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Acartia tonsa grazing on the harmful dinoflagellate *Dinophysis acuminata* reduces copepod survival and increases extracellular toxin concentrations

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ABSTRACT: *Dinophysis* spp. synthesize lipophilic toxins and form harmful algal blooms (HABs) across the globe. Zooplankton can play a role in controlling HABs and be a vector for HAB toxins; however, no study has explored the grazing and survival of copepods fed cultured *Dinophysis*. Here, the copepod *Acartia tonsa* isolated from New York, USA, was fed 3 strains of *D. acuminata* from the eastern USA (Massachutsetts, New York, Virginia), as well as 2 non-toxic prey (*Rhodomonas salina* and *Gymnodinium aureolum*). Grazing and survival rates of *A. tonsa* were quantified along with toxins. *A. tonsa* fed on *D. acuminata* at rates similar to *R. salina* and *G. aureolum.* Mixed-prey experiments suggested that *D. acuminata* was not acutely toxic to *A. tonsa.* Extracellular levels of okadaic acid (OA) significantly increased ($p \leq 0.05$) and extracellular pectenotoxin (PTX2) increased by 50% upon exposure to copepods, suggesting that grazers stimulated extracellular toxin release. During 3 wk survival experiments, copepods consuming *D. acuminata* as a sole food source displayed significantly lower survival rates compared to those fed a control diet of *R. salina* ($p < 0.05$). This depressed survivorship was ameliorated by feeding the copepods a diet of *D. acuminata* mixed with *G. aureolum*, suggesting that nutritional deficiencies drove mortality. Since grazing on *Dinophysis* may be low when prey abundance is low, reduced grazing may contribute to bloom development; however, as blooms intensify, grazing may increase, potentially causing a reduction in copepod survival and continued bloom progression. Finally, grazing-induced increases in OA and PTX2 could enhance the introduction of *Dinophysis*-derived toxins into food webs.

KEY WORDS: *Dinophysis acuminata* · *Acartia tonsa* · Culture · Grazing · Survival · Toxins · Nutrition

1. INTRODUCTION

Harmful algal blooms (HABs) are environmental phenomena that can disrupt ecosystems, economics, and public health (Glibert et al. 2005, Sunda et al. 2006, Anderson et al. 2021). *Dinophysis* spp. are harmful algae known to produce lipophilic toxins such as okadaic acid (OA) and dinophysistoxins (DTXs) that can accumulate in shellfish and cause diarrhetic shellfish poisoning (DSP), and pectenotoxins (PTXs) (Yasumoto et al. 1985, Van Dolah 2000, Reguera et al. 2014). *Dinophysis* is an obligate mixotroph that must consume and retain plastids from a ciliate, *Mesodinium*, that has consumed and retained plastids from the cryptophyte, *Teleaulax* (Mafra et al. 2016, Jiang et al. 2018). Due to this complex series of trophic interactions, *Dinophysis* was not established in culture until 2006 (Park et al. 2006). Since then, many species and strains of *Dinophysis* have been established using different *Mesodinium* and *Teleaulax* pairings (Ayache et al. 2023, Nagai et al. 2008).

While HABs are known to be promoted by excessive nutrient loading (Heisler et al. 2008, Davidson et al. 2014, Anderson et al. 2021), their dynamics can

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also be strongly controlled by zooplankton grazing (Buskey 2008, Smayda 2008, Turner 2014). Others have been shown to possess defenses against grazers that alleviate this top-down control (Turner & Tester 1997, Selander et al. 2006, Xu & Kiørboe 2018). Due to the relatively low abundance of many *Dinophysis* spp. during blooms as well as the difficulty in establishing and maintaining *Dinophysis* in culture, zooplankton grazing on this harmful alga has not been widely studied (Reguera et al. 2014). Field studies have shown that *Dinophysis* grazing is highly dependent on the type of grazer and species of *Dinophysis* present as well as the other co-occurring prey options (Carlsson et al. 1995, Maneiro et al. 2000, Wexels Riser et al. 2003, Jansen et al. 2006, Kozlowsky-Suzuki et al. 2006, Sobrinho-Gonçalves & Moita 2008, Setälä et al. 2009). Although globally distributed, grazing by the copepod *Acartia tonsa* on *Dinophysis* has yet to be studied. In temperate zones, *A. tonsa* increases in abundance during warmer months, which co-occurs with the onset and bloom of *Dinophysis* within NY embayments (Lonsdale et al. 1996, Hattenrath-Lehmann et al. 2013, 2018, Ayache et al. 2023), suggesting that it may influence *Dinophysis* bloom dynamics.

The purpose of this study was to elucidate the interactions between copepods and *Dinophysis*. The precise objectives were to quantify the rates at which *A. tonsa* consumed *D. acuminata* as a function of biomass in mono- and bi-algal cultures and to quantify survival rates of *A. tonsa* fed with *D. acuminata* and non-harmful algal species. Changes in extracellular and intracellular toxin content following grazer exposure were also assessed. Experiments involved 3 strains of *D. acuminata* isolated from 3 estuaries across the eastern USA and used *Rhodomonas salina* and *Gymnodinium aureolum* as a flagellated (swimming) model non-harmful algae. We hypothesized that feeding and survival rates of *A. tonsa* would be significantly higher when fed the non-harmful algae compared to when fed *Dinophysis*. No study to date has quantified *Acartia* grazing or survival when fed *Dinophysis* cultures.

2. MATERIALS AND METHODS

2.1. Culture conditions

Three strains of *Dinophysis acuminata* (diameter: 20.7 ± 3.43 µm; biovolume: 5018 ± 2590 µm^3) from 3 areas of the USA were used for these experiments. One was isolated from Meetinghouse Creek, Riverhead, NY (DANY1; Hattenrath-Lehmann & Gobler 2015), one was isolated from Town Cove, Orleans, MA (DATC03; kindly provided by Michael Brosnahan), and a third was isolated from Chesapeake Bay, Nassawadox, VA (DAVA 01). Taxonomic classification was determined using morphometric and DNA-based analyses as described in Ayache et al. (2023). All strains were fed *Mesodinium rubrum* (JAMR) that was fed *Teleaulax amphioxia* (JATA), both isolated from Inokushi Bay, Oita Prefecture, Japan, in 2007 (Nishitani et al. 2008). The NY *Dinophysis* strain was also separately fed *M. rubrum* (MBL-DK2009) that was fed *T. amphioxia* (K-0434), both isolated from Helsingør Harbor, Denmark, in 2009 and kindly provided by Dr. P. J. Hansen (Nielsen et al. 2012). All cultures were maintained in f/2 media (Guillard & Ryther 1962, Guillard & Hargraves 1993) at salinity 25 except for JAMR, which was maintained in f/6 media. All cultures were exposed to ~80 µEin m^{-2} s^{-1} on a 12 h light:12 h dark cycle; *D. acuminata* and the Japan *M. rubrum* were kept at 18°C while *T. amphioxia* and the Denmark *M. rubrum* were kept at 21°C. To grow cultures for experiments, *M. rubrum* was fed to *D. acuminata* in a 2:1 ratio of prey to predator. *D. acuminata* cultures were inoculated at 1000 cells ml^{-1} and fed every 3 d prior to experimentation. Culture growout was staggered to ensure that cultures for experiments were all in exponential growth phase. *Gymnodinium aureolum* (diameter: 20.8 ± 1.6 µm; biovolume: 4774 ± 1166 µm^3), which was isolated from Meetinghouse Creek, Riverhead, NY, was maintained autotrophically (i.e. with no prey) in the same culture conditions as *D. acuminata*. *Rhodomonas salina* (diameter: 6.44 ± 0.596 µm; biovolume: 141 ± 39.6 µm^3; CCMP1319) was kept in f media at 21°C with ~80 µEin m^{-2} s^{-1} on a 12 h light:12 h dark cycle. *Acartia tonsa* copepods were isolated from Shinnecock Bay, NY, USA, and maintained in continuous culture being fed ad libitum *Tisochrysis lutea*, *Tetraselmis suecica*, and *R. salina* (maintained in f/2, or f in the case of *R. salina*, at 21°C with ~80 µEin m^{-2} s^{-1} on a 12 h light:12 h dark cycle) and transferred to new 0.2 µm-filtered and autoclaved seawater every 2 wk. All algae used as prey were within the size range for *A. tonsa* (Berggreen et al. 1988).

