1 ***Heterosigma akashiwo* does not serve as prey and chloroplast donor for the toxic**
2 **dinoflagellate, *Dinophysis acuminata***
3
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17 **Key Words:**
18 *Dinophysis acuminata*
19 *Heterosigma akashiwo*
20 *Mesodinium rubrum*
21 kleptoplasty
22 Delaware Inland Bays

Abbreviations:

DIBs = Delaware Inland Bays, OA = okadaic acid, DSP = diarrhetic shellfish poisoning

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45 **Abstract**

46 In laboratory culture, the toxic dinoflagellate *Dinophysis acuminata* acquires
47 plastids from the ciliate, *Mesodinium rubrum*, which, in turn, acquires plastids from
48 the cryptophyte, *Teleaulax amphioxeia*. Reports of *D. acuminata* from field samples
49 found plastids of the raphidophyte, *Heterosigma akashiwo* within *D. acuminata* cells,
50 suggesting a broader range of prey. *Dinophysis* blooms often co-occur with *H.*
51 *akashiwo* in Delaware's inland bays. In the study presented here, predation on *H.*
52 *akashiwo* by *D. acuminata* was investigated. Growth rates of *D. acuminata* were
53 measured when cultured with *H. akashiwo* either alone or with its known prey, *M.*
54 *rubrum*. *M. rubrum* was also cultured with *H. akashiwo* to examine predation by the
55 ciliate as a vector for *Heterosigma* plastids. Ingestion rates were measured in
56 individual cells of *D. acuminata* when presented with *H. akashiwo* as prey. Retention
57 of plastids from *H. akashiwo* was investigated by measuring chlorophyll *a*
58 fluorescence intensities in cells presented with *H. akashiwo* as prey compared to *M.*
59 *rubrum*. Additionally, a fluorescence-based method was developed to identify the
60 presence of the accessory pigment fucoxanthin from *H. akashiwo* plastids in cells of
61 *D. acuminata*. Results showed that the growth rate of *D. acuminata* was significantly
62 lower when offered *H. akashiwo* as prey compared the growth rate when offered *M.*
63 *rubrum* as prey. Likewise, no predation was observed when *D. acuminata* was offered
64 *H. akashiwo* as prey. Intensity of chlorophyll *a* fluorescence was lower when *H.*
65 *akashiwo* was offered as prey compared to *M. rubrum,* and fucoxanthin was not
66 detected in any of the *Dinophysis* cells after incubation with *H. akashiwo*. Results of

67 this investigation do not support the hypothesis that *D. acuminata* preys on *H.*
68 *akashiwo* and highlight the need for further research on factors that stimulate the
69 growth of *Dinophysis* in field populations.
70
71

72 **1. Introduction:**

73 *Dinophysis acuminata* is a predatory dinoflagellate that produces okadaic acid
74 (OA) and dinophysistoxins (DTX), toxins associated with diarrhetic shellfish
75 poisoning (DSP) (Yasumoto et al., 1985). In laboratory culture, *D. acuminata* is
76 known to prey on the ciliate, *Mesodinium rubrum*, harboring plastids that originated
77 from one of several cryptophytes, such as *Teleaulax amphioxeia*, which are then
78 retained by *D. acuminata* in the process of kleptoplasty (Park et al., 2006; Takishita et
79 al., 2002). Since *D. acuminata* does not produce chloroplasts of its own, kleptoplasty
80 allows *D. acuminata* to collect energy through photosynthesis in addition to the energy
81 collected from consumed prey, giving it a unique competitive advantage. Due to its
82 obligate kleptoplastic nature, recently established protocols for culturing *D. acuminata*
83 strictly use *M. rubrum* as prey (Park et al., 2006; Burkholder et al., 2008).

84 *Dinophysis acuminata* has been shown to divide prolifically in the presence of
85 *M. rubrum*, suggesting that blooms of this species are driven in part by the presence of
86 prey (Burkholder et al., 2008; Hattenrath-Lehmann et al., 2013). However, wild *D.*
87 *acuminata* cells have been found to contain plastids of multiple algal origin, including
88 those originating not only from *Teleaulax amphioxeia,* but also *Teleaulax acuta*,
89 *Rhodomonas* sp., *Chroomonas* sp., *Pyramimonas* sp., and the raphidophyte
90 *Heterosigma akashiwo* (Díaz et al., 2020; Kim et al., 2012).

