of dissolved nutrients or reduction of less effective competitors (Thingstad et al. 1996; Jost et al. 2004).

In conjunction with heterotrophic forms, phagotrophic phytoplankton (i.e., mixotrophs) are prominent constituents and substantial bacterivores in freshwater food webs (Sanders 1991; Hansson et al. 2019). Mixotrophy has been identified in many eukaryotic taxa, including dinoflagellates (Hitchman & Jones 2000), cryptophytes (Palsson & Graneli 2003), chrysophytes (Holen & Boraas 1995), and chlorophytes (Bell & Laybourn–Parry 2003). The combination of phototrophy and phagotrophic ingestion of bacteria may allow mixotrophic taxa to persist during periods that are otherwise unsuitable for metabolic specialists (i.e., photoautotroph or heterotroph). Field and laboratory studies have demonstrated that bacterivory by mixotrophic protists may be most advantageous when there is a limited availability of dissolved nutrients or particulate prey in combination with ample light environment (Fischer et al. 2017). However, the activity of mixotrophic protists as a group is often difficult to predict in nature due to the spectrum of nutritional strategies that range from primarily phototrophic to primarily heterotrophic. In addition, mixotrophs

often respond in a species–specific manner to environmental conditions based on their own unique physiology, which can dictate the balance between phototrophy and phagotrophy (WILKEN et al. 2020).

Numerous members of the Chrysophyceae have been identified as mixotrophs, defined here as an innate ability to photosynthesize in combination with phagotrophic uptake of particulate prey (HOLEN 1999; LIE et al. 2017). This includes several species of *Dinobryon* Ehrenberg 1834, members of which are primarily phototrophic but with great potential for bacterivory (GEREA et al. 2019), and which have been identified as key grazers on bacteria in both marine (UNREIN et al. 2010) and freshwater environments. However, presence of a mixotroph is not necessarily indicative of activity (e.g., bacterivory) and is best described as potential for mixotrophy (MILLETTE et al. 2021). The purpose of this work is to examine the relationship between abundance and bacterivory of *Dinobryon* spp., with focus on seasonal patterns. Although numerous studies have documented the activity of *Dinobryon* in situ, many lack spatial (i.e., vertical) resolution (but see companion study; PRINCIOTTA & SANDERS 2017). It was predicted that abundance and grazing by *Dinobryon* would reach a maximum in surface waters, driven by ample availability of photosynthetically active radiation (PAR), a requirement for activity in this mixotroph (CARON et al. 1993). Bacterivory was expected to be reduced, but still present, within the thermocline, where attenuation of PAR may also stimulate grazing, but was not expected to be dominant in the hypolimnion, where a significant reduction in PAR would restrict growth and activity. We expected to see increased bacterivory during periods characterized by reduced macronutrient concentrations or shortened day length (i.e., surface waters in fall), during which *Dinobryon* might compensate for reduced photosynthetic activity through bacterivory.

Materials and Methods

Study site and sampling regime. Lake Lacawac (41°22.912' N, 75°17.543' W, 439 m altitude) is a 13,000–yr–old mesotrophic lake of glacial origin located in the Pocono Mountains of northeastern Pennsylvania, U.S.A. This 21–ha lake resides within the forested watershed of a nature preserve and has been protected from excess anthropogenic input since the 1960s, making it a suitable reference site for exploration of ecological patterns. A stable thermocline typically develops during late spring and summer, and the 13–m water column undergoes turnover in early autumn. During the study period, Lake Lacawac exhibited ice–cover for 115 days from mid–December through early April (BRUCE HARGREAVES, unpubl. data). Phytoplankton in Lake Lacawac tend to be most abundant during winter and spring, with dominance of flagellated chrysophytes (BERNINGER et al. 1992). Subsurface phytoplankton peaks have been observed during summer months, as the euphotic zone often extends beyond the surface mixed layer (KNOLL et al. 2016). Procedures for collection of water samples and measurement of physiochemical characteristics were previously reported in PRINCIOTTA et al. (2017). In brief, whole water samples were collected monthly from a fixed, central location over the deepest point in Lake Lacawac between May 2013 and September 2014. Unstable ice conditions prevented sampling during December 2013 and February 2014. Water was collected with a vertical Van Dorn bottle from three depths corresponding to the summer epilimnion (1 m), metalimnion (3–5 m), and hypolimnion (7–8 m) as determined by a concurrent temperature profile. Samples were taken as close to midday as possible, though it is known that bacterivory and vertical distribution may vary within a diel period (ANDERSON et al. 2017). Hypolimnetic samples were always taken several meters above the sediment–water interface, but temperature and oxygen conditions were generally unchanging below 7 m on a given date.

