



High Densities of a Prochlorophyte (Unresolved Species) Inhibit Grazing by the Herbivorous Copepod *Parvocalanus crassirostris*

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OPEN ACCESS

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Specialty section:

This article was submitted to
Marine Ecosystem Ecology,
a section of the journal
Frontiers in Marine Science

Received: 04 February 2021

Accepted: 20 September 2021

Published: 10 November 2021

Citation:

Ma X, Jacoby CA and
Johnson KB (2021) High Densities
of a Prochlorophyte (Unresolved
Species) Inhibit Grazing by
the Herbivorous Copepod
Parvocalanus crassirostris.
Front. Mar. Sci. 8:664153.
doi: 10.3389/fmars.2021.664153

Harmful algal blooms (HABs) are increasing in frequency and severity, underscoring the importance of understanding potential top-down controls of blooms. In the Indian River Lagoon (IRL), a shallow subtropical estuary, one harmful bloom was co-dominated by an unresolved prochlorophyte in the Family Prochlorothricaceae, which reached densities of $> 10^6$ cells ml^{-1} engendering the term “Superbloom.” Experiments were conducted to evaluate grazing rates and the potential for top-down control by an abundant herbivorous copepod, *Parvocalanus crassirostris*, on the prochlorophyte. Those grazing rates were lower than the rates on a palatable alternative algal food, *Isochrysis galbana*, when both algal species were presented in monocultures with identical densities. Grazing on the prochlorophyte decreased or ceased at densities over 4.8×10^5 cells ml^{-1} . When the prochlorophyte and the palatable alternative each comprised half of the total density, both species were consumed, but grazing on *I. galbana* was reduced compared to the grazing rates in a monoculture of this species, especially at higher cell densities. Copepod mortality was observed in treatments with high concentrations of the prochlorophyte, and these treatments contained mucilage. Experiments simulating viscosities produced by prochlorophyte mucilage yielded results consistent with the original grazing experiments (i.e., copepods showed lower grazing rates and higher mortality rates in higher viscosity treatments). Results reveal potential limitations of top-down controls by this grazer on prochlorophyte blooms and HABs that produce mucilage.

Keywords: prochlorophyte, harmful algal blooms, mucilage, top-down control, Indian River Lagoon

INTRODUCTION

Harmful algal blooms (HABs) are increasing in estuaries around the world (Smayda, 2008; Berry et al., 2015; Philips et al., 2015; Cao et al., 2017). Synchronicity at the global scale has been attributed to climate change and eutrophication brought on by increased loads of nutrients (Smayda, 2008), whereas important local influences on the magnitude and composition of HABs include longevity, toxicity, and differential losses due to grazing (Turner, 2006, 2010; Smayda, 2008; Philips et al., 2010, 2015; Berry et al., 2015; Cao et al., 2017).

An unprecedented sequence of intense and long-lasting algal blooms have afflicted the Indian River Lagoon (IRL) in Florida, starting in 2011 (Phlips et al., 2010, 2011, 2015; Lapointe et al., 2015), with the initial bloom being dubbed the “superbloom” because of its ~12-month duration and concentrations of chlorophyll-*a* that reached $136 \mu\text{g L}^{-1}$ ($2\text{--}4 \times$ the concentrations recorded from 1996 to 2010). In contrast to blooms dominated by dinoflagellates or diatoms (Phlips et al., 2015), the superbloom was co-dominated by cyanobacteria and a nanoplanktonic chlorophyte. Both taxa were documented at > 1 million cells ml^{-1} during the event (IRL Consortium, 2015). The bloom caused harm by decreasing light penetration for a duration that led to the loss of tens of thousands of acres of seagrass, and the resulting decreased competition for nutrients may have promoted subsequent blooms of phytoplankton (Phlips et al., 2015). In addition to bottom-up stimulation by nutrients, the relatively small size and unusually high densities of the species in the superbloom led to questions about top-down control by herbivores like zooplankton.

The alga targeted in this study was an unresolved prochlorophyte in the Family Prochlorothricaceae, which was isolated from the 2011 superbloom. The prochlorophyta are a group of cyanobacteria abundant in nutrient-poor tropical waters and possessing a light-harvesting apparatus composed of the higher plant pigments chlorophylls *a* and *b* (Partensky et al., 1993; Post and Bullerjahn, 1994; Lewin, 2002). Studies of prochlorophytes are sparse, but no toxic species have been reported. The strain used in this study was an oval-shaped unicellular cyanobacterium, with cells approximately $\sim 5 \mu\text{m}$ in diameter. Heavy mucus production was observed during the bloom and in monocultures of the prochlorophyte. During the superbloom, concentrations of the prochlorophyte exceeded 5×10^6 cells ml^{-1} . This exceptionally high density is unusual for a natural bloom, even a HAB, and it raises concerns about how such densities might impact grazers. Grazing on the prochlorophyte was evaluated to determine the potential for a key herbivore to exert top-down control. Grazing on *Isochrysis galbana*, a palatable microalga, was examined to contrast with grazing on the prochlorophyte. *I. galbana* is roughly spherical, with a diameter of 4–6 μm , and it is commonly used as a food for culturing copepods (Shields et al., 2005).