91 *Dinophysis acuminata* blooms occur in Delaware and the surrounding mid-
92 Atlantic region and have been confirmed via morphological analysis and rDNA
93 sequence analysis (Wolny et al., 2020). *Heterosigma akashiwo* is also of interest in

94 Delaware where blooms of *H. akashiwo* have co-occurred with blooms of *D.*
95 *acuminata* in Torquay Canal, a tributary of Rehoboth Bay, Delaware (University of
96 Delaware Citizen Monitoring Program, n.d.). Cell densities of both *H. akashiwo* and
97 *D. acuminata* have been recorded in the millions of cells per liter, with *Dinophysis*
98 toxin levels from extracted shellfish tissue exceeding the US FDA recommended
99 exposure levels (Wolny et al., 2020). With shellfish aquaculture also in Rehoboth Bay,
100 DE, an understanding of factors that contribute to bloom dynamics of *D. acuminata* in
101 Torquay Canal is important to preserve human health.

102 Plastids originating from *H. akashiwo* found in wild *D. acuminata* cells (Kim
103 et al., 2012) raises the question of whether *H. akashiwo* plays a role in initiating or
104 sustaining *D. acuminata* blooms. The main objective of this study was to determine if
105 *H. akashiwo* is a source of prey for *D. acuminata*. Laboratory culture experiments
106 measured ingestion and growth rates of *D. acuminata* when offered *H. akashiwo* as
107 prey. Since *D. acuminata* is known to retain functioning chloroplasts from its prey
108 (Díaz et al., 2020; Kim et al., 2012; Park et al., 2006), individual *D. acuminata* cells
109 that were cultured with *H. akashiwo* were also examined to measure chlorophyll
110 content. The source of plastids in individual *D. acuminata* cells was investigated using
111 a novel dual ratiometric approach via confocal microscopy. This technique measured
112 the relative contribution of the accessory pigments fucoxanthin (Ex: 488 nm) and
113 phycoerythrin (Ex: 561 nm) to chlorophyll fluorescence. Since fucoxanthin is present
114 in *H. akashiwo* but not *M. rubrum*, and phycoerythrin is present in *M. rubrum* but not
115 *H. akashiwo*, the ratio of chlorophyll fluorescence intensity when excited at 488 vs.

116 561 nm in *D. acuminata* would provide evidence for the presence of plastids
117 originating from *H. akashiwo* in *D. acuminata* cells (Yutaka et al., 2001; Guiry, 2003).
118 Finally, historical data was examined to identify factors that may have contributed to
119 co-occurring blooms of *H. akashiwo* and *D. acuminata* in Torquay Canal.

120 **2. Methods:**

121 **2.1 Algal and ciliate cultures**

122 Cultures of *D. acuminata* (strain DADE) originally isolated from Torquay Canal,
123 Rehoboth Beach, DE (Lat. = 38.699031, Long. = -75.112409) were shared by Dr.
124 Juliette Smith (Virginia Institute of Marine Science, Gloucester Point, VA). Cultures
125 of *M. rubrum* (strain CBJR05, clade G) and *T. amphioxeia* (strain GCEP01) were
126 shared by Dr. Matthew Johnson (Woods Hole Oceanographic Institution, Woods Hole,
127 MA). *H. akashiwo* (strain CCMP 2393, National Center for Marine Algae and
128 Microbiata, Bigelow, ME) was originally isolated from Torquay Canal. All species
129 were cultured in 20 PSU f/6 media at 18 °C with a 12:12 light: dark regime (Goa et al.,
130 2019). Cultures of *D. acuminata* were maintained with *M. rubrum* at a predator: prey
131 ratio of 1:4 and cultures of *M. rubrum* were fed *T. amphioxeia* at a predator: prey ratio
132 of 1:4, both on a weekly basis. As needed, cultures of *D. acuminata* were filtered onto
133 a 10 µm mesh sieve to prevent overgrowth of the cryptophyte, *T. amphioxeia*.