Sample processing and grazing experiments. The proximity of Lake Lacawac to a biological field station allowed experimentation to initiate within 30 minutes of collection. Grazing experiments were conducted onshore to determine bacterivory rates by mixotrophic nanoflagellates. Samples from the epi– and metalimnion were conducted in semi–shaded conditions, whereas those from the hypolimnion were conducted inside a cooler. Here, we report data for the genus *Dinobryon.* Water samples were first filtered through a 150 µm mesh screen to remove zooplankton, and replicate (n=4) subsamples of 100 ml from each depth were dispensed into Whirl–Pack® bags. Fluorescent polycarbonate microspheres (0.6 µm diameter, excitation maximum 441 nm, emission maximum 485 nm, Polysciences Inc.) were added to each subsample at a constant, tracer level (approximately 20% of natural bacterial abundance, assumed to be approximately 10^6 ml^{-1}). It should be noted that there were likely differences in the size of natural bacteria with season and depth that were not accounted for with polycarbonate microspheres. Ratio of bacteria to microspheres during the study period was 2.7±0.3 SE. Fixation and preservation by the Lugol's–formalin technique was conducted immediately after addition of bacterial surrogates to account for background ingestion (T_0) and again after 20 minutes of incubation (SHERR et al. 1987). Preserved samples were stored in the dark at 4 °C for a maximum of 24 hours until further processing.

Microscopic enumeration and assessment of bacterivory. Background bacterial abundance and concentration of microspheres were determined by filtration of a single 500 µl subsample from each depth and sampling date onto a 25 mm, 0.2 µm black polycarbonate membrane (GE Water and Process Technologies). Filters were mounted onto slides with Vectashield mounting media containing DAPI stain (4'6–diamidino–2–phenylindole, Vector Laboratories) and visualized at 1000× on a Zeiss Axiovert inverted fluorescence microscope. DAPI–stained bacteria were counted using a Zeiss 48–77–02 filter set (G365 exciter filter/FT 395 dichromatic beam splitter/LP 420 barrier filter). Fluorescent microspheres were counted from the same mounted filters as bacteria, but under a Zeiss 48–77–09 filter cube (BP 450–490 exciter filter/ FT 510 dichromatic beam splitter/LP 520 barrier filter). All slides were kept frozen until microscopic analyses.

Dinobryon abundances and genus–specific bacterivory rates were determined from replicate 75 ml aliquots (n=4) of preserved samples from the Whirl–Pack® bags

settled in darkened 100 ml graduated cylinders overnight. Water was then aspirated from the top of the samples with a J–shaped tube until approximately 10 ml remained, which was stored at 4 °C in darkness to preserve chlorophyll autofluorescence. Subsequent enumeration and identification were performed by epifluorescence microscopy on a Zeiss Axiovert microscope at 400×. *Dinobryon* cells were first visualized for chlorophyll autofluorescence with the 48–77–09 filter cube as noted above and incidence of bacterivory was determined based on latex microspheres within the cell. Ingestion rate was calculated by multiplying rates of microsphere uptake and the ratio of ambient bacteria to microspheres after subtraction of background (T_0, microspheres potentially associated with cells but not ingested). We assumed no selective bias against fluorescently labeled tracers (Princiotta et al. 2016), and constant grazing rates over a diel cycle. Background was negligible within all depths and dates. Grazing impact was calculated by multiplying cell abundance (cell.ml^{-1}) by ingestion rate (ingestion cell^{-1}.day^{-1}) and used to estimate the percentage of bacterial standing stock removed by grazing.

Data analyses. Statistical tests were conducted in JMP Pro version 16.2.0 (SAS Institute). Due to failure to achieve normality, non–parametric Kruskal–Wallis rank sum tests were used to examine abundance and grazing rate of *Dinobryon* across seasons and depths in Lake Lacawac. Seasons were categorized according to the following: winter (December, January, February), spring (March, April, May), summer (June, July, August), fall (September, October, November). Where appropriate, post–hoc Dunn multiple comparison tests were used.

Results

Environmental conditions

A full description of environmental conditions during this study can be found in Princiotta et al. (2017). Briefly, collections and field experimentation began in mid–May 2013 when water column stratification was present and increasingly stable. Surface water temperature during summer months ranged from 21 °C to 27 °C. The thermocline weakened in September and October 2013, after which the water column remained isothermal through the winter sampling period. By May 2014 thermal stratification was reestablished and continued until the end of the study in September 2014. The hypolimnion of Lake Lacawac was hypoxic (< 2 mg.l^{-1} DO) during summer months. Lake Lacawac was ice–covered from December through early April and was inaccessible in December and February (2014) due to unstable ice conditions. Light attenuated considerably with depth in Lake Lacawac throughout the year, with PAR levels < 9 µmol photons m^{-2}.s^{-1} below 6 m and <1% surface PAR available below 5 m. Although nutrients were not measured during this study, Knoll et al. (2016) reported average summer total phosphorus as 3.5 µg.l^{-1}.

