Mixotrophy in *Heterocapsa rotundata*: A mechanism for dominating the winter phytoplankton

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Abstract

*Heterocapsa rotundata* is a mixotrophic dinoflagellate that can ingest picoplankton, including bacteria, and is known to form large blooms in temperate estuaries during wet winters, particularly when grazing pressure on phytoplankton is low. We hypothesized that phagotrophy gives *H. rotundata* an advantage over other phytoplankton species during low light conditions. We used laboratory and field experiments to investigate changes in phagotrophy by *H. rotundata* in response to changes in light availability. Prey removal experiments with a non-axenic culture of *H. rotundata* were used to determine changes in *H. rotundata*’s ingestion rates in response to changes in irradiance. Fluorescent microspheres were used to measure in situ ingestion rates of *H. rotundata* collected on 20 different occasions from the Choptank River during the winter of 2016. In situ *H. rotundata* ingestion rates were tested for correlation with inorganic nutrient concentrations and irradiance levels. Ingestion rates measured with cultured and in situ *H. rotundata* followed similar patterns and ingestion rates increased as irradiance decreased. *H. rotundata* has the potential to obtain nutrients from multiple nutrient sources, switching from phototrophy to partial heterotrophy as irradiance decreases. This response may allow *H. rotundata* to survive and to potentially form blooms when growth rates of most other estuarine phytoplankton species is low.

*Heterocapsa* is a genus of dinoflagellates that contains numerous bloom forming and toxic species (Salas et al. 2014). One particular species, *Heterocapsa rotundata* (Loehmann) Loeblich (Hansen 1995), is ubiquitous and occasionally forms large blooms. *H. rotundata* has been reported in a range of environments all over the world including Chesapeake Bay, U.S.A. (Millette et al. 2015), Keum Estuary, South Korea (Seong et al. 2006), Manori Creek and Manim Bay, India (Shahi et al. 2015), Baltic Sea, Germany (Jacobinski et al. 2015), and Kangaroo Island, Australia (Balzano et al. 2015). *H. rotundata* tends to either dominate or be a prominent part of the phytoplankton community for at least part of the year in some of these areas (Seong et al. 2006; Balzano et al. 2015; Millette et al. 2015). Yet, relatively few studies have focused on the ecology of *H. rotundata*.

Some studies have focused on the formation and decline of *H. rotundata* winter blooms in Chesapeake Bay tributaries (Cohen 1985; Sellner et al. 1991; Millette et al. 2015). These researchers found abundant *H. rotundata* blooms in wet, cold winters when salinity is low (Cohen 1985) and when there is a release in grazing pressure from microzooplankton and copepods (Millette et al. 2015). Although wet, cold winters and a release in grazing pressure are factors that impact every phytoplankton species, it is unknown how *H. rotundata* can take advantage of these conditions over other species to bloom.

One possibility is that *H. rotundata* may use their capacity as a mixotroph to overcome light limitation. This would give *H. rotundata* an advantage over other phytoplankton in the winter. *H. rotundata* has been shown to consume heterotrophic bacteria, cyanobacteria such as *Synechococcus*, and small diatoms (Seong et al. 2006; Jeong et al. 2010). Their bacterial ingestion rate is known to increase with increasing bacteria abundances (Seong et al. 2006), but their phagotrophic response to changes in environmental factors has never been examined. Their maximum bacterial ingestion rate has been calculated to be 11.2 bacteria *H. rotundata*2 h, which is equivalent to ingestion of 76% of their body carbon per day (Seong et al. 2006).

Many phagotrophic phototrophs, such as *H. rotundata*, use mixotrophy to acquire limiting nutrients. In some nutrient limited mixotrophs, grazing increases with irradiance levels because at higher irradiance more nutrients are required to keep up with increased photosynthetic rates (Stoecker 1998).
Other phagotrophic phototrophs use mixotrophy to acquire organic carbon when light is limited, and their grazing rates increase as irradiance levels decrease (Stoecker 1998). Light is the primary factor limiting phototrophic growth in the Chesapeake Bay during winter when inorganic nutrient limitation is rare (Fisher et al. 2003; Kemp et al. 2005). If *H. rotundata* is using phagotrophy to compensate for light limitation of photosynthesis, then we would expect their ingestion rates to increase as irradiance levels decrease.