134 **2.2 Growth Rate**

135 *Dinophysis acuminata* were maintained for two weeks without prey prior to the
136 start of the experiment. Cells were concentrated onto a 10 µm mesh sieve and
137 enumerated using a Sedgewick rafter chamber (Goa et al., 2019). *D. acuminata* was

138 transferred into 150 mL plastic Nalgene flasks at a cell density of 3,500 cells mL^{-1} and
139 provided with the following prey at a predator: prey ratio of 1:4 (N=3): only *H.*
140 *akashiwo*, both *H. akashiwo* and *M. rubrum*, and only *M. rubrum.* On days 1, 3, 6, 8,
141 and 10, samples were fixed with Lugol's for counting using light microscopy (Guillard
142 and Sieracki, 2005). The cell density of *D. acuminata* was determined using a
143 Sedgewick rafter chamber, and cell densities of *M. rubrum* and *H. akashiwo* were
144 measured using a hemocytometer counting at least 100 cells. The following
145 calculation was used to determine growth rate (μ) of algae:

146

147
$$\mu = \frac{\ln(cell\ abundance\ T_{final} / cell\ abundance\ T_{initial})}{\text{days}}$$

148 **2.3 Predation**

149 Predation by *M. rubrum* (1,444 cells mL^{-1}) on prey species *H. akashiwo* or *T.*
150 *amphioxeia* (control) was investigated using a predator: prey ratio of 1: 20 in separate
151 wells of a 12-well plate (n=6). At 24 hours (t=24), prey cell density in treatments and
152 controls was enumerated using a hemocytometer, counting at least 100 cells. The
153 experiment was then repeated with *D. acuminata* at 2,743 cells mL^{-1} (n=6) as predator
154 and *H. akashiwo* or *M. rubrum* (control species) as prey using a predator: prey ratio of
155 1:20. Prey controls were included in both predation experiments with only prey
156 species (predators omitted) at the same concentration as in the predator: prey
157 treatments (n=6). Grazing constant, clearance rate, and ingestion rate were calculated
158 as in Frost (1972), Heinbokel (1978) and Jeong and Latz (1994).

159 **2.4 Microscopic analysis of chlorophyll emission**

160 During the growth rate experiment, a 2 mL aliquot from each flask was
161 removed at 48 hours. Samples were then filtered onto 25 mm 0.2 µm black
162 polycarbonate filters and mounted on a microscope slide. Each filter received one drop
163 of Citifluor AF1 mountant media (Ted Pella, Inc., Redding, CA) and was covered with
164 a coverslip (Martin-Cereceda et al., 2008; Martinez et al., 2014).

165 Cells were imaged on a Zeiss LSM 710 inverted confocal microscope fitted
166 with a 40x C-Apochromat water immersion objective (NA = 1.2) (Carl Zeiss Inc.,
167 Thornwood, NY). The 633 nm laser line was used to excite chlorophyll *a* in individual
168 cells (n=30 cells for each treatment). Since chlorophyll *a* and fucoxanthin have similar
169 absorbance spectra, the 633 nm laser line was used to avoid excitation of fucoxanthin,
170 allowing the emission from only chlorophyll *a* to be observed. Fluorescence emission
171 from chlorophyll *a* was collected between 649 and 698 nm. Z-stack images with a step
172 of 0.3 µm were acquired from bottom to top of the cells. Mean intensity values of
173 chlorophyll *a* emission were determined using ImageJ/Fiji (NIH, Bethesda, MD)
174 software. Briefly, maximum intensity projections of the z-stacks were segmented to
175 measure intensities from single cells.

176 **2.5 Contribution of accessory pigments to chlorophyll emission**

177 Cultures of *H. akashiwo*, *T. amphioxeia*, and *D. acuminata* were used initially
178 to investigate cell-specific chlorophyll fluorescence emission when excited at 488 nm,
179 the excitation wavelength of fucoxanthin. Cells were filtered onto 25 mm 0.2 µm
180 black polycarbonate filters and mounted on a microscope slide. Each filter received

181 one drop of Citifluor AF1 mountant media (Ted Pella, Inc., Redding, CA) and was
182 covered with a coverslip (Martin-Cereceda et al., 2008; Martinez et al., 2014). The
183 488 nm laser line was used to excite fucoxanthin in individual cells (Papagiannakis et
184 al., 2005). Fluorescence emission of chlorophyll when excited at these wavelengths
185 was collected between 649-698 nm and emission intensity determined using
186 ImageJ/Fiji (NIH, Bethesda, MD) software. To account for differences in plastid
187 number, the chlorophyll emission intensity when excited at 488 nm was normalized to
188 chlorophyll emission intensity when excited at 633 nm, the excitation wavelength of
189 chlorophyll *a*.