The grazer selected for this study is *Parvocalanus crassirostris*, a small (~ 2 mm long), holoplanktonic, calanoid copepod that dwells in the upper 6–20 m of tropical and subtropical estuarine, coastal, and oceanic waters (Milstein, 1979; Turner and Dagg, 1983; Wong et al., 1993; Tang et al., 1994; Almeida et al., 2012; Sun et al., 2012; Liu et al., 2013). It is common for calanoid copepods to reside permanently in the mid- to upper water column (Ma and Johnson, 2017), where they feed on microalgae and nanoplankton. *P. crassirostris* is a cosmopolitan copepod and tolerates a wide range of temperatures (15–31°C), salinities (20–37), and eutrophic or turbid conditions (Milstein, 1979; Wong et al., 1993; Almeida et al., 2012). *P. crassirostris* mainly grazes on nanophytoplankton (Calbet et al., 2000) and, according to a 3-year record of mesozooplanktonic abundances, it was the dominant herbivorous copepod in the region of the aforementioned superbloom (Sweat unpublished data).

This study aims to (1) determine the grazing rates of *P. crassirostris* on the prochlorophyte, (2) compare grazing rates on the HAB species with those on a palatable alternative (*I. galbana*), and (3) examine selective grazing when the HAB species is presented together with the palatable alternative at equal densities. Additionally, when it was discovered that the prochlorophyte produced heavy mucilage, altering culture media, this study investigated potential impact of that mucilage on copepod grazing rates and mortality.

MATERIALS AND METHODS

Culturing Copepods and Algae

Copepods (*Parvocalanus crassirostris*) and algae (The prochlorophyte and *Isochrysis galbana*) were cultured by AlgaGen LLC (Vero Beach, FL, United States). *P. crassirostris* were initially isolated from the IRL. Starter cultures of the prochlorophyte were isolated from the IRL superbloom by the Phlips Laboratory (University of Florida). Cultures used seawater filtered through a 0.45- μm filter. All algae were cultured in 2-L glass jars held at 26°C, with continuous aeration and 1,100 lux of light provided continuously by fluorescent bulbs. Guillard's (F/2) marine water enrichment solution was used as culture medium. The best yield of the prochlorophyte came from cultures held at a salinity of 32. Around 1,000–2,000 adult *P. crassirostris* were cultured at 26°C and a salinity of 25 in 2-L glass jars that were aerated gently. Pre-experiment cultures of copepods were fed *I. galbana* at 1.5×10^4 cells ml^{-1} (Hoff and Snell, 2007). The sizes of the algal cells were measured through a microscope with scales.

For 24 h immediately preceding experiments, copepods were maintained without food in gently aerated, 2-L glass jars under a 12/12 light/dark cycle. To reduce stress in the experiments involving the prochlorophyte, *P. crassirostris*, and *I. galbana* were gently acclimated to a salinity of 32 in advance of feeding experiments according to the plankton Culture Manual (Hoff and Snell, 2007).

Grazing Experiments

To our knowledge, copepod grazing has not been tested with cell densities on par with the IRL superbloom. Exploring this unique phenomenon necessitated a compromise with chamber size in order to have target algal densities acclimated to appropriate salinities while constrained by the limits of culture volume production. The challenge was to conduct replicated trials using monocultures and 50:50 mixtures of the appropriate algae at densities of 5.8×10^4 , 1.2×10^5 , 2.3×10^5 , 4.8×10^5 , 9.6×10^5 , and 1.9×10^6 cells ml^{-1} . Five adult copepods were placed in a 3-ml incubation chamber (with 2-ml incubation volume) along with algae at a variety of densities, including superbloom densities of 10^6 cells ml^{-1} . According to our preliminary experiments, grazer densities of 2.5 individuals ml^{-1} were necessary in order to observe measurable effects in a reasonable timeframe. Copepods used in the experiment were adult females. Adult males of *P. crassirostris* do not feed due to their reduced feeding appendages (Lawson and Grice, 1973). Morphologically, the body size of

P. crassirostris females (up to 450 μm) is larger than that of the males (up to 350 μm). In preparation for experiments, adult copepods were pre-selected using a 200 μm mesh. Then the large active females were gently selected using a pasteur pipet under stereomicroscopy. Although individuals of different ages could have an influence on the results, replication ($n = 5$) was increased in an attempt to counteract this potential impact. Controls to estimate algal growth and mortality not due to grazing had the same densities, but lacked copepods. Experiments were conducted in filtered seawater held at 26°C for 20 h under 1,100 lux of artificial light.