2.2. Single-prey grazing experiments

Experiments were conducted in triplicate 50 ml tissue culture flasks filled with 40 ml of culture in f/2 media. Two copepods were added to flasks (50 l^{-1}) representing environmentally relevant densities (Peterson 1985). To isolate copepods, they were gently fil-

tered onto a 64 µm sieve that was then washed onto a 200 µm sieve to separate the adult copepods from the juveniles and eggs. Adult copepods (>200 µm) were then separated into wells filled with filtered seawater, sexed, and left for 24 h prior to experimentation to ensure viability and minimize transfer of food to the experimental setup. Female copepods are the gender that is the most affected by a lack of certain nutrients, given they produce the eggs (White & Roman 1992, Lacoste et al. 2001, Shin et al. 2003). They are considered to be more important for population dynamics, and negative impacts on females would be more detrimental to the population as a whole (Ohman et al. 1996, Marcus et al. 2004). Therefore, female copepods are used primarily in grazing and survival studies. Following past harmful alga–copepod experimental practices, only female adult *A. tonsa* were used for experiments (Colin & Dam 2002, Dam & Colin 2005). *D. acuminata* strains were sieved over a 20 µm sieve prior to experimental setup to ensure no prey or extracellular materials were transferred over. Two experimental series were performed using single culture incubations with adult *A. tonsa* (see Table A1 in the Appendix). Experiments with strain DANY1 were performed at 4 densities (100, 500, 1000, and 2000 cells ml^{-1}) and carbon-equivalent *R. salina* densities (3600, 18 000, 36 000, and 72 000 cells ml^{-1}). In addition, *D. acuminata* strains DATC03, DAVA, and DANY1 (fed MBL-DK2009 strain of *M. rubrum*) were used for grazing experiments at 500 and 2000 cells ml^{-1} and carbon-equivalent *R. salina* densities (18 000 and 72 000 cells ml^{-1}). *D. acuminata* densities used were representative of densities found during blooms along the east coast of the USA (Hattenrath-Lehmann et al. 2013, Ayache et al. 2023). *Rhodomonas* was selected as a control as it is known to be a good non-toxic food source for *A. tonsa*. Triplicate flasks were also filled with sieved *D. acuminata* cultures following the procedure above at the same densities as experimental treatments in fresh f/2 media with no copepods as controls. Controls were used to calculate grazing rates (see below) as well as for the toxin content and concentrations with no copepods.

All treatment and control flasks were placed on an orbital rotation incubator at 30 rpm and 18°C for 24 h on a 12 h light:12 h dark cycle with light levels at 100 µEin m^{-2} s^{-1}. Samples (3 ml initial and final [$t = 24$]) were preserved with 5% Lugol's iodine solution and enumerated via light microscopy. Growth of the algal species was calculated using the equation $g = \ln(C_f / C_i) / t$, where C_f is the final cell concentration, C_i is the initial cell concentration, and t is time in days (Frost 1972). Grazing was calculated using the equation $G = (g_c - g_t) / n$, where g_c is growth in the control, g_t is growth in the treatment, and n is the number of copepods added (Frost 1972). Ingestion was calculated using the equation $I = V \times [\ln(C_{fc} / C_{ft})] / n \times [C]$, where V is volume, C_{fc} is the final cell concentration in the control, C_{ft} is the final cell concentration in the treatment, and $[C]$ is equal to $(C_{ft} + C_i) / 2$ (Frost 1972, Båmstedt et al. 1999). For the calculation of indirect ingestion rate, cell concentration was converted to ng carbon using the biovolume of the cells and 884 pg C $cell^{-1}$ for *Dinophysis* and 24 pg C $cell^{-1}$ for *Rhodomonas* (Putt & Stoeker 1989, Menden-Deuer & Lessard 2000).

Toxin samples were obtained at the final time point for the highest *D. acuminata* density treatment after 24 h exposure to the copepods as well as without exposure (i.e. the no-copepod control). A total of 3 ml was taken from each replicate and centrifuged at 3000 × *g* for 15 min at 4°C. The supernatant was then separated from the pellet, representing the extracellular and intracellular fraction, respectively. The intracellular samples were then resuspended in 1 ml of 100% methanol. Both samples were stored at −20°C until extraction. Samples were extracted and an aliquot was base-hydrolyzed following the protocol of Ayache et al. (2023). The toxins, pectenotoxin-2 (PTX2) and free and esterified forms of dinophysistoxin-1 (DTX-1) and OA, were quantified by liquid chromatography coupled with tandem mass spectrometry following the method described in Ayache et al. (2023). Toxin data are presented as intracellular toxin content (toxins per cell) in *D. acuminata* cells and extracellular toxin concentrations (toxins per ml).

2.3. Mixed-prey grazing experiment

Mixed algal diet grazing experiments were conducted by combining 2 prey species: the toxigenic *D. acuminata* (DANY1 fed *M. rubrum* JAMR) and a non-toxic algal species with similar motility and biovolume, *G. aureolum* (Tang et al. 2008, Jeong et al. 2010, Yoo et al. 2010). Combined *D. acuminata* and *G. aureolum* densities were 100, 500, 1000, and 2000 cells ml^{-1} with the proportions of toxic and non-toxic prey at 0, 25, 50, 75, and 100% for each density (5 treatments per density; Fig. A1). Tissue culture flasks (50 ml) were filled with 40 ml of combined sieved *D. acuminata* (described above) and *G. aureolum* culture in new f/2 media in triplicate for each mixed density as described above. Two *A. tonsa* copepods, prepared as described above, were added to each flask (50 l^{-1}) and flasks were incubated at 18°C for 24 h at a 12 h

light:12 h dark cycle in a shaking incubator. Triplicate flasks were also filled with the algal mixtures at each percentage and density in media without the addition of copepods to determine grazing rates. Initial and final samples of 3 ml were preserved in 5% Lugol's iodine for cell enumeration via microscopy. Grazing and ingestion by the copepods were calculated using the equations described above.

Alpha selectivity coefficients were calculated according to Chesson (1978): $\alpha = (C_{sc} / C_{se}) / (C_{sc} / C_{se} + C_{oc} / C_{oe})$ where C_{sc} is the density of a single prey consumed, C_{se} is the density of the single prey in the environment, C_{oc} is the density of the other prey consumed, and C_{oe} is the density of the other prey in the environment. This index ranges from 0 to 1, where 0.5 is no selection; the interpretation was that any value above 0.5 indicated selection for and any value below 0.5 indicated selection against. Prey consumed (C_{sc} or C_{oc}) was calculated by subtracting the final density of the copepod treatment from the final density of the control treatment (no copepods). Percent survival was also plotted against percent *D. acuminata* in each treatment to assess whether the effects of *D. acuminata* on *A. tonsa* are due to toxicity or nutritional deficiency, following a modified method of Colin & Dam (Jónasdóttir et al. 1998, Colin & Dam 2002, Dam & Colin 2005; Fig. A2), where a reference line is drawn connecting the points for 0 and 100% treatment algae. A negative slope indicates that the control algae is more nutritious than the treatment algae. If points fall along the reference line, it indicates that the treatment is non-toxic and also has no additional nutritional value, whereas if points fall below the reference line, it indicates the treatment is toxic and nutritional value was lost with its addition. If points fall above the reference line, it indicates that the treatment has added some nutritional value (Jónasdóttir et al. 1998, Colin & Dam 2002, Dam & Colin 2005).

2.4. Survival experiments

Two longer-term (3 wk) survival experiments were conducted using triplicate 6-well plates (n = 18 wells) with 10 ml of culture in media in each well. One *A. tonsa* copepod, prepared as described above, was added to each well, providing an environmentally realistic final density of 100 individuals l^{-1} (Peterson 1985). *D. acuminata* cultures were sieved prior to experimental setup as described above. Two series of experiments were conducted to assess the survival of *A. tonsa* on toxic and non-toxic in single exposure, and a mixture of toxic and non-toxic algae. The first experiment assessed density-dependent effects of *D. acuminata* on survival of *A. tonsa* with single prey exposure and used DANY1 (fed JAMR strain of *M. rubrum*) at 100, 500, 1000, and 2000 cells ml^{-1} with carbon-equivalent treatments of *R. salina* at 3600, 18 000, 36 000, and 72 000 cells ml^{-1} (known non-toxic food source as the positive control) and copepods in f/2 media with no food as negative controls. The second experiment compared survival of *A. tonsa* on different *D. acuminata* strains, a similar dinoflagellate to *Dinophysis* (*G. aureolum*), and a known non-toxic food source (*R. salina*). This experiment used single exposures of DATC03, DAVA, DANY1 (fed MBL-DK2009 strain of *M. rubrum*), and DANY1 (fed JAMR strain of *M. rubrum*), all at 500 cells ml^{-1} and carbon-equivalent *G. aureolum* (500 cells ml^{-1}). Additionally, a 50/50 mixture of DANY1 (fed JAMR strain of *M. rubrum*) and *G. aureolum* with a final mixed concentration of 500 cells ml^{-1} was also tested. The carbon equivalent of *R. salina* (18 000 cells ml^{-1}) and starved copepods in f/2 media were used as controls for all treatments. Cultures kept in exponential growth in media were replaced daily (80% exchange) after the well had been mixed and survival of copepods was visually assessed.