190 Using the protocol described above for growth rate experiments, a separate
191 experiment was conducted using *Dinophysis acuminata* provided with *H. akashiwo*, a
192 mix of *H. akashiwo* and *M. rubrum*, or only *M. rubrum* as prey. Samples were
193 collected at 48 hours, mounted onto microscope slides, and the 488 nm and 633 nm
194 laser lines were used to excite fucoxanthin and chlorophyll as above. A second
195 measurement of chlorophyll fluorescence was made when excited at 561 nm, the
196 excitation wavelength of phycoerythrin. Chlorophyll fluorescence emission when
197 excited at each wavelength was collected between 649-698 nm. To determine the
198 relationship between fucoxanthin-containing plastids to phycoerythrin-containing
199 plastids, the ratio of chlorophyll fluorescence when excited at 488 nm to the
200 chlorophyll emission intensity when excited at 561 nm was calculated.

201 **2.6 Relationships between *D. acuminata* and *H. akashiwo* abundances in field**
202 **samples**

203 Between 2011 and 2017, monthly water samples were collected between April
204 and October at Torquay Canal, Rehoboth, DE (38.699015, -75.112394) by the
205 University of Delaware Citizen Monitoring program. Salinity and temperature data
206 were collected using a YSI handheld multi-meter (Xylem Analytics, Yellow Springs,
207 Ohio). Cell densities were determined by microscopic screenings of live samples (two
208 or three 40 µL drops), followed by screenings of preserved (Lugol's) samples (1-2
209 mL) if cell densities of *D. acuminata* were less than 10^5 cells L^{-1}. Samples in which
210 both *H. akashiwo* and *D. acuminata* exceeded 10^4 cells L^{-1} (n = 16) were selected for
211 data analysis.

212 **2.7 Statistical Analysis**

213 Statistical analysis was performed using a one-way ANOVA test in excel on
214 individual data collected during the following experiments: growth curve, ingestion
215 rate, chlorophyll *a* intensity, and the ratio of intensity of emission of both fucoxanthin:
216 chlorophyll *a* and fucoxanthin: phycoerythrin. If data were found to be significantly
217 different ($p \leq 0.05$), then a Tukey honestly significant difference (HSD) *post hoc* test
218 was conducted. Pearson's correlation coefficient was determined between *D.*
219 *acuminata* and *H. akashiwo* cell density in Torquay Canal between 2011 and 2017.
220 The relationship between salinity, temperature and *H. akashiwo* cell density and co-
221 occurring blooms of *D. acuminata* between 2011 and 2017 were analyzed using a
222 Pearson's correlation (Data shared by UDCMP).

223 **3. Results**

224 **3.1 Growth Rates**

225 Changes in cell abundance for *D. acuminata* over 10 days incubation when
226 offered *M. rubrum*, *H. akashiwo*, or a mix of *M. rubrum* and *H. akashiwo* are shown in
227 Fig. 1A. Changes in cell abundance for prey are shown in Fig. 1B. The mean growth
228 rate of *D. acuminata* when offered only *H. akashiwo* as prey was -0.037 (+/- 0.028) d^{-1}
229 and was not significantly different from the growth rate of *D. acuminata* without prey
230 (-0.12 +/- 0.14 d^{-1}), or when offered *M. rubrum* and *H. akashiwo* together [-0.0076
231 (+/- 0.015) d^{-1}]. In control cultures with only *M. rubrum* as prey, the growth rate of *D.*
232 *acuminata* was 0.050 (+/- 0.0032) d^{-1} and significantly higher ($p < 0.005$) than the
233 growth rate without prey or when in the presence of both *H. akashiwo* and *M. rubrum*
234 or *H. akashiwo* alone. During this experiment, the growth rate of *H. akashiwo* in
235 treatments with *D. acuminata* was 0.28 (+/- 0.0035) d^{-1} and was not significantly
236 different from the growth rate of *H. akashiwo* when in the presence of both *M. rubrum*
237 and *D. acuminata* [0.30 (+/- 0.012) d^{-1}]. *M. rubrum* cells were not detected after 3
238 days in flasks containing only *D. acuminata* or those containing *H. akashiwo* and *D.*
239 *acuminata* (Fig. 1).

240 **3.2 Ingestion Rates**

241 In 24-hour predation experiments, ingestion of *H. akashiwo* by either *M.*
242 *rubrum* or *D. acuminata* was not observed. Control experiments in which *T.*
243 *amphioxeia* was provided as prey for *M. rubrum*, or *M. rubrum* as prey for *D.*
244 *acuminata* exhibited positive ingestion rates (12.3 +/- 1.98 cells d^{-1} and 14.7 +/- 3.38

245 cells d^{-1}, respectively). Cell density of *M. rubrum* decreased when given *H. akashiwo*
246 as prey but not when given *T. amphioxeia* as prey, suggesting potential allelopathic
247 interactions. Control cultures with prey alone demonstrated positive growth for both
248 *M. rubrum* (μ=0.0623 +/-0.0068 h^{-1}) and *H. akashiwo* (μ=0.0474 +/-0.0088 h^{-1}).