Behavioral artifacts due to containment always have been a concern in laboratory feeding experiments (Elmore, 1982; Johnson and Shanks, 1997, 2003), but the extreme algal densities required to mimic observed superbloom conditions, near and exceeding 10^6 cells ml^{-1} , dictated final experimental volumes. In fact, standard culture procedures (Hoff and Snell, 2007) did not produce the required densities, a testament to the extreme nature of the IRL superbloom. AlgaGen LLC, a company specializing in high-density production, used a proprietary culture procedure to produce the required densities of the prochlorophyte and *I. galbana*.

Simulating the Effects of Mucilage

In cultures and during the experiments, the prochlorophyte produced mucilage that affected the viscosity of the water. Therefore, an additional experiment was designed wherein *P. crassirostris* were fed palatable algae (*I. galbana*) in water with different viscosities to test for a physical effect of mucilage on grazing. In the mucilage effect experiment, the algal densities were held constant at 1.8×10^4 cells ml^{-1} , a common density used in culturing copepods (Hoff and Snell, 2007). The viscosity of the water was manipulated by adding polyvinyl pyrrolidone (PVP, average Mw 360,000). This polymer was chosen because it has been used in analogous experiments with no detectable toxicity to delicate planktonic larvae (Podolsky and Emler, 1993). The polymer was added until the water reached the desired kinematic viscosity as measured using a falling-ball viscometer (Gilmont model # GV-2100). The experimental viscosities were 0.923, 1.45, 1.98, 2.50, 3.03, and 3.56×10^{-6} $\text{m}^2 \text{s}^{-1}$, with untreated water (seawater at the same temperature and salinity) having a viscosity of 0.923×10^{-6} $\text{m}^2 \text{s}^{-1}$.

Mucilage experiments were not constrained by the need for extreme phytoplankton densities, so they were carried out in larger volumes with lower densities of grazers (i.e., ten adult copepods within 200 ml of filtered seawater) and prey algae. This approach is comparable to other laboratory experiments (Abu-Rezq et al., 1997; Jungbluth et al., 2017), and should reduce artifacts, if such were occurring. These experiments were conducted at $25 \pm 0.1^\circ\text{C}$ (controlled by a micro-temperature controller) and a salinity of 25 for 11 h under 1,100 lux artificial light with gentle aeration. Preliminary work showed that a minimum of 11 h was required to generate reliable reductions due to grazing. Controls involved the same sized containers, algal density and viscosities as the treatments, but they lacked copepods. Five replicate treatments and corresponding controls were prepared for each experiment ($n = 5$).

Analysis of Data

The initial and final densities of microalgae were measured *via* flow cytometry (BD Accuri C6), and copepod survival was scored *via* stereo microscopy. Grazing rates (cells copepod $^{-1}$ h $^{-1}$) were determined using equations from Frost (1972), with the mean of replicate controls used for comparison. Statistically significant differences in grazing rates among treatments were determined *via* analysis of variance (ANOVA) and *post hoc* Tukey pairwise comparisons ($\alpha = 0.05$). When mortality of copepods occurs, grazing rates for all densities were calculated using the assumption that they had lived for half of the experiment (Turner et al., 2012), since we do not know when ailing copepods ceased grazing or died during the experiment.

RESULTS

When copepods were fed the prochlorophyte, some mortality was observed (Figure 1). Copepod mortality rates (less than 20%) were similar between less dense monocultures of the prochlorophyte (less than 2.3×10^5 cells ml^{-1}) and mixed treatments (Figure 1). However, mortality rates were over 40% in monocultures containing the prochlorophyte at high densities ($\geq 4.8 \times 10^5$ cells ml^{-1}), and the rates increased substantially with densities $\geq 4.8 \times 10^5$ cells ml^{-1} (Figure 1A). Copepod mortality was not observed in monocultures of *I. galbana*.