We used a combination of laboratory and field experiments to measure *H. rotundata*’s phagotrophic and cellular response to changes in light and nutrients to understand how it can bloom during temperate winters in a coastal plain estuary. We hypothesize that as light decreases, *H. rotundata*’s ingestion on bacteria increases, giving the dinoflagellate an advantage over other phytoplankton and allowing them to dominate the nanoplanckton community in the winter. If this is the case, the impact of *H. rotundata*’s grazing may keep bacteria and small phytoplankton populations at low abundances during a winter bloom (Seong et al. 2006).

**Methods**

Our overall approach combined laboratory and field experiments to determine how the ingestion of *H. rotundata* was affected by changes in environmental conditions, primarily irradiance levels. We conducted laboratory experiments to identify how changes in irradiance impacted *H. rotundata*’s ingestion rate and cellular content under controlled conditions. We then conducted high frequency field sampling and in situ experiments to track how natural variation in environmental factors impacted *H. rotundata*’s ingestion rate in situ and compared it to our laboratory data. The

![Fig. 1. Location of sampling site in Choptank River, MD on the Bill Burton Fishing Pier.](image)
field experiments also allowed us to estimate the impact of grazing by these mixotrophs on the bacterial population.

Laboratory experiments

A culture of *H. rotundata* (K-0483) from the Scandinavian Culture Collection of Algae and Protozoa at the University of Copenhagen in Denmark was used in all laboratory experiments. *H. rotundata* was reared at the Horn Point Laboratory in Cambridge, Maryland, U.S.A. on enriched f/2 – Si medium (Guillard and Ryther 1962) in autoclaved Choptank River water at 10.8 salinity and 4°C on an 8 : 16 light : dark cycle at 85 mol photons m⁻² s⁻¹. The culture conditions were the same conditions used for the laboratory experiments unless otherwise noted. The culture was non-axenic and unidentified bacteria in the culture were used as prey for the laboratory experiments. The bacteria in the culture had an average length (± SE) of 0.251 (± 0.01) μm and average width (± SE) of 0.251 (± 0.01) μm (N = 30). The average volume of the bacterial cells was estimated with the equation for a cylindrical rod (Lee 1993) and converted to carbon biomass with the equation pg C cell⁻¹ = 0.12 × Y⁰.⁷ (Norland 1993), with volume expressed in μm³. The average volume of the bacterial cells (± SE) was 0.032 ± 0.004 μm³ and average carbon content per cell (± SE) was 11.0 ± 0.9 fg C cell⁻¹.

We tested *H. rotundata*’s ingestion rates at five different irradiance levels. Different light levels were achieved by incubating bottles without mesh (~ 40 μmol photons m⁻² s⁻¹, Iₙ), with 1 (~ 58% Iₙ), 2 (~ 15% Iₙ), or 4 (~ 4% Iₙ) layers of black nylon mesh, or one layer of black electric tape covering the container (completely dark). Each treatment consisted of four 250 mL Nalgene polycarbonate bottles with 100 mL of f/2 – Si media, with one bottle for a control (only bacteria) and three bottles for experimental replicates (bacteria + *H. rotundata*). Bacteria cells in controls were isolated from the *H. rotundata* culture through gentle reverse filtration using a 3 μm nucropore filter. For the duration of the experiment air was gently bubbled into the containers, similar to culture conditions used to maintain the cells in suspension.

Based on preliminary experiments, we ran these experiments for 48 h. The first 24 h were used as an adaptation period to experimental conditions during which *H. rotundata* division and grazing rates were not measured. Bacteria samples of 1 mL were taken at 24 h and 48 h and 5 mL of water was collected at 0 h, 24 h, and 48 h to estimate *H. rotundata* abundance. The 1 mL aliquots from each bottle were preserved in 4% buffered paraformaldehyde (PFA) and then stored in cryovials at −80°C. The bacteria were then stained with SYBR green (Van Nevel et al. 2013) and counted using a BD Accurri C6 flow cytometer within a week after preservation. *H. rotundata* samples were preserved in Lugol’s solution and counted with a Nikon Eclipse E800 microscope at 20X.