249 **3.3 Analysis of chlorophyll emission**

250 It was hypothesized that if *D. acuminata* was retaining plastids from prey, cell-
251 specific chlorophyll *a* intensity would increase. Chlorophyll *a* emission intensity was
252 significantly lower ($p > 0.0001$) in *D. acuminata* that were presented with only *H.*
253 *akashiwo* as prey (140.3 ± 15.5) or a combination of *H. akashiwo* and *M. rubrum* as
254 prey (192.8 ± 15.9) compared to cells of *D. acuminata* that were presented with only
255 *M. rubrum* as prey (361.0 ± 16.2) (Fig. 2).

256 **3.4 A ratiometric approach to evaluate plastid source**

257 The ratiometric approach was used to further determine if *D. acuminata* preys
258 on *H. akashiwo* by searching for fucoxanthin in *D. acuminata* cells that were given *H.*
259 *akashiwo* as prey. It was hypothesized that if *D. acuminata* preys on *H. akashiwo*, the
260 pigment fucoxanthin, which is present in *H. akashiwo* plastids but not *M. rubrum*
261 would be detected after feeding (Yutaka et al., 2001; Rial et al., 2012). Fucoxanthin
262 was detected by obtaining a ratio of the relative contribution of fucoxanthin and
263 phycoerythrin to chlorophyll fluorescence. This hypothesis was tested using
264 monocultures of *H. akashiwo*, *T. amphioxeia* and *D. acuminata* previously fed with *M.*
265 *rubrum.* The chlorophyll *a* emission intensity when excited at 488 nm (for

266 fucoxanthin), normalized to emission intensity when excited at 633 nm (for
267 chlorophyll *a*), was significantly higher in *H. akashiwo* cells (1.31 ± 0.12; $p < 0.00001$)
268 than in *T. amphioxeia* (1.21 ± 0.01) or in *D. acuminata* that were offered *M. rubrum* as
269 prey (0.98 ± 0.28) (Suppl. Fig. S1). This data confirms the hypothesis that a higher
270 ratio of fucoxanthin to phycoerythrin is seen in the fucoxanthin containing plastids of
271 *H. akashiwo.*

272 In order to determine if *D. acuminata* retains plastids from *H. akashiwo* when
273 offered as prey, the same ratiometric test was conducted. There was no statistically
274 significant difference ($p = 0.752$) between the ratio of chlorophyll *a* emission intensity
275 when excited at 488 nm (for fucoxanthin) to 561 nm (for phycoerythrin) in cells of *D.*
276 *acuminata* that were presented with only *H. akashiwo, H. akashiwo* and *M. rubrum,* or
277 only *M. rubrum* as prey (Fig. 3). The lack of a significant difference between ratios of
278 the two treatment groups suggests that *D. acuminata* is not retaining plastids from *H.*
279 *akashiwo* when offered as prey.

280 **3.5 Relationships between *D. acuminata* and *H. akashiwo* abundances in field**
281 **samples**

282 Pearson's correlation between *D. acuminata* and *H. akashiwo* cell abundances
283 in Torquay Canal between 2011 and 2017 revealed a highly significant correlation
284 when both species were present at $>10^4$ cells L^{-1} ($r = 0.604$, $p = 0.00065$) (Fig. 4).
285 Salinity ranged from 18.5 to 27.6 and temperature ranged from 17.7 °C to 25.9 °C.
286 Cell densities for *D. acuminata* and *H. akashiwo* were significantly correlated to both

287 salinity (p = 0.0070 and 0.0079, respectively) and temperature (p = 0.0070 and 0.0079,
288 respectively).

289 **4. Discussion**

290 Trophic interactions between species has been shown to be an important
291 determinant in blooms of *D. acuminata.* Previous studies showed that blooms occur
292 when *D. acuminata* practices kleptoplasty over autotrophy (Burkholder et al., 2008;
293 Hattenrath-Lehmann et al., 2013). However, laboratory cultures of *D. acuminata* have
294 only been observed to feed on *M. rubrum* and retain plastids originating from its prey
295 *Teleaulax* sp. When offered *M. rubrum* as prey, *D. acuminata* has a growth rate that is
296 three times higher than when no prey is provided, due to the increased trophic
297 efficiency associated with mixotrophy over autotrophy (Park et al., 2006; Sanders,
298 1991).