Copepods grazed on the prochlorophyte in treatments with $\leq 4.8 \times 10^5$ cells ml^{-1} ; grazing rates on the denser prochlorophyte at treatments of 9.6×10^5 and 1.9×10^6 cells ml^{-1} were indistinguishable from zero (Figure 2A). Compared to grazing rates on monocultures of *I. galbana*, rates on the prochlorophyte were significantly lower in treatments with $\geq 2.3 \times 10^5$ cells ml^{-1} ($p < 0.05$) (Figure 2A). Again, water that contained the prochlorophyte was more viscous than filtered seawater alone due to the production of mucilage.

In experiments with mixed cultures, grazing on *I. galbana* was markedly reduced compared to grazing recorded in monocultures (Figure 2), with grazing rates on *I. galbana* decreasing by 80% in the highest density mixed treatment as compared to the monoculture of *I. galbana* at the same density. In mixed cultures, the grazing rates on *I. galbana* were statistically indistinguishable from those on the prochlorophyte (Figure 2B). Conversely, grazing rates on the prochlorophyte in mixed cultures were equal to or higher than rates on the same species in monoculture (Figure 2). This was especially true for the highest algal density treatments ($\geq 9.6 \times 10^5$ cells ml^{-1}).

When exposed to *I. galbana* in PVP treated water, copepod mortality increased at higher viscosities (Figure 3A). Copepods grazed on *I. galbana* in low viscosity treatments, but grazing rates declined significantly with increasing viscosity, dropping from 6.8×10^3 cells copepod $^{-1}$ h $^{-1}$ to zero (Figure 3B).

DISCUSSION

Top-down control *via* grazing has been shown to vary with the characteristics of grazers, their algal food, and environmental