### Table 1. Laboratory Experiment 2- *Heterocapsa rotundata* ingestion rates (I, bacteria *H. rotundata*⁻¹ h⁻¹), cellular chlorophyll a concentration (Chl a, pg Chl a *H. rotundata*⁻¹), population growth rate (μ, d⁻¹), cellular carbon concentration (pg carbon *H. rotundata*⁻¹), and cellular carbon : Chl a at the different irradiance levels (PAR, μmol photons m⁻² s⁻¹) for each treatment. The average (± SE) of each factor in each treatment is given.

| Treatment | Replicate | PAR | I   | Chl a | μ    | Carbon | C : Chl |
|-----------|-----------|-----|-----|-------|------|--------|--------|
| L         | A         | 44  | 5.35| 1.81  | 0.20 | 55.9   | 30.9   |
|           | B         | 28.9| 8.32| 2.18  | −0.24| 84.1   | 38.6   |
|           | C         | 43.8| 6.77| 1.49  | 0.24 | 53.9   | 36.2   |
| Average   |           | 38.9±6.12| 6.81±0.62| 1.83±0.24| 0.07±0.19| 64.6±11.9| 35.2±2.8|
| L1        | A         | 37.3| 4.72| 1.68  | 0.21 | 53.6   | 31.9   |
|           | B         | 34.3| 5.14| n/a   | 0.05 | 54.3   | n/a    |
|           | C         | 10.8| 10.57| 2.28  | 0.04 | 66.2   | 29.0   |
| Average   |           | 27.47±10.26| 6.81±1.97| 1.98±0.42| 0.10±0.07| 58.0±5.0| 30.5±1.4|
| L2        | A         | 10.8| 9.79| 2.27  | 0.14 | 58.3   | 25.7   |
|           | B         | 21.1| 8.68| 3.1   | −0.14| 76.7   | 24.7   |
|           | C         | 13.3| 8.01| 3.1   | 0.22 | 64.1   | 20.7   |
| Average   |           | 15.07±3.80| 8.22±0.52| 2.82±0.34| 0.07±0.13| 66.3±6.7| 23.7±1.9|
| L4        | A         | 6.3 | 5.55| 2.09  | 0.02 | 59.0   | 28.2   |
|           | B         | 4.8 | 12.13| 2.43  | 0    | 76.1   | 31.3   |
|           | C         | 1.2 | 4.93| 3.17  | −0.27| 100.2  | 31.6   |
| Average   |           | 4.11±4.11| 7.54±2.58| 2.56±0.55| −0.08±0.11| 78.4±14.6| 30.4±1.3|
| D         | A         | 0   | 13.93| 2.92  | −0.28| 72.5   | 24.8   |
|           | B         | 0   | 12.25| 1.67  | 0.07 | 40.3   | 24.1   |
|           | C         | 0   | 15.40| 2.92  | −0.31| 85.7   | 29.4   |
| Average   |           | 0±0.00| 13.86±1.11| 2.50±0.51| −0.17±0.15| 66.2±16.5| 26.1±2.0|
magnification on a Sedgewick rafter slide (Sherr and Sherr 1993). A minimum of 300 cells were counted per sample, resulting in \(< 11.5\%\) counting error (Lund et al. 1958).

\[ H. \text{rotundata}'s \text{ ingestion rate (bacteria } H. \text{rotundata}^{-1} \text{ hr}^{-1}) \]

was calculated at 24–48 h using the Frost (1972) equations. Recently, the equations by Frost (1972) have been used by Kang et al. (2011) and Lee et al. (2014) to measure the ingestion and prey removal rates of various mixotrophic dinoflagellate species. We calculated the geometric mean \( H. \text{rotundata} \) concentration in each bottle throughout the run of the experiment (Båmstedt et al. 2000) to estimate per capita ingestion rate with the following equation,

\[ n = \frac{(n_t - n_0)}{\ln(n_t/n_0)} \]

where \( n \) is the mean \( H. \text{rotundata} \) concentration throughout the experimental run and \( n_0 \) and \( n_t \) are the initial and final concentrations of \( H. \text{rotundata} \). Calculated average concentrations of \( H. \text{rotundata} \) were used to estimate the amount of bacteria consumed per \( H. \text{rotundata} \) in laboratory experiments.

To measure cellular concentration of chlorophyll \( a \) (Chl \( a \)) and carbon in \( H. \text{rotundata} \), 15–30 mL of water was filtered onto 25 mm GF/F (glassfiber filters) at 0 h and 48 h. Filters used to estimate Chl \( a \) were extracted in 90\% acetone for 24 h in the freezer (Arar and Collins 1997). The chlorophyll fluorescence of the acetone extract was measured with a calibrated Turner Designs AU-10 fluorometer. Carbon samples were filtered onto pre-combusted GF/Filters and stored in the freezer. The filters were then dried at 60\°C and analyzed on an Exeter Analytical CE-440 Elemental Analyzer at Horn Point Laboratory Analytical Services.