2.5. Statistical analysis

A Shapiro-Wilk test was used to test for normality, and Levene's test was used to test for equal variance prior to statistical analyses that require those prerequisites (ANOVA, *t*-test). To assess differences between treatments in the single-prey experiments, a 2-way ANOVA was performed followed by a Tukey's HSD test, where algal strain and biomass level were the main effects, using R statistical software v.4.2.0 (R Core Team 2022). To assess differences between toxins, a 1-way ANOVA with a Tukey's HSD post hoc test was performed where the dependent variable was treatment with and without copepod additions for each toxin (OA and PTX2 separately; independent variable) where intracellular and extracellular fractions were statistically analyzed separately. A Kruskal-Wallis test was used when normality and equal variance were not met. To assess differences between grazing or ingestion rates on *D. acuminata* and *G. aureolum* at each percentage for each density, a 2-way ANOVA with a Tukey's HSD post hoc test was performed. The percentage of *D. acuminata* and prey species were used as the main effects for each density level. A *t*-test was used to determine if the selectivity value was significantly different from the no-selection value (0.5) in the mixed prey experiments. A Kaplan-

Meier pairwise comparison with log-rank was used to assess differences in survival of copepod among treatments using the 'survival' (v.3.5-8; Therneau & Grambsch 2000) and 'survminor' (v.0.4.9; Kassambara et al. 2021) libraries. The Benjamini-Hochburg procedure was used to adjust the p-values for multiple comparisons. All statistical tests were performed in R v.4.2.0 (R Core Team 2022).

3. RESULTS

3.1. Single-prey experiments: grazing on individual *Dinophysis acuminata* strains

The single-prey grazing experiment with *Acartia tonsa* adults showed no significant difference ($p > 0.05$) between grazing or ingestion rates when fed *Dinophysis acuminata* or carbon equivalent densities of *Rhodomonas salina* at any of the algal densities examined. Grazing rates of the copepod on *D. acuminata* decreased with increasing prey density for DANY1 (fed JAMR strain of *Mesodinium rubrum*), with rates ranging from 0.08 ± 0.02 to 0.27 ± 0.04 ind.$^{-1}$ d^{-1} (mean ± SE; Fig. 1A). This pattern persisted with each strain of *D. acuminata* as well as the DANY1 strain fed the other strain of *M. rubrum* (strain MBL-DK2009). Grazing rates by the copepods on 500 and 2000 cells ml^{-1} *D. acuminata* respectively were 0.22 ± 0.04 and 0.08 ± 0.03 ind.$^{-1}$ d^{-1} for DATC03 (Fig. 2A), 0.12 ± 0.02 and 0.02 ± 0.04 ind.$^{-1}$ d^{-1} for DAVA (Fig. 2B), and 0.17 ± 0.04 and 0.10 ± 0.02 ind.$^{-1}$ d^{-1} for DANY1 fed *M. rubrum* (MBL-DK2009) (Fig. 1B). Ingestion rates increased with increasing DANY1 (fed JAMR strain of *M. rubrum*), with rates ranging from 965 ± 138 to 6189 ± 1369 ng C ind.$^{-1}$ d^{-1} (Fig. 1C). This pattern also persisted for each strain tested and for DANY1 fed *M. rubrum* MBL-DK2009. Ingestion on 500 and 2000 cells ml^{-1} respectively was 3126 ± 445 and 5340 ± 1925 ng C ind.$^{-1}$ d^{-1} for DATC03 (Fig. 2C), 1883 ± 410 and 1100 ± 3162 ng C ind.$^{-1}$ d^{-1} DAVA (Fig. 2D), and 2348 ± 475 and 6793 ± 1077 ng C ind.$^{-1}$ d^{-1} for DANY1 (fed MBL-DK2009 strain of *M. rubrum*) (Fig. 1D). *A. tonsa* fed *R. salina* exhibited similar pat-

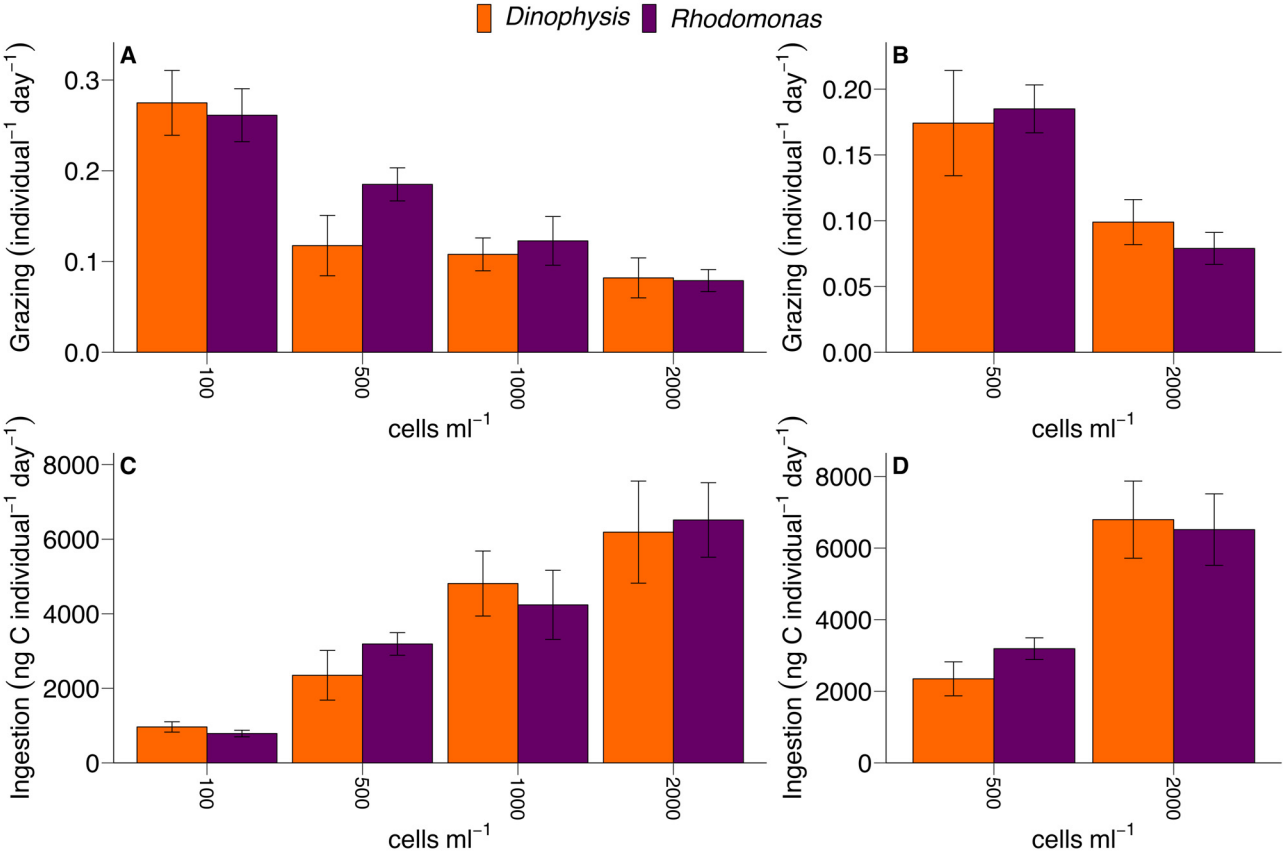

Fig. 1. Grazing and ingestion rates of *Acartia tonsa* in the single-prey experiments on (A,C) DANY1 fed JAMR strain of *Mesodinium rubrum* and (B,D) DANY1 fed MBL-DK2009 strain of *M. rubrum* with carbon equivalents of *Rhodomonas salina* at multiple densities. Error bars: ±SE