General characteristics of *Dinobryon* in vertical space and patterns in seasonal succession

Abundance of *Dinobryon* was greatest in the epilimnion of Lake Lacawac (Fig. 1, Table 1), with abundances ranging from 0 to 9,900 cells.ml^{-1} and a mean of 1,500 cells.ml^{-1} ± 365 SE during the study period. Although we did not collect data by species, we observed a dominance of *D. cylindricum* and *D. divergens*. The greatest abundance of *Dinobryon* was observed in January (8,500 cells.ml^{-1} ± 689 SE) and September (3,000 cells.ml^{-1} ± 1,100 SE), though a significant amount of monthly variability was observed. There was a significant effect of season on *Dinobryon* abundance in surface waters, with the greatest observations in winter (X^2 (3) = 12.54, p = 0.006).

Dinobryon was less commonly observed in the meta– and hypolimnion of Lake Lacawac (Fig. 1, Table 1). Though extremely variable, the greatest abundance in the metalimnion occurred during spring with a mean of 1,175 cells.ml^{-1} ± 1,492 SE. There was a significant effect of season on *Dinobryon* abundance in the metalimnion (X^2 (3) = 13.38, p = 0.004), with key differences between spring–fall (p = 0.007) and spring–summer (p = 0.004). *Dinobryon* did not exceed 350 cells.ml^{-1} in the hypolimnion and there were no significant trends in seasonal abundance.

Bacterivory by *Dinobryon* with season and depth

Bacterial abundance ranged from 3.5×10^4 bacteria.ml^{-1}

Table 1. Mean values for abundance, daily grazing rate, and daily grazing impact by *Dinobryon* in Lake Lacawac across depth horizons (Epi = epilimnion, Meta = metalimnion, Hypo = hypolimnion). Statistical effect of depth or season within each depth horizon on each parameter is represented by p–value from non–parametric Kruskal–Wallis test (ns = not significant, defined as p < 0.05).

Parameter	Mean ± SE			Effect of depth	Effect of season within each depth layer		
	Epi	Meta	Hypo		Epi	Meta	Hypo
Abundance (cells.ml^{-1})	1,513 ± 365	529 ± 130	225 ± 72	0.008	0.006	0.004	ns
Grazing rate (bac.cell^{-1}.day^{-1})	26 ± 7	13 ± 3	5 ± 2	0.01	ns	ns	ns
Grazing impact (bac.ml^{-1}.day^{-1})	5.0×10^4 ± 1.5×10^4	2.1×10^4 ± 7.6×10^3	5.1×10^3 ± 2.8×10^3	0.005	ns	ns	ns

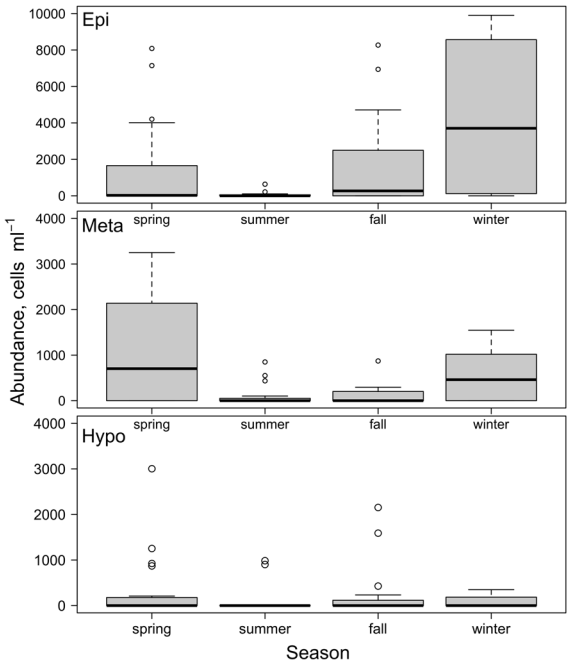

Fig. 1. Seasonal abundance (cells.ml^{-1}) of *Dinobryon* in the epilimnion (Epi), metalimnion (Meta), and hypolimnion (Hypo) of Lake Lacawac during the 15–month study period. Median value at mid–point of data is represented by a solid line within the quartile range. Open circles above quartiles represent outliers beyond 1.5 inter–quartile range. Note change of scale on y–axis for meta– and hypolimnion.