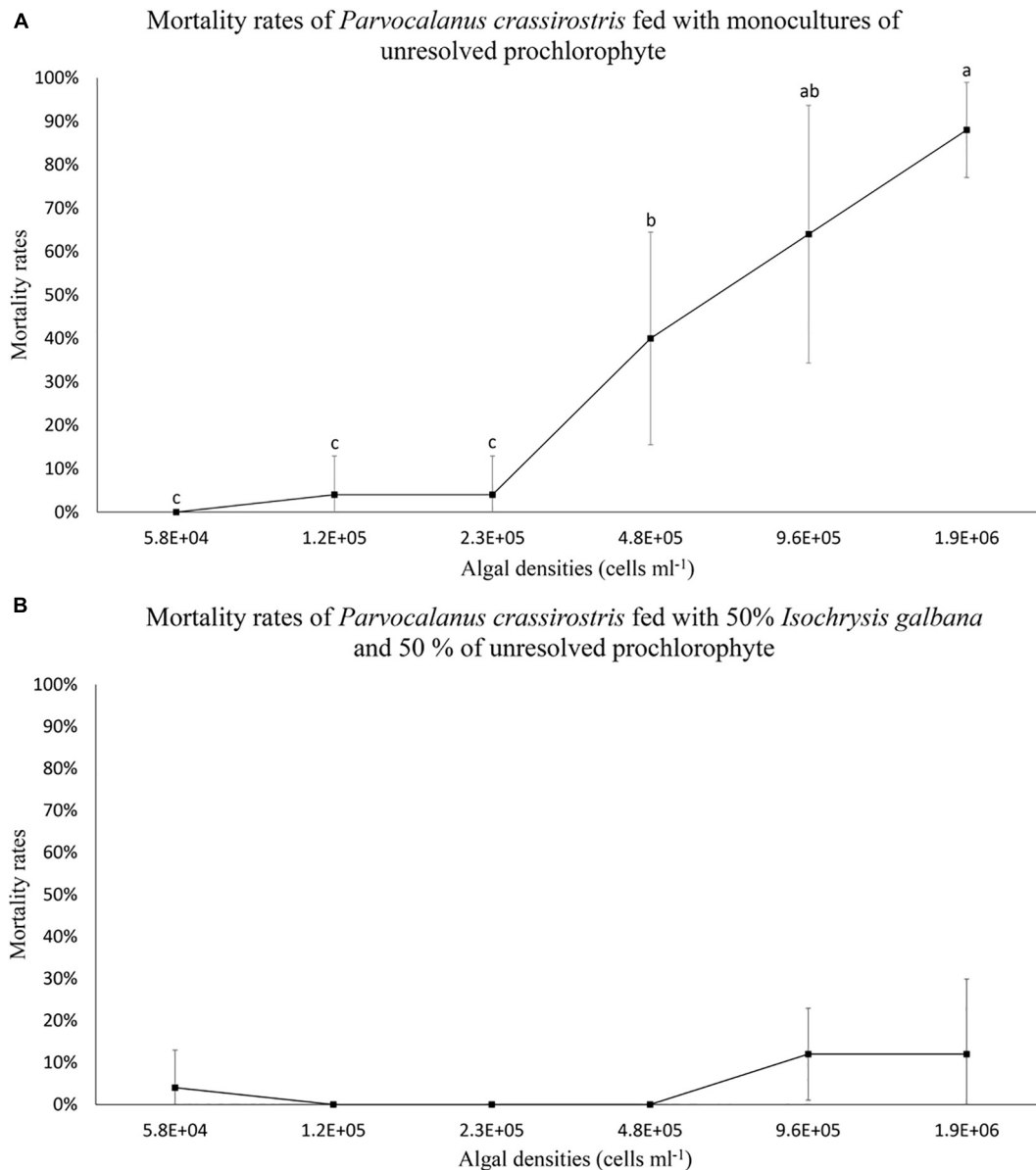
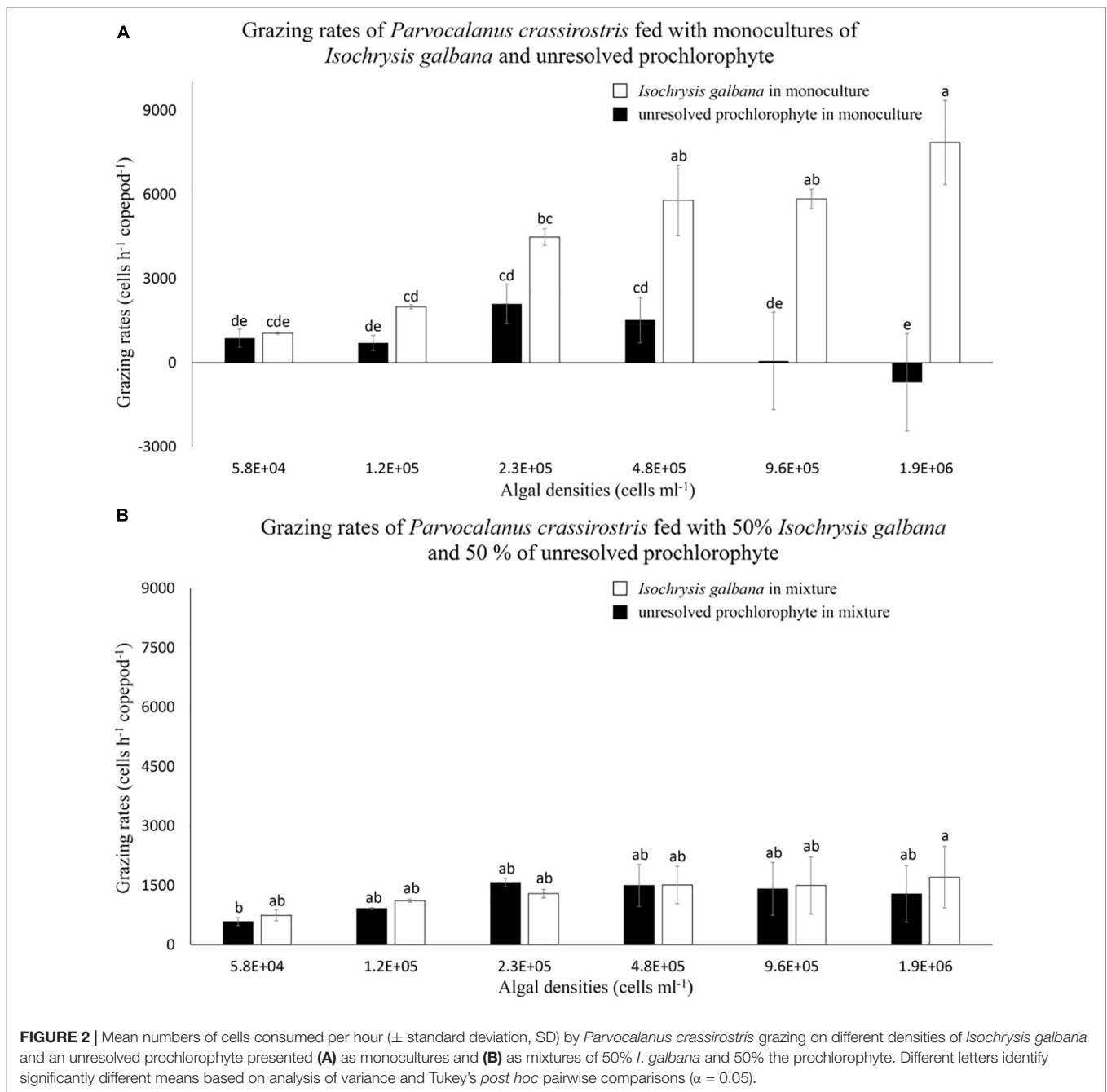