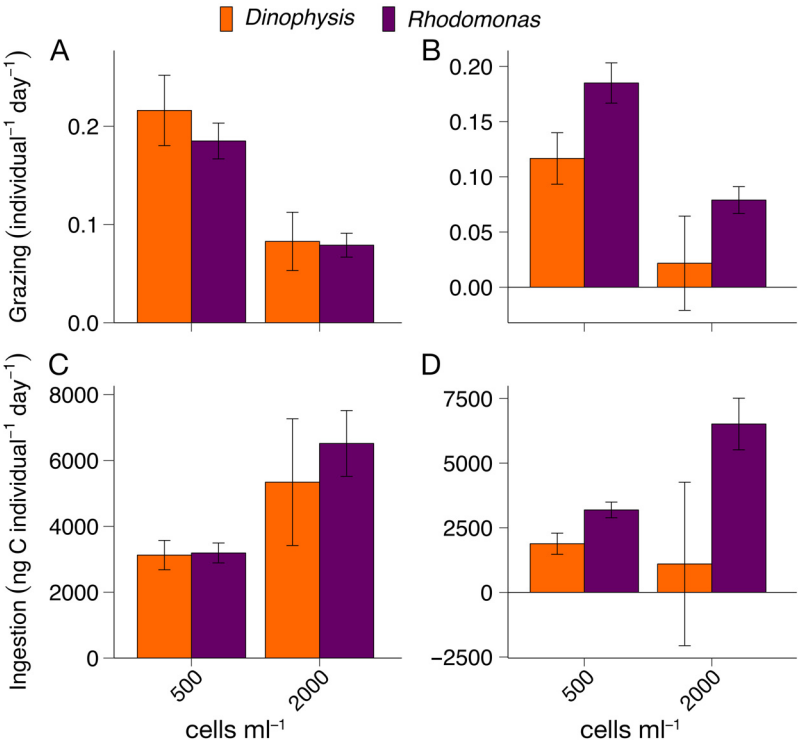

Fig. 2. Grazing and ingestion rates of *Acartia tonsa* in the single-prey experiment fed (A,C) DATC03 fed JAMR strain of *Mesodinium rubrum* and (B,D) DAVA fed JAMR strain of *M. rubrum*, with carbon-equivalent densities of *Rhodomonas* at 2 densities. Error bars: ±SE

terns in which grazing decreased with increasing density and ingestion increased with increasing density (Figs. 1 & 2). Grazing rates ranged from 0.08 ± 0.01 to 0.26 ± 0.03 ind.$^{-1}$ d^{-1} and ingestion rates ranged from 788 ± 89 to 6515 ± 998 ng ind.$^{-1}$ d^{-1} (Figs. 1 & 2).

3.2. Intra- and extracellular toxins following exposure to *A. tonsa*

The intracellular toxin content and extracellular toxin concentrations of OA, DTX1, and PTX2 in the single-prey 2000 cells ml^{-1} *D. acuminata* (DANY1) treatments (with copepods, *A. tonsa*) and controls (no copepods) were quantified following a 24 h co-incubation. Neither intracellular nor extracellular DTX1 was detected in any treatments or control. Total toxin content (intracellular and extracellular) of PTX2 (copepod: 16596.4 pg ml^{-1}; no copepod: 20 599 ± 1897.2 pg ml^{-1}) and OA (copepod: 217 ± 58.1 pg ml^{-1}; no copepod: 246.1 ± 34.8 pg ml^{-1}) was not significantly different ($p > 0.05$) between copepod and no copepod (data not shown). The intracellular PTX2 content changed little after exposure to *A. tonsa* (control: 8.69 ± 0.6 pg PTX2 cell^{-1} vs. treatment: 7.35 ± 1 pg PTX2 cell^{-1}) whereas the extracellular concentrations doubled (213.3 ± 20 pg PTX2 ml^{-1} vs. 405.6 ±153 pg PTX2 ml^{-1}; Fig. 3A). Total intracellular OA was 0.04 ± 0.02 pg OA cell^{-1} when copepods were present and 0.1 ± 0.03 pg OA cell^{-1} when copepods were absent (Fig. 3B). While extracellular OA was undetectable when copepods were absent, concentrations significantly increased to 147.5 ± 63.1 pg toxin ml^{-1} when copepods were present ($p = 0.012$; Fig. 3B). Percent esterified intracellular OA relative to free OA was 81 ± 8% when copepods were present, and decreased to 60 ± 13% when copepods were absent.

3.3. Mixed-prey experiments: grazing on mixtures of *D. acuminata* and *G. aureolum*

Similar to the single-prey grazing experiment, the mixed-prey grazing experiment showed no significant differences in grazing between treatments with the exception of the 50% *Dinophysis* and 50% *Gymnodinium aureolum* mixture at 100 cells ml^{-1}, when the grazing rate on *Dinophysis* (0.05 ind.$^{-1}$ d^{-1}) was significantly lower than the grazing rate on *G. aureolum* (0.4 ind.$^{-1}$ d^{-1}; $p < 0.001$; Fig. 4A). At the lowest density tested (100 cells ml^{-1}), the grazing rates were higher on *G. aureolum* at all percentages (Fig. 4A). The ingestion rate also followed this pattern, with the exception of the 75% *D. acuminata* treatment (Fig. 5A).

A. tonsa showed selectivity for *G. aureolum* at the lowest density (100 cells ml^{-1}) at all percentages, with 50 and 25% *G. aureolum* being significantly different ($p < 0.001$) from the non-selectivity value (0.5), suggesting true selectivity (Table 1). Selectivity was exhibited for *D. acuminata* in the 50% mixture at 500 cells ml^{-1}, all percentages for 1000 cells ml^{-1}, and 75% at 2000 cells ml^{-1}; however, these were not statistically different from the no selection value ($p > 0.05$; Table 1). Plotting the short-term survival (24 h) of copepods against the percentage of *D. acuminata* indicated that the presence of *G. aureolum* enhanced survival in all densities except for the 500 cells ml^{-1} treatment (Fig. 6).

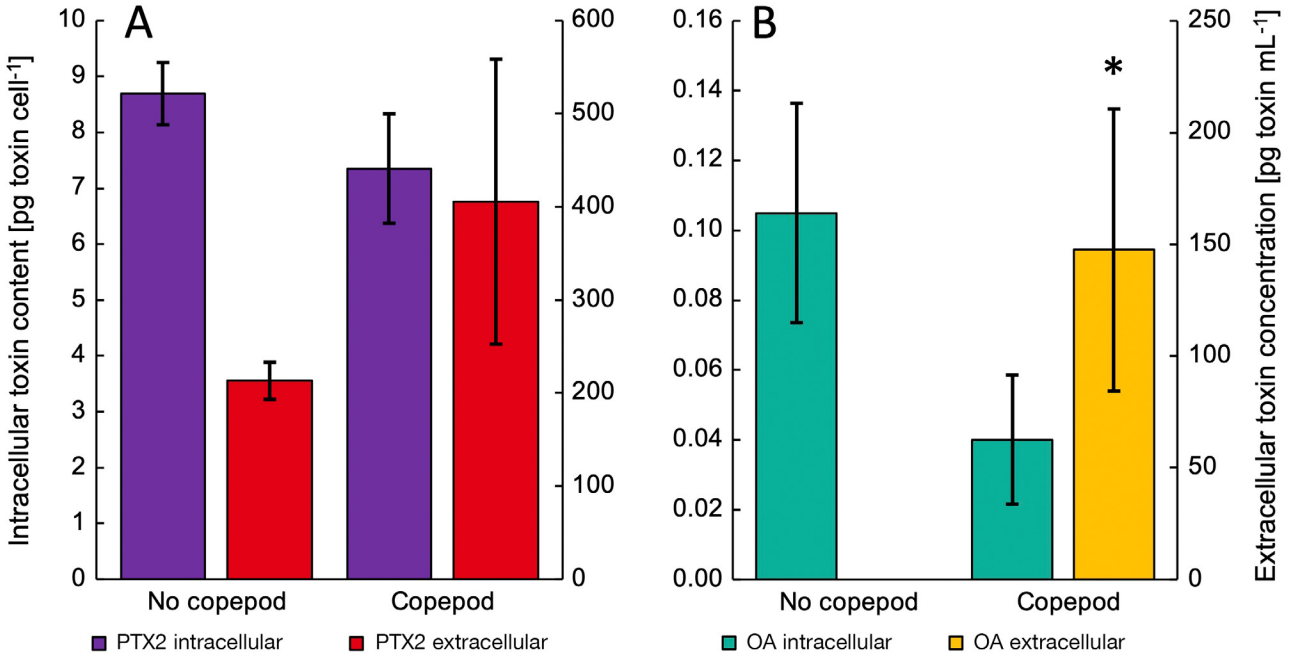

Fig. 3. Toxin data from single-prey grazing experiment at 2000 cells ml^{-1} *Dinophysis acuminata* with *Acartia tonsa* and without *A. tonsa* added, showing intracellular and extracellular (A) PTX2 and (B) okadaic acid (esterified and free). Error bars: ±SE; asterisk: significant difference ($p \leq 0.05$) between treatment and control (no copepod)

