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High Grazing Rates on Cryptophyte Algae in Chesapeake Bay

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Cryptophyte algae are globally distributed photosynthetic flagellates found in freshwater, estuarine, and neritic ecosystems. While cryptophytes can be highly abundant and are consumed by a wide variety of protistan predators, few studies have sought to quantify *in situ* grazing rates on their populations. Here we show that autumnal grazing rates on *in situ* communities of cryptophyte algae in Chesapeake Bay are high throughout the system, while growth rates, particularly in the lower bay, were low. Analysis of the genetic diversity of cryptophyte populations within dilution experiments suggests that microzooplankton may be selectively grazing the fastest-growing members of the population, which were generally *Teleaulax* spp. We also demonstrate that potential grazing rates of ciliates and dinoflagellates on fluorescently labeled (FL) *Rhodomonas salina, Storeatula major*, and *Teleaulax amphioxeia* can be high (up to 149 prey predator^{-1} d^{-1}), and that a *Gyrodinium* sp. and *Mesodinium rubrum* could be selective grazers. Potential grazing was highest for heterotrophic dinoflagellates, but due to its abundance, *M. rubrum* also had a high overall impact. This study reveals that cryptophyte algae in Chesapeake Bay can experience extremely high grazing pressure from phagotrophic protists, and that this grazing likely shapes their community diversity.

Keywords: cryptophytes, mixotrophy, grazing, Chesapeake Bay, dinoflagellates, *Mesodinium rubrum*

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INTRODUCTION

Cryptophyte algae are predominantly a photosynthetic lineage of flagellated protists in aquatic ecosystems (Mallin et al., 1991; Gervais, 1997; Marshall et al., 2005), capable of thriving in turbid and low light environments due to their highly efficient green light harvesting phycobiliproteins (Spear-Bernstein and Miller, 1989). Several species have been shown to use dissolved organic carbon (DOC) to supplement their growth requirements (Lewitus et al., 1991; Lewitus and Kana, 1995; Gervais, 1997), while others, particularly in freshwater and polar habitats, ingest bacterial prey (Marshall and Laybourn-Parry, 2002; Yoo et al., 2017). Collectively, these traits allow cryptophytes to thrive in diverse environmental conditions.

Cryptophyte algae are either preferred or optimal prey for numerous protist and mesozooplankton grazers. Most non-constitutive mixotrophic (i.e., acquired phototrophic or kleptoplastidic) dinoflagellates selectively graze on cryptophytes for their plastids, in both marine (Larsen, 1988; Skovgaard, 1998) and freshwater (Fields and Rhodes, 1991; Onuma and Horiguchi, 2016) environments. Many constitutively mixotrophic (i.e., phagotrophic phototrophs) and heterotrophic dinoflagellates have also been shown to selectively graze cryptophyte algae (Li et al., 1999; Jeong et al., 2007; Johnson, 2015). Numerous studies have also demonstrated that

heterotrophic and mixotrophic ciliates are important grazers of cryptophyte algae (Stoecker and Silver, 1990; Jakobsen and Hansen, 1997; Weisse and Kirchhoff, 1997). Several studies have also shown that certain mesozooplankton grazers may also selectively graze cryptophyte algae (Liu et al., 2010; Tõnno et al., 2016).

In Chesapeake Bay the cryptophyte pigment alloxanthin peaks within the southern Bay during autumn, but is present throughout the Bay year-round (Adolf et al., 2006). High concentrations of cryptophyte algae are commonly found in many of the tidal regions of Chesapeake Bay tributaries (Marshall et al., 2005), and are sometimes associated with blooms of the organelle stealing ciliate *Mesodinium rubrum* (Johnson et al., 2013) or the mixotrophic dinoflagellate *Karlodinium veneficum* (Li et al., 2000; Adolf et al., 2008). While cryptophytes are known to be ecologically important in estuarine ecosystems, few studies have directly measured their *in situ* growth rates or grazing pressure on their populations. Here we provide estimates of growth and grazing rates of *in situ* cryptophyte communities, the effects of grazing on community diversity, as well as potential loss rates on fluorescently labeled cryptophyte prey added to natural samples.