Linear regressions were used to analyze the data from this experiment to look for relationships between irradiance levels, \( H. \text{rotundata} \) bacterial ingestion rates, division rates, cellular carbon concentration, cellular Chl \( a \) concentration, and carbon : Chl \( a \).

### Winter field experiments

We estimated in situ ingestion rates of \( H. \text{rotundata} \) on bacteria at natural nutrient concentrations and irradiance levels throughout winter using water collected at the field sampling site. We collected water at 08:00 20 times from 27
January 2016 to 18 March 2016 from a fishing pier on the Choptank River in Cambridge, MD USA (38°8′34″0″N 76°4′6″W) (Fig. 1). At this location *H. rotundata* is known to dominate the winter phytoplankton community and form large winter blooms (Millette et al. 2015). On each sampling date we collected 10 L of surface water with a bucket, and immediately filtered it through 20 μm mesh to remove larger plankton. Temperature and salinity were measured with a hand-held YSI-30 immediately after the bucket was retrieved. Samples for nutrients (dissolved nitrate $\text{NO}_3^-$ nitrite $\text{NO}_2^-$, ammonium, and ortho-phosphate) were filtered through a Whatman’s 0.45 μm nylon sieve with a glass microfiber filter and frozen until analyzed. Nutrient samples were analyzed at Horn Point Laboratory Analytical Services with a Technicon AutoAnalyzer II. PAR data were recorded with a hand-held 4τ LI-COR light sensor and day length was downloaded from the website http://www.sunrisesunset.com/usa/Maryland.asp. Water for ingestion experiments was transported to the UMCES Horn Point Laboratory in Cambridge, Maryland.

We used fluorescent microspheres to estimate *H. rotundata* ingestion rates. Three 250 mL Nalgene polycarbonate bottles were filled with 100 mL of 20 μm filtered Choptank water. Fluorescent microspheres (0.5 μm diameter, Fluoresbrite® YG Carboxylate Microspheres from Polysciences) were added to each bottle at a concentration of $2.5 \times 10^5$ mL$^{-1}$, equivalent to approximately 25% of the mean natural bacterial abundance (unpublished data), and bottles were gently rotated to mix them. At the start of experiments 12–15 mL of water was collected from each bottle and preserved with acid Lugol’s solution to estimate phytoplankton abundance, and 1 mL of water was collected and preserved with 4% buffered PFA to estimate bacterial and microsphere abundance. These samples were analyzed as described for the laboratory experiments.

The bottles were placed in black mesh bags that allowed 55% of natural light exposure and were then incubated in floats in a small, protected cove of the Choptank River. Based on preliminary experiments (data not shown), the *H. rotundata* culture linearly took microspheres for 60 min, so we incubated the bottles for each experiment for 30 min, typically starting experiments between 08:30 and 09:00. After 30 min, 50 mL of water were fixed using the Lugol’s/formaldehyde/Na$_2$S$_2$O$_3$ method to prevent regurgitation of microspheres (Sherr and Sherr 1993). Samples were filtered onto 2 μm membrane polycarbonate filters (Poretics Corp.) and mounted with immersion oil and cover slips on glass slides. To eliminate loss of microsphere fluorescence, specimens were frozen until counted. A Zeiss epifluorescence microscope at 1000X using a 43 HE Red Fluorescent filter was used to count the microspheres within a *H. rotundata* cell. When a cell was located, the number of microspheres cell$^{-1}$ was counted.

### Table 2. Temperature (°C), salinity, NH$_4^+$ (μM-N), NO$_3^+$ + NO$_2^-$ (μM-N), PO$_4^{3-}$ (μM-P), NH$_4^+$ + NO$_3^-$ : PO$_4^{3-}$ (N : P), Irradiance at 0900 h (PAR) (μmol photons s$^{-1}$ m$^{-2}$), and day-length (hour:minute) in the Choptank River each day an experiment was set-up between 1/27/2016 and 3/18/2016. The average (± SE) of each factor is given.