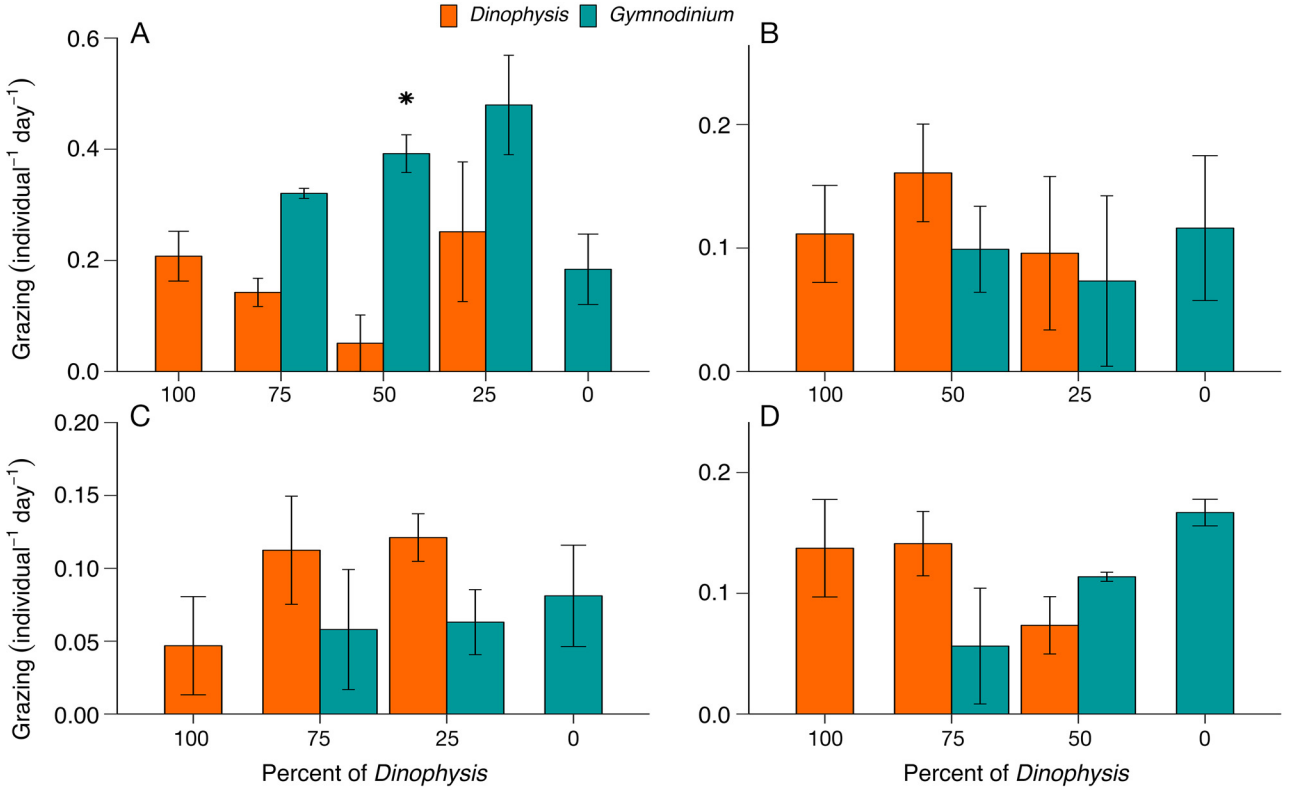

Fig. 4. Grazing rate of *Acartia tonsa* on mixed-prey diet of *Dinophysis acuminata* (DANY1 fed JAMR strain of *Mesodinium rubrum*) and *Gymnodinium aureolum* at (A) 100, (B) 500, (C) 1000, and (D) 2000 cells ml^{-1}. Asterisk indicates significant difference ($p \leq 0.05$) between grazing rate on *G. aureolum* compared to *D. acuminata* within that percentage. Error bars: ±SE

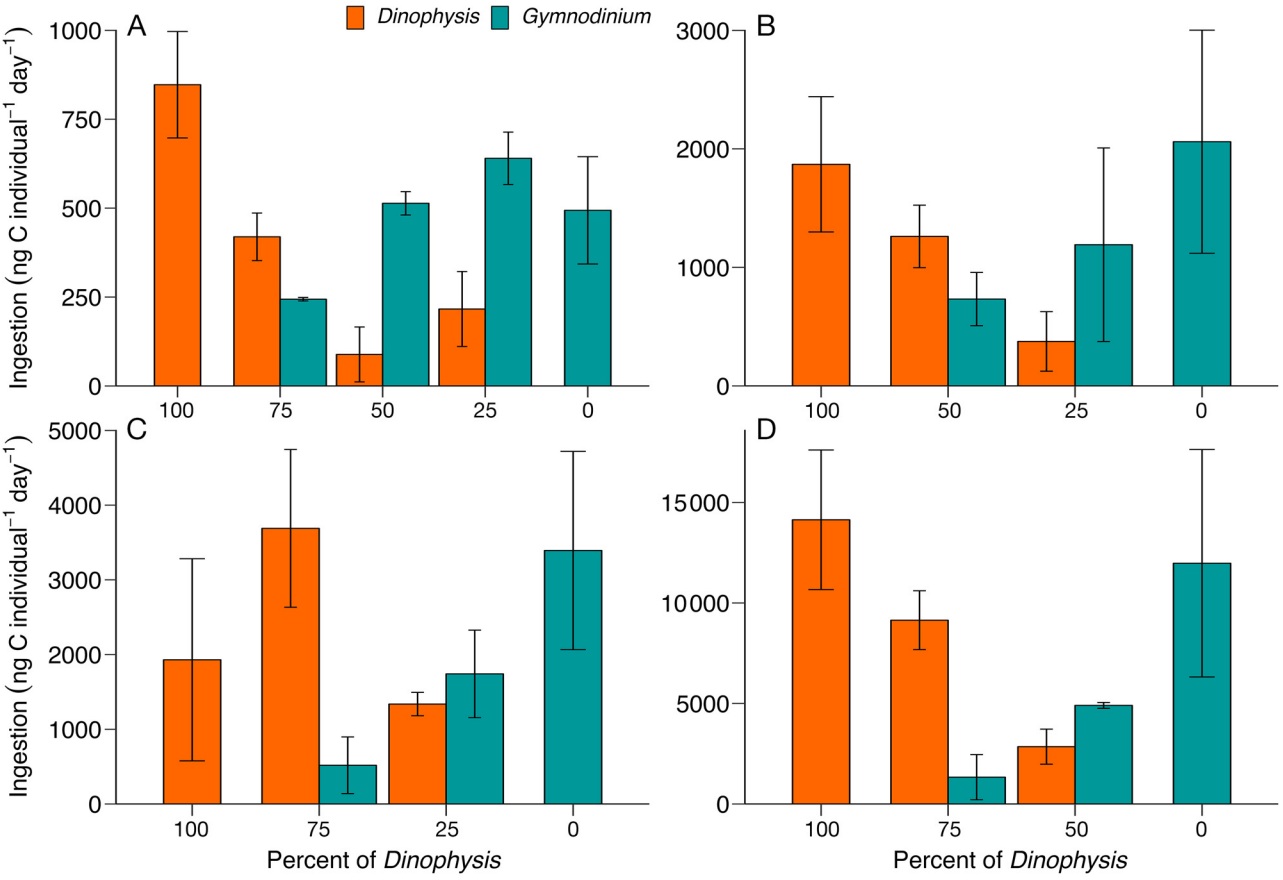

Fig. 5. Ingestion rate of *Acartia tonsa* on mixed-prey diet of *Dinophysis acuminata* (DANY1 fed JAMR strain of *Mesodinium rubrum*) and *Gymnodinium aureolum* at (A) 100, (B) 500, (C) 1000, and (D) 2000 cells ml^{-1}. Error bars: ±SE

3.4. Long-term survival experiment

The survival of copepods decreased significantly faster when fed *D. acuminata* at multiple densities compared to carbon equivalents of *Rhodomonas* over the course of 3 wk (100 cells ml^{-1}, p = 0.029; 500 and 1000 cells ml^{-1}, p < 0.001; 2000 cells ml^{-1}, p = 0.002; Fig. 7, Table 2). Survival decreased to less than 40% in all *D. acuminata* treatments by Day 2, whereas survival remained above 50% in all treatments of *R. salina* until Day 9 (Fig. 7). Survival was not significantly different from starved *A. tonsa* when fed all densities of DANY1 (fed JAMR strain of *M. rubrum*; Fig. 7).