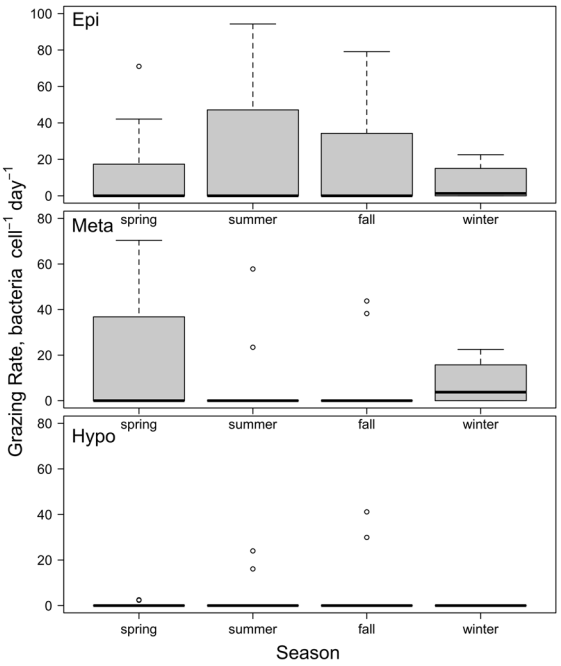

Fig. 2. Grazing rate (bacteria.cell^{-1}.day^{-1}) of *Dinobryon* populations in the epilimnion (Epi), metalimnion (Meta), and hypolimnion (Hypo) of Lake Lacawac during the 15–month study period. Median value at mid–point of data is represented by a solid line within the quartile range. Open circles above quartiles represent outliers beyond 1.5 inter–quartile range. Note change of scale on y–axis for meta– and hypolimnion.

(recorded in May 2014 metalimnion) to a maximum of 9.3×10^5 bacteria.ml^{-1} in August 2014, fluctuating widely during the study period. Daily grazing rate by *Dinobryon* was highly variable with both season and depth during the study period, ranging from undetectable to 40 bacteria.cell^{-1}.day^{-1} ± 18 SE observed in the summer epilimnion (Fig. 2). Despite the highest observed abundance in the winter epilimnion, grazing rate by *Dinobryon* was relatively low in the colder waters (7 bacteria.cell^{-1}.day^{-1} ± 3 SE). Grazing rates in the surface waters were approximately 2× and 5× higher than the means for the meta– and hypolimnion, respectively. In fact, an effect of depth was observed for grazing rate (X^2 (2) = 9.17, p = 0.01) with the most significant differences between the epi– and hypolimnion (p = 0.0025). There was no statistically significant seasonal trend in daily bacterial grazing rate by *Dinobryon* within any depth horizon, likely due to the high variability.

Peak impacts on the bacterial assemblage by *Dinobryon* were observed in the epilimnion (Fig. 3). Grazing impact by *Dinobryon* in the hypolimnion was negligible, removing less than 2% of the bacterial community per day, with significant differences between the hypolimnion with the other two depth horizons (X^2 (2) = 10.52, epi–hypo; p = 0.001, meta–hypo; p = 0.04), as might be expected from the relatively low abundances of the alga there. However, within each depth horizon there were no significant seasonal patterns in grazing impact. Grazing by *Dinobryon* contributed to approximately 15% of heterotrophic bacteria turnover during the study period. In the epi– and metalimnion, the highest contribution to bacterial removal by *Dinobryon* was observed during spring (25% ± 9 SE, 43% ± 17 SE).

DISCUSSION

The highest abundances of the mixotrophic genus *Dinobryon* were observed in the surface waters of Lake Lacawac. This is not surprising given the light–dependence that has been measured in laboratory cultures (CARON et al. 1993; PRINCIOTTA et al. 2016). In a companion study within Lake Lacawac, distribution and bacterivory by mixotrophic nanoflagellates (MNAN, defined as < 4 μm) were also positively influenced by PAR (PRINCIOTTA & SANDERS 2017). Light has been cited as a key determinant in the competitive success of mixotrophic protists because bacterivory can be used to support photosynthesis, especially when dissolved nutrients are limiting (PTACNIK et al. 2016). However, in the event of significant attenuation of PAR (i.e., in the hypolimnion), bacterivory cannot always fully support the metabolic demands of mixotrophic