| Date    | Temperature | Salinity | NH$_4^+$ | NO$_3^+$ + NO$_2^-$ | PO$_4^{3-}$ | N : P | PAR (h:m) |
|---------|-------------|----------|----------|---------------------|-------------|-------|-----------|
| 1/27    | 1.1         | 13.3     | 2.8      | 16.7                | 0.14        | 140   | 480       | 10:4     |
| 1/29    | 1.3         | 12.5     | 3.1      | 15.2                | 0.15        | 122   | 520       | 10:8     |
| 2/1     | 2.5         | 12.8     | 2.0      | 17.3                | 0.12        | 160   | 1250      | 10:14    |
| 2/3     | 4           | 11.1     | 4.9      | 32.0                | 0.23        | 160   | 260       | 10:18    |
| 2/5     | 4.5         | 10.6     | 4.5      | 36.6                | 0.16        | 257   | 240       | 10:22    |
| 2/8     | 4.3         | 10.3     | 3.1      | 42.0                | 0.13        | 347   | 440       | 10:29    |
| 2/10    | 4.1         | 11.9     | 2.6      | 20.4                | 0.12        | 192   | 720       | 10:33    |
| 2/12    | 2.1         | 11.6     | 1.7      | 25.6                | 0.12        | 227   | 2000      | 10:38    |
| 2/15    | −0.1        | 10.6     | 2.8      | 36.1                | 0.10        | 389   | 620       | 10:44    |
| 2/17    | 1.6         | 9.5      | 3.4      | 50.3                | 0.08        | 672   | 2000      | 10:49    |
| 2/19    | 1.7         | 8.6      | 3.1      | 66.0                | 0.10        | 691   | 1400      | 10:54    |
| 2/29    | 5.2         | 10.4     | 1.9      | 36.7                | 0.14        | 276   | 425       | 11:18    |
| 3/2     | 6.5         | 10.9     | 1.9      | 28.7                | 0.13        | 235   | 1500      | 11:23    |
| 3/4     | 5.9         | 7.9      | 1.8      | 57.5                | 0.11        | 539   | 630       | 11:28    |
| 3/7     | 5.7         | 9.5      | 1.6      | 41.0                | 0.11        | 387   | 2150      | 11:35    |
| 3/9     | 7.3         | 9.3      | 1.5      | 42.7                | 0.09        | 492   | 2200      | 11:40    |
| 3/11    | 9.1         | 10.4     | 1.7      | 31.9                | 0.10        | 336   | 270       | 11:45    |
| 3/14    | 9.8         | 9.6      | 2.1      | 37.7                | 0.09        | 442   | 225       | 11:53    |
| 3/16    | 10.3        | 9.3      | 2.3      | 39.5                | 0.11        | 380   | 1750      | 11:58    |
| 3/18    | 11.2        | 8.8      | 1.5      | 38.8                | 0.09        | 447   | 1900      | 12:3     |
| Average | 4.9 ± 0.8   | 10.4 ± 0.3 | 2.5 ± 0.2 | 35.6 ± 3.0 | 0.1 ± 0.01 | 345 ± 38 | 1049 ± 169 | 11:1     |
inside the cell; at least 100 individual cells were examined for each sample to count ingested microspheres.

The ingestion rate of microspheres \((I_m)\) was calculated by dividing the total number of microspheres ingested by the number of \(H.\ rotundata\) counted for each replicate. Using these ingestion rates, we calculated the actual ingestion rate on bacteria \((I_b)\) based on equations from Domaizon et al. (2003). These equations calculate a cell’s clearance rate on microspheres to estimate how much of the available bacteria a cell could ingest at that clearance rate:

\[
I_b = \frac{I_m}{MS} \times B
\]

where MS is the abundance of microspheres mL\(^{-1}\) and \(B\) is the abundance bacteria mL\(^{-1}\). We assumed that \(H.\ rotundata\) had no preference for bacteria or microspheres and their clearance rates on microspheres were the same as on bacteria.

We fitted a logarithmic curve to the response of \(H.\ rotundata\) ingestion rates from both field and laboratory experiments to irradiance.

**Results**

**Laboratory experiments**

In the laboratory, we used five different treatments with three replicates per treatment, but we found that each bottle received slightly different irradiance levels. Some of the replicates in a treatment had irradiance levels that were closer to other treatments (Table 1). As a result, we treated each replicate as an individual data point and did not group data by treatments, and analyzed the data using regression techniques.