This pattern persisted when copepods were fed different strains of *D. acuminata* in the second long-term experiment (Fig. 8). Survival was significantly lower for copepods fed any *D. acuminata* strain compared to the control *R. salina* and was reduced when fed a sole diet of *G. aureolum* (DANY1 fed MBL-DK2009 strain of *M. rubrum*, p = 0.037; DANY1 fed JAMR strain of *M. rubrum*, DATC03 and DAVA, p = 0.019; Fig. 8, Table 3). For example, copepod survival was reduced to below 50% by Day 8 for all 3 *D. acuminata* strains and by Day 9 for *G. aureolum*. Survival remained above 50% until Day 11 for *R. salina*

Table 1. Chesson's selectivity index for the percent of *Dinophysis acuminata* in the mixed grazing experiments. Dashes: negative grazing rates; numbers in parentheses: SE. A value of 0.5 indicates no selective grazing, 0–0.5 indicates negative selectivity, and 0.5–1 indicates positive selectivity. **Bold** numbers indicate significant differences (p ≤ 0.05) from non-selective value. *Gymnodinium aureolum* selectivity values are the reverse of the *D. acuminata* selectivity values

D. acuminata in the diet (%)	100 cells ml^{-1}	500 cells ml^{-1}	1000 cells ml^{-1}	2000 cells ml^{-1}
75	**0.267 (0.03)**	–	0.686 (0.23)	0.745 (0.19)
50	**0.085 (0.06)**	0.638 (0.05)	–	0.382 (0.09)
25	0.268 (0.12)	0.506 (0.87)	0.675 (0.10)	–

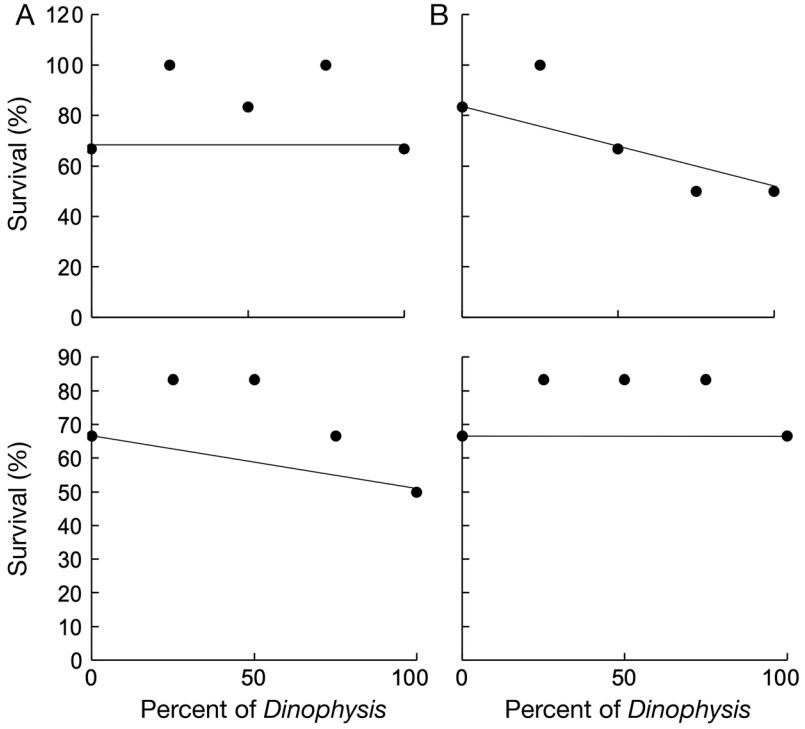

Fig. 6. Percent survival of *Acartia* copepods during the 24 h mixed-prey grazing experiment plotted against percent of *Dinophysis acuminata* (DANY1 fed JAMR strain of *Mesodinium rubrum*) in the diet for (A)100, (B) 500, (C) 1000, and (D) 2000 cells ml^{-1}. Line between 0 and 100% indicates the reference line between toxic (below) and non-toxic (above) following Colin & Dam (2002)

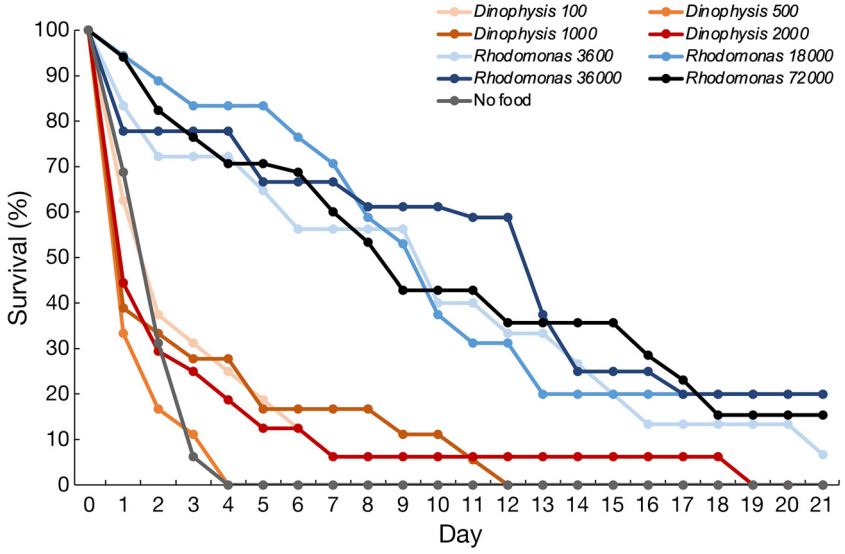

Fig. 7. Percent survival of *Acartia tonsa* feeding on *Dinophysis acuminata* (DANY1 fed JAMR strain of *Mesodinium rubrum*) or on carbon equivalents of *Rhodomonas salina* at multiple densities. Numbers in legend represent density of *D. acuminata* or non-toxic *R. salina* in cells ml^{-1}, where 3600 cells ml^{-1} of *R. salina* is equivalent to 100 cells ml^{-1} of *D. acuminata*; 18000 cells ml^{-1} *R. salina* is equivalent to 500 cells ml^{-1} *D. acuminata*; 36000 cells ml^{-1} of *R. salina* is equivalent to 1000 cells ml^{-1} *D. acuminata*; and 72000 cells ml^{-1} of *R. salina* is equivalent to 2000 cells ml^{-1} of *D. acuminata*. Differences were assessed using a log-rank Kaplan-Meier test (Table 2)

and until Day 19 for the 50:50 mixture of *D. acuminata* and *G. aureolum* (Fig. 8). Survival was lowest for copepods exposed to the MA strain of *D. acuminata* (DATCO3), followed by the VA strain (DAVA), then DANY1 (fed JAMR strain of *M. rubrum*), and finally DANY1 (fed MBL-DK2009 strain of *M. rubrum*; Fig. 8). Survival on *G. aureolum* alone was not significantly different than *D. acuminata* or *R. salina* (Table 3). In contrast to the first experiment, survival on DANY1 (fed JAMR strain of *M. rubrum*) was significantly different from the no-food control ($p = 0.045$; Fig. 8, Table 3). Additionally, survival on DAVA was significantly different from the no-food control ($p = 0.022$; Fig. 8, Table 3). Copepod survival within 50:50 mixture of *G. aureolum* and *D. acuminata* was significantly greater than all treatments and the no-food control except the control *R. salina* and sole *G. aureolum* (no food, $p = 0.001$; DANY1 fed MBL-DK2009 strain of *M. rubrum*, $p = 0.019$; DANY1 fed JAMR strain of *M. rubrum*, DATCO3 and DAVA, $p = 0.008$; Fig. 8, Table 3).

4. DISCUSSION

4.1. Overview

In this study, experiments were performed with strains of *Dinophysis acuminata* from the eastern US and *Acartia tonsa* from NY, USA. *A. tonsa* copepods were able to ingest and graze on all *D. acuminata* strains at rates similar to those for other flagellated non-toxic prey (*Rhodomonas salina* and *Gymnodinium aureolum*) in single- and mixed-prey experiments, indicating *D. acuminata* and the surrounding medium contained no immediate grazer deterrents. When fed any of the *D. acuminata* strains over longer time periods (3 wk), however, copepods exhibited significantly more mortality than when offered the non-toxic food source, *R. salina*. This effect on survival was mitigated by giving copepods a mixed prey

Table 2. Log-rank Kaplan-Meier test with censoring of the *Acartia tonsa* copepod survival experiment using *Dinophysis acuminata* (DANY1)-fed JAMR strain of *Mesodinium rubrum*. Shown are p-values; **bold** numbers indicate statistical significance ($p \leq 0.05$). *Italicized* densities are DANY1 densities; non-italicized are *Rhodomonas salina*

	Control	*100 cells ml^{-1}*	*500 cells ml^{-1}*	*1000 cells ml^{-1}*	*2000 cells ml^{-1}*	3600 cells ml^{-1}	18000 cells ml^{-1}	36000 cells ml^{-1}
100 cells ml^{-1}	0.305	–	–	–	–	–	–	–
500 cells ml^{-1}	0.765	0.113	–	–	–	–	–	–
1000 cells ml^{-1}	0.538	0.814	0.242	–	–	–	–	–
2000 cells ml^{-1}	0.982	0.468	0.617	0.837	–	–	–	–
3600 cells ml^{-1}	**0.002**	**0.033**	**<0.001**	**0.006**	**0.016**	–	–	–
18000 cells ml^{-1}	**<0.001**	**0.003**	**<0.001**	**0.005**	**0.001**	0.765	–	–
36000 cells ml^{-1}	**0.001**	**0.006**	**<0.001**	**0.001**	**0.003**	0.503	0.692	–
72000 cells ml^{-1}	**0.001**	**0.020**	**<0.001**	**0.010**	**0.007**	0.765	0.837	0.692