299 There is evidence that wild populations of *D. acuminata* may consume other
300 species in addition to *M. rubrum*. Examination of *D. acuminata* cells during a bloom
301 along the coast of South Korea, for example, revealed the presence of plastids of
302 multiple algal origins, including from *H. akashiwo* (Kim et al., 2012). The observation
303 of both *D. acuminata* and *H. akashiwo* blooms in Torquay Canal, DE suggested the
304 need for further research to understand interactions between these species in local
305 environments.

306 Results of this study indicated that *D. acuminata* does not prey on *H. akashiwo*
307 in controlled laboratory culture experiments, and that *M. rubrum* does not provide a
308 conduit for plastids from *H. akashiwo* to *D. acuminata* as it does for *Teleaulax*. The

309 growth rate of *D. acuminata* when provided with *H. akashiwo* alone (-0.037 d^{-1}) was
310 not significantly different from the growth rate of starved *D. acuminata* without prey.
311 Additionally, growth rates of *D. acuminata* when provided a combination of *M.*
312 *rubrum* and *H. akashiwo* (-0.0076 d^{-1}) as prey was significantly lower ($p<0.005$) than
313 when provided with *M. rubrum* as sole prey (0.050 d^{-1}) (Fig. 1). This suggests that
314 grazing and growth of *D. acuminata* may be inhibited by the presence of *H.*
315 *akashiwo*, at least in laboratory culture, even when its preferred prey (*M. rubrum*) is
316 present. Further evidence that *D. acuminata* selectively feeds on *M. rubrum* is the
317 disappearance of *M. rubrum* and increase in *H. akashiwo* cell abundance in predation
318 experiments. It should be noted here, however, that grazing studies to investigate the
319 potential for *H. akashiwo* to serve as prey for *M. rubrum* also resulted in a decline of
320 *M. rubrum* cells. The interactions between *H. akashiwo* and *M. rubrum* require
321 further study, but research has demonstrated allelopathic interactions between *H.*
322 *akashiwo* and other ciliates (cf. Frederickson et al. 2011). Elevated pH of the mixed
323 prey culture may have also had a negative impact on cell densities of *M. rubrum*
324 (Smith & Hansen 2007; Nielsen et al 2012). Although *M. rubrum* was routinely
325 grown to high density in cell culture with *T. amphioxia* provided as prey, previous
326 studies have shown negative impacts of elevated pH (>8.8) on growth of this ciliate
327 (Smith and Hansen 2007). The low tolerance to elevated pH may have contributed to
328 the decline in cell densities of *M. rubrum* when provided to *D. acuminata* either alone
329 or in mixed prey experiments with *H. akashiwo* (Fig. 1).

330 In addition to growth experiments, 24-hour grazing experiments were
331 conducted with *H. akashiwo* to evaluate the potential for this species to serve as prey
332 for *D. acuminata.* Several factors can affect ingestion rates. Riisgaard and Hansen
333 (2009), for example, showed a correlation between *D. acuminata* ingestion rates and
334 *M. rubrum* prey abundance, resulting in rates that varied from 1 to 11 *M. rubrum*
335 (prey) per *D. acuminata* d^{-1}. Kim et al. (2008) observed highest ingestion rates of *M.*
336 *rubrum* by *D. acuminata* (3.2 *M. rubrum* d^{-1}) when grown at higher light intensities.
337 Temperature has also been shown to affect the activity and feeding rate of plankton
338 (Hansen et al., 1997; Kamiyama et al., 2009). For example, reported average ingestion
339 rates of *M. rubrum* by *D. acuminata* increased from 1.18 d^{-1} at 4 °C to 2.06 d^{-1} at 10
340 °C (Tong et al., 2010). Results of this study yielded an ingestion rate of control
341 species *M. rubrum* at 14.7 cells d^{-1}. Despite the high grazing activity of *D. acuminata*,
342 there was no measurable predation on *H. akashiwo* in laboratory culture experiments,
343 which were conducted in parallel with control experiments where *M. rubrum* served as
344 prey. It is important to note here that *D. acuminata*, along with other members of this
345 genus have been observed to form mucus traps which could impact ingestion rates
346 (Ojamae et al., 2016; Mafra et al., 2016; Papiol et al 2016); however, mucus traps
347 were not observed for this strain of *D. acuminata*.
348 The presence of plastids from *H. akashiwo* in *D. acuminata* cells described by
349 Kim et al. (2012) may have occurred through “sloppy” predation, resulting in rare
350 and/or accidental ingestion that could be missed by measuring ingestion rates in bulk