The minimum and maximum hourly ingestion rates on bacteria were 4.7 and 15.4 bacteria cell\(^{-1}\) h\(^{-1}\), respectively (Table 1). As irradiance decreased, ingestion rates increased (linear regression, \(df = 11, r^2 = 0.419, p = 0.009\)) (Fig. 2). The \(H.\ rotundata\) minimum and maximum population division rates were \(-0.31\) d\(^{-1}\) and 0.24 d\(^{-1}\), respectively (Table 1). \(H.\ rotundata\) division rates were positively correlated to irradiance (linear regression, \(df = 11, r^2 = 0.292, p = 0.04\)) but not correlated to ingestion rates (linear regression, \(df = 11, r^2 = 0.177, p = 0.12\)).

The mean (± SE) initial cellular content of cultured \(H.\ rotundata\) raised at 85 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) was 1.28 (± 0.12) pg Chl \(a\) cell\(^{-1}\) and 84.77 (± 1.18) pg carbon cell\(^{-1}\), with a carbon : Chl \(a\) (C : Chl \(a\)) ratio of 67.07 (± 5.44) (Fig. 3). \(H.\ rotundata\) cellular Chl \(a\) concentration was negatively correlated with irradiance levels (linear regression, \(df = 12, r^2 = 0.576, p = 0.004\)) (Fig. 3). C : Chl ratio was positively correlated to irradiance levels (linear regression, \(df = 12, r^2 = 0.810, p < 0.001\)) (Fig. 3). \(H.\ rotundata\) cellular carbon
concentration was not related to irradiance levels (linear regression, df = 12, \( r^2 = 0.046, p = 0.39 \)) (Fig. 3).

**Winter field experiments**

During the winter in the Choptank River between 1/27/2016 and 3/18/2016 the mean (± SE) temperature and salinity was 4.9 (± 0.8) °C and 10.4 (± 0.3), respectively (Table 2). Average ammonium concentration was 2.5 (± 0.2) \( \mu \)M-N, average NO\(_x\) concentration was 35.6 (± 3.0) \( \mu \)M-N, average phosphate concentration was 0.1 (± 0.01) \( \mu \)M-P, and average N : P ratio of NH\(_4\)+, NO\(_x\), PO\(_4\)\(^{3-}\) was 345 (± 38) (Table 2). The average *H. rotundata* abundance was 3924 (± 865) cells mL\(^{-1}\) and range from 297 cells mL\(^{-1}\) to 11475 cells mL\(^{-1}\).

The average (± SE) calculated *H. rotundata* ingestion rate was 4.1 (± 0.6) bacteria h\(^{-1}\) and ranged from 1.17 to 12.34 bacteria h\(^{-1}\). We fitted a logarithmic equation (MATLAB Statistics Toolbox) to the field and laboratory *H. rotundata* ingestion rates at different irradiance levels (Fig. 4, \( r^2 = 0.64 \)):

\[
I_b = -1.37 \log_e (P+1) + 12.1
\]

where \( I_b \) is *H. rotundata* bacterial ingestion rates and \( P \) is irradiance. In the laboratory experiments, we measured the level of irradiance each treatment received but in the field we measured the amount of available irradiance reaching the water surface (Table 2). Experimental bottles were incubated in mesh bags that allowed 55% of the light to reach the bottles, so we multiplied our surface irradiance measurements (Table 2) by 0.55.

Above 400 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) changes in irradiance resulted in negligible changes in bacterial ingestion by *H. rotundata* (Fig. 4). Below 400 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) decreases in irradiance levels resulted in significantly higher ingestion rates (Fig. 4). A comparison of field ingestion rates (mean ± SE) above (2.5 ± 0.9 bacteria h\(^{-1}\)) and below (5.3 ± 0.3 bacteria h\(^{-1}\)) 400 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) showed that *H. rotundata* bacterial ingestion was significantly higher at irradiance levels below 400 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) (unequal variance t-test, \( p = 0.006 \)).

Table 3. Linear regression analysis of *H. rotundata* bacterial ingestion rates collected in the Choptank River during the 2016 winter compared to ammonium (\( \mu \)M-N), nitrate + nitrite (\( \mu \)M-N), and phosphate (\( \mu \)M-P) concentrations and N : P. All \( r^2 \) were non-significant.

| Factor   | df | \( r^2 \) | \( p \)-value |
|----------|----|----------|--------------|
| NH\(_4\)+ | 19 | 0.001    | 0.92         |
| NO\(_x\)  | 19 | 0.02     | 0.55         |
| PO\(_4\)\(^{3-}\) | 19 | 0.01     | 0.69         |
| N : P     | 19 | 0.03     | 0.44         |
There was no relationship between bacteria concentration and either *H. rotundata* clearance rate (linear regression, df = 19, $r^2 = 0.08$, $p = 0.19$) or ingestion rate (linear regression, df = 19, $r^2 = 0.09$, $p = 0.22$) (Fig. 5). There was also no relationship between *H. rotundata* ingestion rate and ammonium, NO$_3$, phosphate, and N : P ratio (Table 3).