FIGURE 1 | Mean mortality rates (\pm standard deviation, SD) for *Parvocalanus crassirostris* exposed to different densities of an unresolved prochlorophyte presented **(A)** as a monoculture and **(B)** as a mixture of 50% *Isochrysis galbana* and 50% the prochlorophyte. Different letters identify significantly different means based on analysis of variance and Tukey's *post hoc* pairwise comparisons ($\alpha = 0.05$). Mean mortality rates in the mixed culture **(B)** were statistically equivalent and not significantly different from zero.

conditions. Our experiments involved consistent environmental conditions and life stage (adult) of one grazer, which limited the influence of body size, foraging speed, feeding efficiency, and life history stage (Peters, 1984; Hansen et al., 1997; Atkinson, 1998; Nejstgaard et al., 2007; Seuront and Vincent, 2008). Our focus was on characteristics of phytoplankton, including cell density, size, and apparent palatability (Peters, 1984; Hansen et al., 1994; Kiørboe et al., 1996; Norberg and DeAngelis, 1997; Calbet, 2001; Seuront and Vincent, 2008).

Algal density had a substantial influence on grazing by *P. crassirostris*, regardless of the species of algae being offered

or whether they were offered as monocultures or mixed cultures containing the prochlorophyte. Mean grazing rates on *I. galbana* in monocultures ranged from 1.1×10^3 to 1.8×10^4 cells copepod⁻¹ h⁻¹, and are similar to rates reported for *P. crassirostris* feeding on other nanoplankton (Calbet et al., 2000), *Tisbe furcata* feeding on a flagellated haptophyte and a flagellated cryptophyte of similar size (Abu-Rezq et al., 1997), and *Pseudodiaptomus euryhalinus* feeding on an *Isochrysis* sp. (Anzueto-Sánchez et al., 2014). *P. crassirostris* has been documented to be a suspension feeder, with no evidence of raptorial behavior (McKinnon and Klumpp, 1998).