351 culture experiments. Further evaluation of predation by *D. acuminata* then considered
352 the function of kleptochloroplasts on a cell-by-cell basis when offered either *M.*
353 *rubrum* or *H. akashiwo* as prey. An increase in the photosynthetic rate and chlorophyll
354 *a* concentration has been previously documented in *D. acuminata* when fed *M. rubrum*
355 (Nielsen et al., 2012; Hansen et al., 2016). Consistent with grazing and growth
356 experiments, chlorophyll *a* intensity was significantly lower ($p < 0.0001$) in *D.*
357 *acuminata* cells when provided with *H. akashiwo* or combined *H. akashiwo* and *M.*
358 *rubrum* as prey, than in cells offered only *M. rubrum* as prey (Fig. 2).

359 Despite the lower chlorophyll *a* intensity, it was still possible that plastids from
360 *H. akashiwo* may have been retained. In order to evaluate this hypothesis, a
361 ratiometric approach to evaluate energy transfer from accessory pigments present in
362 plastids retained by individual cells was developed. Here, cell-specific chlorophyll *a*
363 emission was measured using fluorescence confocal microscopy after excitation at 488
364 nm to measure energy transfer from fucoxanthin, the accessory pigment in *H.*
365 *akashiwo* plastids, and at 561 nm to measure energy transfer from phycoerythrin, the
366 accessory pigment in *Teleaulax* plastids. In validating this method, the normalized
367 chlorophyll emission after excitation at 488 nm was significantly higher in cultures of
368 *H. akashiwo* than cultures of *T. amphioxeia* or *D. acuminata* (Suppl. Fig. S1). *D.*
369 *acuminata* was then offered *H. akashiwo* alone, both *H. akashiwo* and *M. rubrum,* or
370 *M. rubrum* alone as prey, and chlorophyll emission in *D. acuminata* cells was
371 measured after excitation at 488 nm and compared to chlorophyll emission when
372 excited at 561 nm (for phycoerythrin). There was no significant difference ($p = 0.21$)

373 in the ratio of chlorophyll emission intensities in any of the treatment groups (Fig. 3),
374 suggesting that fucoxanthin-containing plastids were not present in *D. acuminata*.
375 Overall, results of this analysis in which individual cells were evaluated for changes in
376 *in vivo* chlorophyll fluorescence and the presence of fucoxanthin-containing plastids,
377 do not support the hypothesis that sloppy feeding results in the ingestion of *H.*
378 *akashiwo*.

379 Blooms of *D. acuminata* co-occur with other phytoplankton species, including
380 the raphidophyte, *H. akashiwo* (Burkholder et al., 2008; Ma et al., 2006).
381 Retrospective analysis of cell abundance data between 2011-2018 for samples in
382 which *D. acuminata* and *H. akashiwo* cell densities exceeded 10^4 cells L^{-1} demonstrate
383 a highly significant correlation between *D. acuminata* and *H. akashiwo* cell densities
384 in Torquay Canal, Rehoboth, DE (Fig. 4), suggesting that these species respond to
385 similar biotic and/or abiotic factors. Bloom co-occurrence of *D. acuminata* and *H.*
386 *akashiwo* in Torquay Canal is likely driven by nutrient inputs and/or other predator-
387 prey dynamics (Connell & Jacobs, 1997; Hattenrath-Lehman et al., 2013; Hattenrath-
388 Lehman et al., 2015). In grazing experiments conducted here, the growth rate of *H.*
389 *akashiwo* actually increased when in the presence of *D. acuminata*, suggesting the
390 possibility that exudates such as dissolved organic matter from *D. acuminata* (Gao et
391 al., 2019) may have fueled growth of *H. akashiwo*. Seasonal fluxes in temperature
392 may also constrain bloom dynamics. Blooms examined here, for example, occurred
393 when water temperatures were between 17.7 °C and 25.9 °C, the optimal temperature

394 range for blooms of each species (Zhang et al., 2006; Handy et al., 2008; Hattenrath-
395 Lehmann, 2013).