Between 1/27/2016 and 2/19/2016 the average *H. rotundata* abundance (± SE) was 1021 (± 211) cells mL$^{-1}$ and we estimated they consumed of 4.8 (± 0.5) % of bacteria’s standing stock d$^{-1}$ at $I =$ 55% $I_0$ (Fig. 6). Between 2/29/2016 and 3/18/2016 the average *H. rotundata* abundance (± SE) was 7472 (± 967) cells mL$^{-1}$ and we estimated they consumed 52.9 (± 12.0) % of bacteria’s standing stock d$^{-1}$ at 55% $I_0$ (Fig. 6).

**Discussion**

We ran laboratory experiments and a series of 20 in situ experiments which all suggested that *H. rotundata* uses phagotrophy as a primary means to partially compensate for light limitation. As light levels decreased *H. rotundata*’s ingestion rate on bacteria increased. *H. rotundata* phagotrophic response to decreasing irradiance levels likely gives them an ecological advantage over other phytoplankton in the winter that allows them to survive and sometimes bloom under light limited conditions. A similar use of phagotrophy to overcome light limitation has been demonstrated in other mixotrophs that dominate in low light conditions (Czyżpionka et al. 2011; McKie-Krisberg et al. 2015). There was no relationship between in situ *H. rotundata* ingestion rates and nutrients (nitrate + nitrite, ammonium, and ortho-phosphate) in the field experiments. Average nitrate + nitrite and ammonium concentrations in the Choptank River were high in winter, and it is unlikely that nitrogen limitation was an important factor regulating bacterivory in our field experiments. Based on high N : P driven by low phosphate concentrations, phosphorus limitation was possible, but we saw no evidence that phosphate concentrations effected ingestion rates.

*H. rotundata*’s cellular Chl $a$ concentrations increased as irradiance decreased. Cohen (1985) also found *H. rotundata* responded to low light by increasing Chl $a$ concentrations. Phytoplankton are known to increase their concentrations of Chl $a$ in order to trap more light energy and maintain photosynthesis as light becomes limiting (Perry et al. 1981). This response to a decrease in irradiance suggests that autotrophy is *H. rotundata*’s preferred method to acquire energy.

Our data suggest that below approximately 400 $\mu$mol photons m$^{-2}$ s$^{-1}$ light becomes limiting to *H. rotundata* and that it responds by increasing ingestion of bacteria. There was a weak negative relationship between irradiance levels and growth and no relationship between growth and ingestion rates. Ingestion on bacteria does not appear to support *H. rotundata*’s growth in the absence of phototrophy, but it likely reduces the rate at which growth decreases as irradiance levels decrease. At some critical light level, even with the ingestion of bacteria, it appears that *H. rotundata* cannot maintain positive division rates because more energy is required for cell maintenance than is produced by photosynthesis and derived from phagotrophy. Based on our laboratory experiments, this critical light level for *H. rotundata* appears to be $\sim$ 10 $\mu$mol photons m$^{-2}$ s$^{-1}$. We propose that *H. rotundata* uses phagotrophy to partially compensate for light limitation to improve its winter survival and growth compared to other cold adapted estuarine phytoplankton species. Future research identifying the critical light levels of other winter phytoplankton species and how quickly *H. rotundata* and other winter phytoplankton species recover from exposure to subcritical light levels is necessary to confirm whether or not *H. rotundata* is better adapted to low light compared to other co-occurring species.