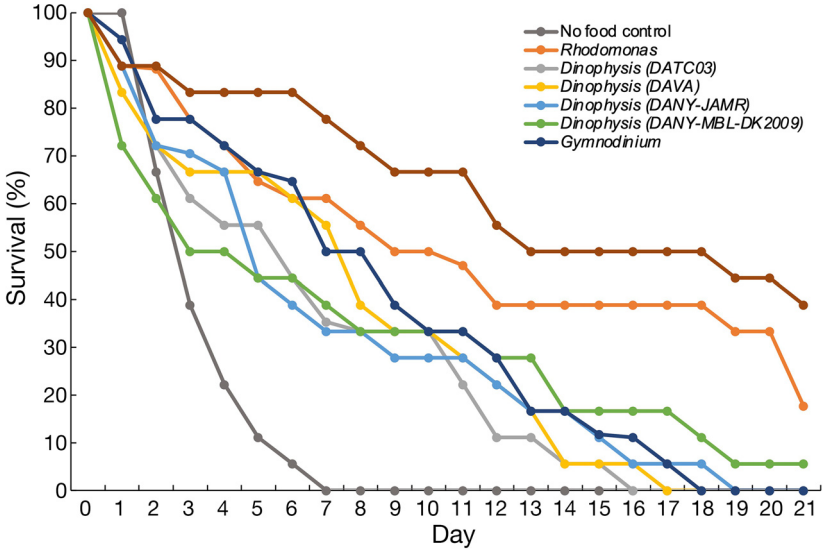

Fig. 8. Percent survival of *Acartia tonsa* fed *Dinophysis acuminata* strains DAVA, DATC03, or DANY1 (fed either MBL-DK2009 or JAMR strains of *Mesodinium rubrum*), carbon equivalents of non-toxic *Gymnodinium aureolum* or *Rhodomonas salina*, and a 50:50 mixture of DANY1 (fed JAMR strain of *M. rubrum*) and *G. aureolum*, all at 500 cells ml^{-1}. Differences were assessed using a log-rank Kaplan-Meier test (Table 2)

diet of *D. acuminata* and non-toxic *G. aureolum*. Collectively, these findings bring new insight toward understanding *D. acuminata* HABs.

To date, no study has examined *Acartia* spp. grazing on cultured *D. acuminata*. While *Acartia* spp. have been shown to co-exist with *D. acuminata* in natural populations, conclusions regarding their consumption of this HAB in an ecosystem setting have been mixed. *A. hudsonica* in Perch Pond, MA, USA (Turner & Anderson 1983), *A. clausi* in Ría de Pontevedra, Spain (Maneiro et al. 2000), and adult *A. tonsa* in Long Island, NY, USA (Ladds 2023) were shown to not feed on bloom populations of *D. acuminata*. *A. clausi* on the French Atlantic coast within the Partuis d'Antioche (Carlsson et al. 1995) and within the Aegean Sea (Frangoulis et al. 2022), however, fed

Table 3. Log-rank Kaplan-Meier test of the *Acartia tonsa* copepod survival experiment using multiple strains of *Dinophysis acuminata*, *Gymnodinium aureolum* and *Rhodomonas salina*. DANY1, the New York strain (NY) of *D. acuminata*, was fed either JAMR (Japan) or MBL-DK2009 (Denmark) strains of *Mesodinium rubrum*. DATC03 is the Massachusetts (MA) strain, and DAVA is the Virginia (VA) strain of *D. acuminata*; both were fed JAMR strain of *M. rubrum*. Mixed treatment is 50% *G. aureolum* and 50% DANY1 fed JAMR. All treatments were at 500 cells ml^{-1}. Shown are p-values; **bold** numbers indicate statistical significance ($p \leqslant 0.05$)

	Control	*Rhodomonas*	DANY1, JAMR	DANY1, MBL-DK2009	DATC03	DAVA	*Gymnodinium*
Rhodomonas	**0.003**	–	–	–	–	–	–
DANY, JAMR	**0.045**	**0.019**	–	–	–	–	–
DANY, MBL-DK2009	0.255	**0.037**	0.856	–	–	–	–
DATC03	0.069	**0.019**	0.701	0.701	–	–	–
DAVA	**0.022**	**0.019**	0.856	0.701	0.701	–	–
Gymnodinium	**0.008**	0.114	0.626	0.701	0.304	0.521	–
Mixed	**0.001**	0.701	**0.008**	**0.019**	**0.008**	**0.008**	0.052

on *D. acuminata in situ*. Of note, *A. clausi* on the French Atlantic coast also exhibited reduced survival while feeding on *Dinophysis* (Carlsson et al. 1995) and in the Aegean Sea, *A. clausi* grazing of *Dinophysis* was minimal (Frangoulis et al. 2022). Based on these prior studies as well as the results given here in culturing experiments, it is likely that *Acartia* are able to consume *Dinophysis* but may suffer adverse effects as a result. While other prey options may mitigate the inhibitory effects of *Dinophysis* and/or allow for selection of other prey over *Dinophysis*, alternate prey may become rare as blooms progress and *Dinophysis* becomes dominant. For example, in the current study, there was selection for *G. aureolum* when total prey abundance was low (100 cells ml^{-1}) and the relative abundance of *D. acuminata* was high (25–75%), although this selection decreased when total prey density increased, possibly due to increased encounter rates with *D. acuminata*. While *Dinophysis* is often considered a low-abundance HAB (Reguera et al. 2014), dense (10^6–10^8 cells l^{-1}) and persistent (1 mo) blooms have been observed in NY estuaries, with some blooms being close to monospecific among microphytoplankton (Hattenrath-Lehmann et al. 2013, 2018, M. Ladds & C. J. Gobler pers. obs.). Likewise, dense *Dinophysis* blooms have been observed in southern Brazil (Mafra et al. 2019) and the Chilean fjord system (Díaz et al. 2021), with a clear dominance in the total phytoplanktonic community and maximum cell densities of 2.2×10^6 *D. acuminata* and 6.6×10^5 *D. acuta*, respectively.

When offered as a sole source of food, *D. acuminata* caused a reduction in copepod survival to a level indistinguishable from the unfed treatment and could be associated with *Dinophysis*-derived toxins. Many HAB species have been shown to increase their toxin production in the presence of copepods (Selander et al. 2006, Tammilehto et al. 2015, Lundholm et al. 2018), which has been attributed to the presence of compounds such as copepodamides (Selander et al. 2015, Trapp et al. 2021). The Trapp et al. (2021) study demonstrated that copepodamides in shellfish were highly correlated with OA levels, suggesting that copepodamide presence may induce or enhance *Dinophysis* toxin production. In the present study, after 24 h of co-incubation of copepods and *Dinophysis*, extracellular OA concentrations significantly increased in the medium from undetectable to over 100 pg OA ml^{-1} and extracellular PTX2 concentrations of *Dinophysis* cultures doubled, suggesting that toxin release and/or toxin production may be stimulated by exposure to copepods. Given that intracellular toxin content did not significantly change, this suggests that there was an increase in production and subsequent extracellular release. Additionally, the percentage of esterified (metabolized forms) OA increased with the addition of copepods. This suggests that copepods were metabolizing the OA and releasing it or that *D. acuminata* were releasing an esterified form into the media (Windust et al. 2000). The exact composition of these esterified forms is unknown; however, esterified forms have been found in *Dinophysis* assemblages (Vale & Sampayo 2002, Moroño et al. 2003) as well as shellfish when they consume *Dinophysis* (Vale & Sampayo 2002, Torgersen et al. 2005). Zooplankton fractions have been shown to contain OA (Maneiro et al. 2000) and esterified forms of OA (Mafra et al. 2019), and crustaceans are suspected of being able to esterify OA (Torgersen et al. 2005, Mafra et al. 2019); therefore, there is potential for copepods to esterify these toxins and release these forms into the environment. Further study is needed to fully determine the potential for esterified forms within copepods. OA is an anti-miotic compound (Gliksman et al. 1992), and dissolved OA has been shown to be both toxic and a feeding deterrent to the harpacticoid copepod *Tigriopus californicus* (Shaw et al. 1997) as well as having lethal effects on other crustacean grazers by specifically disrupting molting and reproductive processes (Gong et al. 2021). Similarly, PTXs were toxic to the zooplankton species *Artemia salina*, causing increased mortality (Zhang et al. 2023). Hence, it is possible that grazer-induced OA and PTX2 release may have contributed to the enhanced copepod mortality observed during the longer-term experiment. Consistent with this hypothesis, DATC03 contains the highest amount of OA and PTX2 per cell (toxin content) among the *D. acuminata* strains examined here (Ayache et al. 2023) and resulted in the lowest survival rates in copepods in long-term experiments.