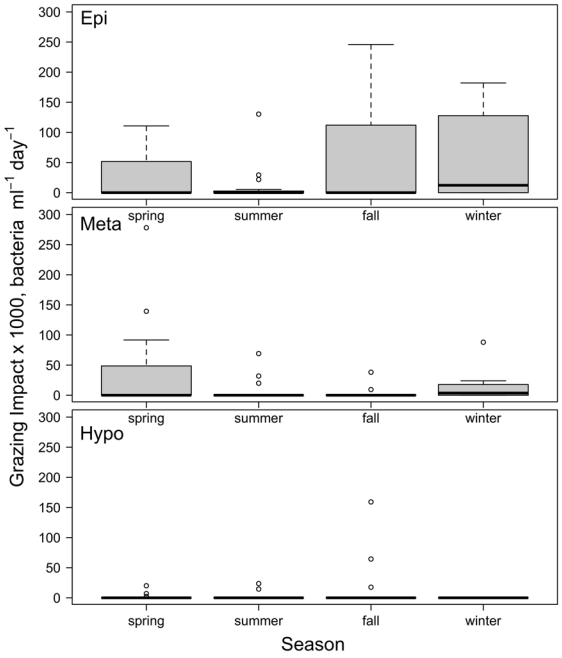

Fig. 3. Daily grazing impact (bacteria.ml^{-1}.hr^{-1} ×1000) of *Dinobryon* in the epilimnion (Epi), metalimnion (Meta), and hypolimnion (Hypo) of Lake Lacawac during the 15–month study period. Median value at mid–point of data is represented by a solid line within the quartile range. Open circles above quartiles represent outliers beyond 1.5 inter–quartile range.

metabolism, at least in *Dinobryon,* which is considered a predominantly phototrophic mixotroph. Our work in Lake Lacawac generally supports this concept, as abundance and impact on the bacterial community was greater in the surface waters than in the hypolimnion. However, mixotrophy is considered a spectrum of nutritional strategies, ranging from obligate phototrophy to obligate heterotrophy, and different depth–related trends are to be expected.

Dinobryon abundance and grazing were insignificant in the hypolimnion. Whereas sustained growth by some mixotrophic genera has been demonstrated at low light levels without substantial bacterivory (Gasol et al. 1993), *Dinobryon* ceases bacterivory and declines in abundance after a period of several days in darkness (Caron et al. 1993; Princiotta et al. 2016), suggesting that it is an obligate phototroph. In this study, bacterivory by *Dinobryon* was observed during all seasons at all depths within the photic zone. The mismatch between peak abundances and grazing observed in this study suggests that photosynthetic carbon fixation can support the *Dinobryon* population and bacterivory provides a supplement. Interestingly, however, few studies exist that attempt to culture *Dinobryon* under axenic conditions (but see Caron et al. 1993 in which axenic growth of laboratory culture was poor).

Dinobryon bacterivory was an important feature of the planktonic community under ice, as was also observed with mixotrophic flagellates < 4 μm (Berninger et al. 1992). Though winter plankton has been historically portrayed as dormant, sampling has revealed that key ecosystem processes still occur under ice (Hampton et al. 2015; Grosbois et al. 2017). Mixotrophy has been hypothesized to promote survival during conditions of reduced light and temperature; it is not uncommon in polar ecosystems (Charvet et al. 2012; Stoecker & Lavrentyev 2018). The ability to ingest bacteria in primary phototrophs can serve as an additional nutrient and carbon source, particularly under light–limited conditions presented by ice (Vick–Majors et al. 2014). A dominance of *Dinobryon* was not surprising given the ability to reach a maximum photosynthetic rate under moderate light conditions (Heinze et al. 2013) and other field studies have observed population peaks in winter (Hitchman & Jones 2000; Butts & Carrick 2017). A correlation between abundance of *Dinobryon* with concentration of bacteria in winter suggests that the mixotroph may use bacterivory to survive the under–ice environment. However, in our study, grazing rate of *Dinobryon* was generally low in winter, and these data do not support the proposal by Bird & Kalff (1987) that *Dinobryon* depends on phagotrophy for a dominant proportion of its carbon requirement. Regardless, this mixotroph benefited from the presence of heterotrophic bacteria, potentially through mechanisms other than direction ingestion, such as carbon or nutrient remineralization.

This work highlights the importance of considering both seasonal and spatial (i.e., vertical) scale in studies of planktonic dynamics. Attention to mixotrophy also should be included, but with direct assessments of feeding which varies substantially with these factors. Historically, plankton phenology was examined during peak growing seasons in surface waters and infrequently included measurements of mixotrophic activity. A more complete understanding of the mechanisms underlying planktonic community structure and function will benefit from both winter and subsurface sampling.

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