Our estimates of *H. rotundata* ingestion rates are similar to rates reported in other studies. The minimum and maximum ingestion rate (± SE) measured in the field was 1.2 (± 0.4) and 12.3 (± 2.9) bacteria h$^{-1}$, respectively. The minimum and maximum ingestion rates measured in the laboratory in this study were 4.7 and 15.4 bacteria h$^{-1}$, respectively. In our laboratory experiment, we assumed that bacterial growth rates ($\mu$, d$^{-1}$) in the experimental treatments were the same as the rates we measured in the control treatment at 24–48 h. It is possible that bacterial growth rates were higher in experimental treatments than we assumed as a result of stimulation of growth by DOM released by *H. rotundata*. If this was the case, then our ingestion rates would be underestimated; however, our indirect measurements of *H. rotundata*’s ingestion rate were similar to direct measurements that we made in the field (Fig. 4) and estimates that other scientists have made in the laboratory (Seong et al. 2006). Seong et al. (2006) estimated that *H. rotundata*’s maximum ingestion rate was 11.2 bacteria h$^{-1}$ at saturating bacteria concentrations, illumination of 30 $\mu$mol photons m$^{-2}$ s$^{-1}$, and 20°C, and they measured ingestion rates of 2.2 (± 0.1) bacteria h$^{-1}$ in Kuem Estuary, South Korea in May at 21.5°C. The *H. rotundata* ingestion rates reported here are similar to those reported in Seong et al. (2006)’s, this is unexpected given the 17.5°C difference in temperature. Seong et al. (2006) did not test for environmental factors controlling the dinoflagellate’s ingestion rate, so different factors may be impacting the *H. rotundata* ingestion rates in South Korea.

Prey concentration has been identified as an important factor in controlling plankton clearance and ingestion rates, with clearance rates decreasing and ingestion rates increasing as prey concentration increases until a saturating concentration is reached (Frost 1972). This relationship with prey has also been shown for mixotrophic protists, including *H. rotundata* (Seong et al. 2006), but we did not see this response with *H. rotundata* and bacteria (Fig. 5). Laboratory
experiments by Seong et al. (2006) were run at a constant irradiance level as bacteria concentrations varied, in our field experiments bacteria concentration and irradiance both varied. The range of bacterial concentrations Seong et al. (2006) tested was $>1.0 \times 10^{6}$ to $21.0 \times 10^{6}$ mL$^{-1}$, while bacterial concentrations in the Choptank River during our experiments ranged from $0.9 \times 10^{6}$ to $2.9 \times 10^{6}$ mL$^{-1}$. Based on Seong and colleague’s results we would have expected to see a linear relationship between *H. rotundata* ingestion rates and bacteria concentration; however, we did not. It is possible that if irradiance levels were constant, we could have seen the functional response of ingestion rate to increased bacteria concentrations. Nonetheless, our findings suggest that irradiance likely has a stronger effect on *H. rotundata* ingestion rates than bacterial concentrations in situ.

The benefits and drawbacks of using fluorescent microspheres to measure ingestion rates have been addressed previously in the literature (McManus and Okubo 1991; Vaqué et al. 1994; Domaizon et al. 2003). Some studies have found discrimination against (Sherr et al. 1987; Sanders et al. 1989) and preference for microspheres (Sanders and Gast 2012) compared to fluorescently labeled bacteria, which would underestimate or overestimate ingestion, respectively. Size selectivity appears to be the most important factor in determining which particles are preferentially grazed by nanoflagellates (Domaizon et al. 2003; Sanders and Gast 2012). We selected 0.5 μm diameter microspheres because they were the easiest to see and count inside cells and rarely stuck to the outside of cells, therefore providing the most accurate counts.

*H. rotundata* can form large blooms (Seong et al. 2006; Balzano et al. 2015; Millette et al. 2015). Now that we have robust estimates of how many bacteria an individual *H. rotundata* cell is capable of ingesting per hour and what controls their ingestion rates, the next step is to understand how *H. rotundata* blooms impact the bacteria community and nutrient cycling in the microbial loop. Our data show that when *H. rotundata* reached elevated (3000 cells mL$^{-1}$) or bloom ($10^{4}$ cells mL$^{-1}$) abundances they were capable of ingesting up to 100% of the daily bacteria standing stock. Future research comparing *H. rotundata* ingestion rates and bacterial division rates is necessary to know if *H. rotundata* blooms are capable of controlling field bacteria populations.

*H. rotundata* is a mixotroph that increases its ingestion rate when light is limiting (below 400 μmol photons m$^{-2}$ s$^{-1}$). This likely allows them to out-compete other phytoplankton species and form winter blooms under the low light conditions. Under bloom abundances, the *H. rotundata* population is estimated to ingest $>50\%$ of the bacterial standing stock; however, the impact on bacterial population dynamics is not known. Future research should address whether blooms of *H. rotundata* are capable of controlling or reducing the in situ bacterial population and how this affects the DOM pool and microbial loop in winter.

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Conflict of Interest
None declared.