396 While results presented here also do not support predation of *H. akashiwo* by
397 either *D. acuminata* or *M. rubrum*, other ciliates may provide a conduit for transfer of
398 plastids from *H. akashiwo* to *D. acuminata*. *Strombidinopsis acuminatum*, *Coxliella*
399 sp., *Metacylis* sp., and *Eutintinnus* sp. have all been shown to prey on *H. akashiwo*,
400 and there is evidence that *Strombidinopsis* sp. retains chloroplasts from prey (Clough
401 and Strom, 2005; Frederickson et al., 2011). Furthermore, individual *Dinophysis* cells
402 from wild populations were shown to retain red cryptophyte plastids that were
403 phylogenetically associated with plastids retrieved from tintinnids and heliozoans
404 (Díaz et al., 2020), suggesting the possibility that *D. acuminata* may acquire plastids
405 from *H. akashiwo* retained through predation by other ciliates or protists.

406 **3. Conclusions**

407 Although *D. acuminata* cells isolated from field samples have been found to
408 contain plastids from *H. akashiwo*, direct predation on *H. akashiwo* by *D. acuminata*
409 or by an intermediate, *M. rubrum*, was not observed in the laboratory. Growth rates
410 and ingestion rates of *D. acuminata* when provided with *H. akashiwo* as prey were
411 both negative. Furthermore, chlorophyll *a* intensity when offered *H. akashiwo* as prey
412 was significantly lower than when offered only *M. rubrum* as prey, and there was no
413 evidence of fucoxanthin-containing plastids in *D. acuminata* after co-incubation with
414 *H. akashiwo.*

415 To better understand the ecology of *D. acuminata* and factors that contribute to
416 bloom initiation in the mid-Atlantic region of the US, further research into the
417 predator-prey dynamics in field populations will be necessary. Such analysis could
418 include studies where cell densities in feeding experiments are more representative of
419 natural conditions and could also investigate feeding behavior. While it is known that
420 *D. acuminata* uses a peduncle to feed, for example, the ability to produce mucus traps
421 may decrease prey selectivity. It is unclear what factors signal deployment of mucus
422 traps or how widespread this phenomenon is among *D. acuminata* strains.

423 Results here also suggest that abiotic factors likely provide an environment that
424 favors co-occurrence of *D. acuminata* and *H. akashiwo* in Torquay Canal, while
425 species interactions may play a larger role in *Dinophysis* bloom dynamics. Further
426 analysis of biotic and abiotic factors regulating growth and proliferation of *D.*
427 *acuminata* in this and other mid-Atlantic estuaries will be essential to development of
428 management strategies for prevention and mitigation of toxic blooms of this species.

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642 **Figure Legends**

643

644 Figure 1. (A) Cell abundances of *D. acuminata* over a period of 10 days, when offered
645 only *Heterosigma akashiwo*, both *H. akashiwo* and *Mesodinium rubrum*, or only *M.*
646 *rubrum* as prey. Error bars represent standard error (n=3 for each treatment). (B) Cell
647 abundances of *Heterosigma akashiwo* and *Mesodinium rubrum* over a period of 10
648 days, when offered to *Dinophysis acuminata* as prey. Error bars represent standard
649 error (n=3 for each treatment).

650

651 Figure 2. Fluorescence intensity of chlorophyll *a* in *Dinophysis acuminata* offered
652 only *Heterosigma akashiwo*, both *Heterosigma akashiwo* and *Mesodinium rubrum*,
653 and only *Mesodinium rubrum* as prey (excitation wavelength = 633 nm, emission
654 wavelength range = 649-698 nm). Error bars represent standard error (n=30 for each
655 treatment) and asterisk indicates a significant difference between fluorescence
656 intensity of chlorophyll *a* ($p < 0.0001$).

657

658 Figure 3. Ratio of intensity of emission of chlorophyll *a* when excited using 488 nm
659 to emission intensity when excited using 561 nm wavelengths, for individual cells of
660 *Dinophysis acuminata* when offered only *Heterosigma akashiwo*, *Heterosigma*
661 *akashiwo* and *Mesodinium rubrum*, or only *Mesodinium rubrum* as prey. Error bars
662 represent standard error (n=30 for each treatment).

663 Figure 4. Correlation between *Heterosigma akashiwo* and *Dinophysis acuminata* cell
664 abundances in Torquay Canal between 2011 and 2017 for samples in which both
665 species were present at $>10^4$ cells L^{-1}. Pearson's correlation analysis revealed that
666 *Heterosigma akashiwo* and *Dinophysis acuminata* cell abundance were highly
667 significantly correlated ($p < 0.001$).
668
669