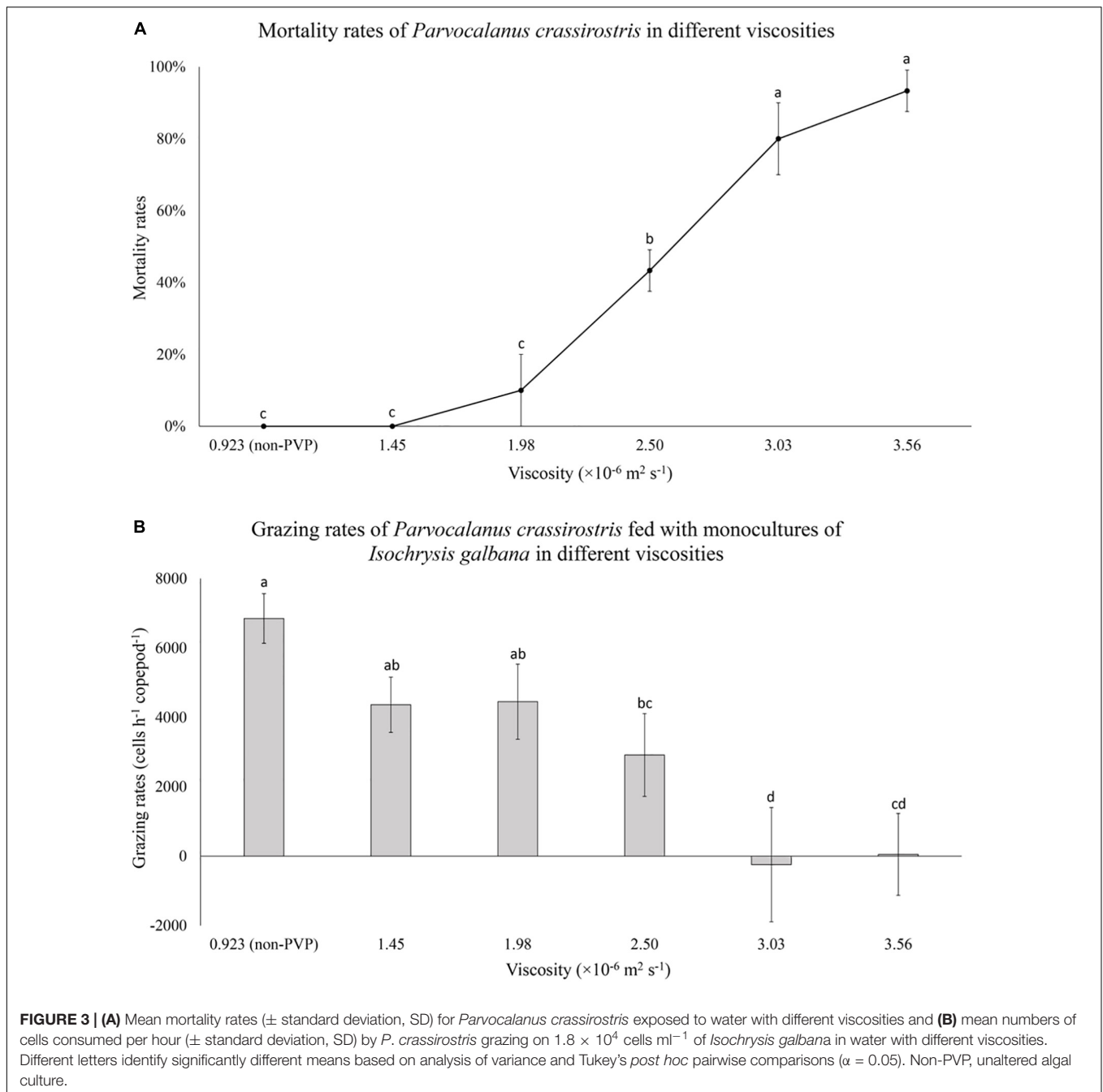


Such copepods typically generate small-scale currents to entrain particles, which subsequently are trapped by feeding appendages (Koehl and Strickler, 1981). It follows that encounter rates and the resulting grazing rates would, to some degree, depend on algal densities, provided there were no other constraints. Given that other studies have also shown correlations of grazing rates with higher algal densities (Frost, 1972), that general pattern does not appear to have been altered by experimental conditions, neither for *I. galbana* nor the prochlorophyte.

Consumption of the prochlorophyte presented as a monoculture or mixed with *I. galbana* varied from 0 to

3.0×10^3 cells copepod⁻¹ h⁻¹ at densities from 5.8×10^4 to 1.9×10^6 cells ml⁻¹. These rates were low compared to consumption in monocultures of *I. galbana*, especially at algal densities of 9.6×10^5 – 1.9×10^6 cells ml⁻¹. Grazing on *I. galbana* in mixed cultures also was depressed, which in combination with the relatively consistent rate of grazing on the prochlorophyte in all cultures, suggests that copepods did not select prey based on cell taste.

Copepod feeding mechanics are predominantly governed by viscous forces (Koehl and Strickler, 1981; Yen, 2000; van Duren and Videler, 2003). Viscosity indicates how well a fluid can resist



deformation under stress and is sometimes equated with fluid “thickness.” Mucilage produced by the prochlorophyte in this study may have reduced grazing rates. Mucilage was unlikely to have been an artifact of containment, because cyanobacteria have been shown to produce it in natural assemblages found in Florida Bay (Berry et al., 2015) and off the coast of Venezuela (Ramos and Reyes-Vazquez, 1990). In previous work, polymeric exudates (a.k.a. mucilage) reduced copepod grazing rates, either *via* chemical inhibition or mechanical interference with filtration or particle capture (Malej and Harris, 1993; Dilling et al., 1998; Nejstgaard et al., 2007). The effects of mucilage were most

obvious above a threshold cell density, because copepods grazed on low densities of the prochlorophyte at rates that were not significantly different from grazing on *I. galbana*. The presence of a threshold has been shown for *Acartia clausi* grazing on the toxic dinoflagellate *Alexandrium minutum*, with consumption decreasing dramatically when *A. minutum* was abundant and producing more toxin (Guisande et al., 2002). In fact, the apparent increase in grazing on the prochlorophyte in higher density mixed cultures could have been due to actual densities of cyanobacteria cells being half of those in monocultures. In general, mucilage may have interfered with handling of cells, with

the increased viscosity rendering the delicate work of particle sorting and selection more difficult. The diameters of *I. galbana* and the prochlorophyte were similar ($\sim 5 \mu\text{m}$), making sorting based solely on size challenging. In addition, the experiments involving PVP indicated that viscosity had a substantial influence on *P. crassirostris* grazing rates. Copepods grazed effectively on *I. galbana* in low viscosity treatments, but grazing rates fell with increasing viscosity (**Figure 3B**). Feeding behaviors of suspension feeders have been shown to be influenced by water viscosity (Podolsky, 1994; Bolton and Havenhand, 1998). In fact, Podolsky (1994) found that high viscosities cut grazing by sand dollar larvae in half, and higher viscosities shifted ingestion to larger particles. “Brown tide,” *Aureoumbra lagunensis*, is another harmful algal bloom species that secretes extracellular polymeric substances (EPS), doing so mainly under hypersaline conditions (Liu and Buskey, 2000). Some species of protozoa had difficulty feeding on high densities of *A. lagunensis*, but they grew faster on cells separated from their EPS exudates via gentle centrifugation (Liu and Buskey, 2000). Given these reports and our results, it seems likely that depressed grazing reflects a natural consequence of increased viscosity.