The phytoplankton cell densities used during this study were within the range found during *Dinophysis* blooms in NY and MA, USA (Hattenrath-Lehmann et al. 2013, 2018, Ayache et al. 2023) and are within the carbon concentrations necessary for optimal growth and survival of *A. tonsa* (Berggreen et al. 1988). The survival of *A. tonsa* at all concentrations of *D. acuminata* and *G. aureolum* as sole food sources was reduced compared to the fed control, carbon-equivalent diet of *R. salina*. This low survival effect was mitigated when both dinoflagellates were presented in a mixture during both the long-term survival experiments as well as the 24 h mixed grazing experiments. This is consistent with the idea that mixed-prey diets support optimal physiology of copepods

(Kleppel 1993, 1995) and suggests that both *D. acuminata* and *G. aureolum* individually may lack essential nutrients needed for the copepods or contain inhibitory compounds that are mitigated by the presence of the other dinoflagellate. Given that different phytoplankton contain different ratios and amounts of essential nutrients including fatty acids, amino acids, and sterols (Klein Breteler et al. 1999, Broglio et al. 2003, Koski & Klein Breteler 2003), when consumed individually, they may lack the essential compounds for survival but when combined, they meet the nutritional needs of the copepods. Consistent with this hypothesis, the reduction in survival when copepods were fed *Dinophysis* alone over short-term, mixed-prey experiments seemed to be due to nutritional deficiency with points in the modified (Colin & Dam 2002, Jónasdóttir et al. 1998) plots falling above the reference line, demonstrating that *Dinophysis* is not immediately toxic to *Acartia*. Future work examining egg production and hatching rates, which are key components of copepod fecundity and often used to determine toxicity or nutritional deficits (Jónasdóttir et al. 1998, Colin & Dam 2002, Prince et al. 2006), will help further advance the understanding of *Dinophysis* impacts on copepods. Additionally, experimentation on how *Dinophysis* would affect adult males as well as different copepod life stages would also aid in understanding the interaction between copepods and *Dinophysis*.

4.2. Ecosystem implications

The results of this study have important implications for food webs and ecosystems experiencing *Dinophysis* blooms. Given that there seems to be some density-dependent selection by *Acartia*, the grazing impacts of this copepod are likely to change across the bloom period. Copepods may graze on alternative food items at the beginning of blooms if their total prey abundance is low, which may promote bloom intensification (Gobler et al. 2004, Irigoien et al. 2005, Mitra & Flynn 2006, Sunda et al. 2006, Smayda 2008, Graham & Strom 2010). However, as blooms intensify, *Dinophysis* would become a more prominent prey item, leading to enhanced grazing on *Dinophysis*, given that this study found no short-term grazing deterrence. Prolonged grazing, however, could lead to reduced copepod survival, potentially enhancing blooms over time and/or allowing a bloom 'rebound' (Mitra & Flynn 2006). There may also be enhancement of OA and PTX2 production by *Dinophysis* when grazing pressure increases, which may, over the longer term, reduce grazing on this HAB (Shaw et al. 1997, Gong et al. 2021, Zhang et al. 2023), potentially facilitating bloom development. The only study using *Acartia* spp. and natural *D. acuminata* blooms that reported active grazing on *Dinophysis* and reduced survival also documented ingestion rates that were higher than some other studies (Carlsson et al. 1995) but similar to the current study, suggesting that ingestion above a threshold may reduce survival. While additional prey options might eliminate this deleterious effect, the precise complement to prey needed to mitigate significantly lowered survival caused by *Dinophysis* is unknown.

The active grazing of *Dinophysis* by *A. tonsa* has important implications for toxin transfer through the food web. Fecal pellets containing *Dinophysis* have been observed in field studies with copepod grazers (Maneiro et al. 2000, Wexels Riser et al. 2003, Jansen et al. 2006, Frangoulis et al. 2022), and toxins within these fecal pellets could be vectors to benthic food webs (Maneiro et al. 2000, Wexels Riser et al. 2003, Kuuppo et al. 2006) — especially shellfish, thus enhancing the potential for DSP events. Moreover, if *Dinophysis* is in large enough absolute or relative quantities, copepod grazing and/or dense packaging of *Dinophysis* cells within fecal pellets could enhance toxin transfer to pelagic food webs. Some studies have found that copepods become enriched in OA (Maneiro et al. 2000, D'Agostino et al. 2019) and PTX2 (Setälä et al. 2009) after feeding on *Dinophysis*, and here we reported increases in extracellular OA and PTX2 caused by copepods. Collectively, these findings suggest that copepods ingesting *Dinophysis* could be toxin vectors to higher trophic levels during blooms.

4.3. Conclusions

Dinophysis was readily consumed *by A. tonsa* across many densities and in the absence and presence of other phytoplankton. Prolonged exposure to this harmful alga as a single prey caused significantly decreased copepod survival. Copepods may induce increased excretion and/or production of the *Dinophysis*-derived toxins OA and PTX-2, which, along with potential nutritional deficiency, may contribute to reduced copepod survival and ultimately contribute to bloom intensification. The active and continued consumption of *Dinophysis* by *A. tonsa* during blooms, specifically dense and near-monospecific blooms, could make these copepods and/or their fecal pellets vectors for DSP toxins in marine food webs.

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Appendix. Detailed information related to the methodology done within this study, specifically the experimental set-up for the single and mixed grazing experiments as well as the modified Colin & Dam (2002) method to determine toxicity in the mixed experiments

Table A1. Starting cell densities for each strain of *Dinophysis* tested in the single-prey experiments. The *Rhodomonas* column is the carbon-equivalent prey concentration that was also used as a single prey to compare to the *Dinophysis* density tested. DANY1 was fed either JAMR or MBL-DK2009 strain of *Mesodinium* prior to experimentation

Test algae	*Dinophysis* density (cells ml^{-1})	*Rhodomonas* density (cells ml^{-1}]
DANY1 fed JAMR	100	3600
	500	18000
	1000	36000
	2000	72000
DANY1 fed MBL-DK2009	500	18000
	2000	72000
DAVA	500	18000
	2000	72000
DATC03	500	18000
	2000	72000

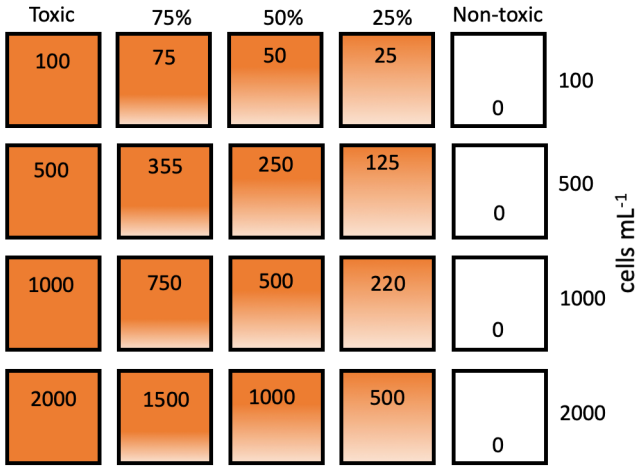

Fig. A1. Mixed experiment setup. The toxic algae is *Dinophysis acuminata* (fed the JAMR strain of *Mesodinium rubrum*) and non-toxic algae is *Gymnodinium aureolum*. Percentages are percent of toxic algae within the mixture; cells ml^{-1} represent the total final density. Numbers within the squares: density (in cells ml^{-1}) of the toxic algae

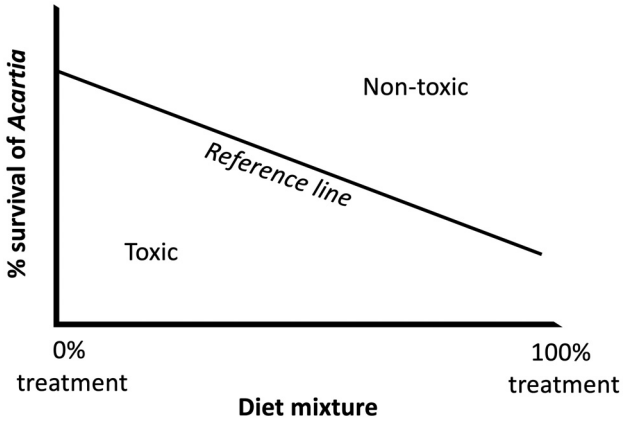

Fig. A2. Modification of Colin & Dam (2002) method to determine toxicity and nutritional sufficiency of a target potentially toxic algae (treatment) in a mixed diet with varying percentages of treatment algae. Reference line connects points at 0 and 100% treatment algae. Points above indicate no toxicity and nutritional enhancement; points below indicate toxicity and nutritional subtraction. Points on the line indicate no added nutritional value, but also no toxicity

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