Mortality was observed when *P. crassirostris* was fed the prochlorophyte, especially in high-density monocultures (**Figure 1**). This result may have been due to toxicity or physical effects associated with mucilage. Although toxic cyanobacteria species are common (Martins et al., 2005, 2007; Valério et al., 2010), many other cyanobacteria may serve as food for copepods (Koski and Klein-Breteler, 2003; Kå et al., 2012). Lower mortality in mixed cultures of the prochlorophyte and *I. galbana*, even at higher total densities, suggested that acute toxicity is unlikely. High mortality rates also were observed when *P. crassirostris* was feeding on palatable algae in water with high viscosity (**Figure 3B**), and PVP has been used in analogous experiments with no evidence of toxicity to delicate zooplankton (Podolsky and Emlet, 1993; Larsen et al., 2008). One possible explanation for copepod mortality is that oxygen diffuses more slowly in viscous water creating a risk of suffocation. Copepods have no gills, and oxygen uptake and carbon dioxide release take place through the integument and in the hindgut (Blaxter et al., 1998). Mucilage and PVP may have interfered with effective exchange of gases. In addition, *I. galbana* occasionally formed aggregates when PVP was added, and aggregates may have been difficult for copepods to grasp or masticate, which may cause stress, or occupy the copepod’s time examining non-consumable aggregate particles. Mortality from starvation was unlikely, however, due to the short duration of the experiments. The presence of non-consumable particles, however, could be considered a form of background plankton that reduces overall grazing rates (Johnson and Shanks, 1997, 2003).

Crowding and container effects have been a common concern when conducting laboratory grazing studies (Folt and Goldman, 1981; Peters, 1984; Helgen, 1987; Burns, 1995, 2000; Preuss et al., 2009; Lee et al., 2012). Potential artifacts can arise from physical interference among zooplankton, interactions between zooplankton and vessel walls or boundary layers (Peters, 1984), or chemically-mediated interactions among competitors (Folt and Goldman, 1981). Some studies demonstrate that crowding or

container effects can lead to depression of zooplankton feeding rates (Folt and Goldman, 1981; Helgen, 1987; Burns, 1995, 2000; Preuss et al., 2009; Lee et al., 2012). However, Mullin (1963) found that container size was not a significant influence on feeding rates when specifically testing copepods. Small containers also can crowd grazers, and we must acknowledge that the influence of such effects cannot be known with certainty. However, Lee et al. (2012) demonstrated that some copepod species could be raised at densities as high as 20 individuals ml^{-1} . In fact, numerous copepod grazing studies have utilized small containers or high densities of grazers (Hong et al., 2013; Motwani and Gorokhova, 2013; Boersma et al., 2016; Ger et al., 2016; Rangel et al., 2016; Svensen and Vernet, 2016; Mathews et al., 2018). Overall, evidence suggests the present experiments yielded useful insights into HAB grazing by copepods.

In conclusion, grazing on the prochlorophyte by *P. crassirostris* declined when algal densities were high and the water was viscous, whereas grazing on palatable algae increased with algal density. Copepod mortality increased with increasing viscosity, whether created by mucilage or a polymer. Copepod mortality and lower grazing rates in the presence of cultured prochlorophyte densities, meant to reflect *in situ* superbloom densities, suggest that *P. crassirostris*, a dominant herbivorous copepod, could be prevented from exerting top-down control in bloom conditions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

XM and KJ conceived the study. XM carried out data collection. XM and CJ analyzed data. All authors contributed to interpretation of the results, preparation and revision of the manuscript, and approved the submission.

FUNDING

This research was funded by contract 27786 from the St. Johns River Water Management District.

ACKNOWLEDGMENTS

We would like to thank E. J. Phlips for supplying the initial HAB cultures. We would also like to thank J. Trefry, S. Fire, K. Hunsucker, and C. Combs for their advice in developing this project and comments on the manuscript. We would further like to thank E. Stenn and AlgaGen LLC Culture Company for culturing the copepods and algae. We would also further like to thank L. H. Sweat and H. Kolb for assistance with laboratory work and helpful suggestions.

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