METHODS

Study Sites, Sampling, and Sample Processing

All research occurred within the main stem of Chesapeake Bay and three of its tributaries (**Table 1**, **Figure 1**). In Chesapeake Bay and the Potomac River, all sampling was conducted during October 2011 on the R/V Sharp. Otherwise tributary sampling was conducted from small boats and occurred in the Choptank River in April 2012 and in the Pocomoke River during May, June, and October 2001–2002 (**Table 1**). Station sampling within the main stem of Chesapeake Bay and the Potomac River was conducted during a cruise that surveyed much of the Bay system, and is part of a series of long-term monitoring locations visited in previous studies (Johnson et al., 2003).

Water was collected from Chesapeake Bay and Potomac River stations at 1 m depth from R/V Sharp using Niskin bottles attached to a rosette equipped with a CTD probe (Seabird 911 plus, conductivity, temperature and density). Water was kept in carboys within a flow-through incubator until used for "on-deck" experiments. From the Choptank River, water was obtained from 1 m depth, using a small boat and a single Niskin bottle affixed to a CTD probe cage. Surface water was collected from the Pocomoke River using small boats and a bucket. Water collected using small boats was stored in carboys on deck or in bottles in coolers until returned to the lab for experiments.

Dilution Experiments

Dilution experiments were used to measure *in situ* cryptophyte community growth (μ) and mortality (g) rates due to grazing by microzooplankton. Pre-screened (<200 μm) whole seawater (WSW), containing phytoplankton and microzooplankton, was prepared by gently passing water through a 200 μm mesh filter and particle free filtered seawater (FSW) was prepared by filtering water through Pall 0.2 μm vented sterile filter capsule. A three-point dilution method was used to create 100, 20, and 5% WSW, diluted with FSW, and all treatments were measured in triplicate bottles (Landry, 1993). All dilution bottles were incubated on deck at *in situ* surface temperature and at ∼50% surface irradiance. Sampling for enumeration of cryptophytes and assessment of cryptophyte diversity was conducted at time 0 and 24 h. Cryptophyte populations from dilution experiments were counted using a BD Accuri C6 flow cytometer equipped with a 488 nm laser. Cryptophyte cells were differentiated based on their autofluorescence properties using bivariate scatter plots of orange (585/40 nm emission filter) for high phycoerythrin and red fluorescence (>670 nm emission filter) for chlorophyll against side scatter. Cultures of the cryptophytes *Teleaulax amphioxeia* (strain GCEP01), *Storeatula major* (strain SM), and *Rhodomonas salina* (strain Q) were used as standards for

TABLE 1 | Station locations, conditions, and experiments conducted.

System	Date	Station	Latitude	Longitude	Sal (PSU)	Temp (°C)	Experiment
Chesapeake Bay	10/17/11	845	38.749667	−76.433167	6.7	18.8	DIL
Chesapeake Bay	10/17/11	834	38.567333	−76.433500	7.1	19.1	DIL
Chesapeake Bay	10/19/11	724	37.400667	−76.082500	19.8	19.3	DIL
Choptank River	4/24/12	S13	38.682000	−75.970000	3.8	16.4	FLC
Choptank River	4/24/12	S11	38.611000	−75.982333	6.3	16	FLC
Choptank River	Various	HPLD	38.593333	−76.128833	NA	NA	FLC
James River	10/19/11	JR6	37.030000	−76.523500	6.7	18.8	DIL
Potomac River	5/1/11	P5	38.03266	−76.38696	6.5	16	FLC
Potomac River	5/1/11	P34	38.03266	−76.38696	3	17.7	FLC
Potomac River	5/1/11	P8	38.07154	−76.47446	6.5	16.5	FLC
Pocomoke River	Various	Poc1	37.985833*	−75.632833*	5*	NA	FLC
Pocomoke River	Various	Poc2	37.961759*	−75.667818*	10*	NA	FLC

*DIL, dilution experiment; FLC, fluorescently labeled cryptophyte experiment; NA, not available; *Approximate value.*

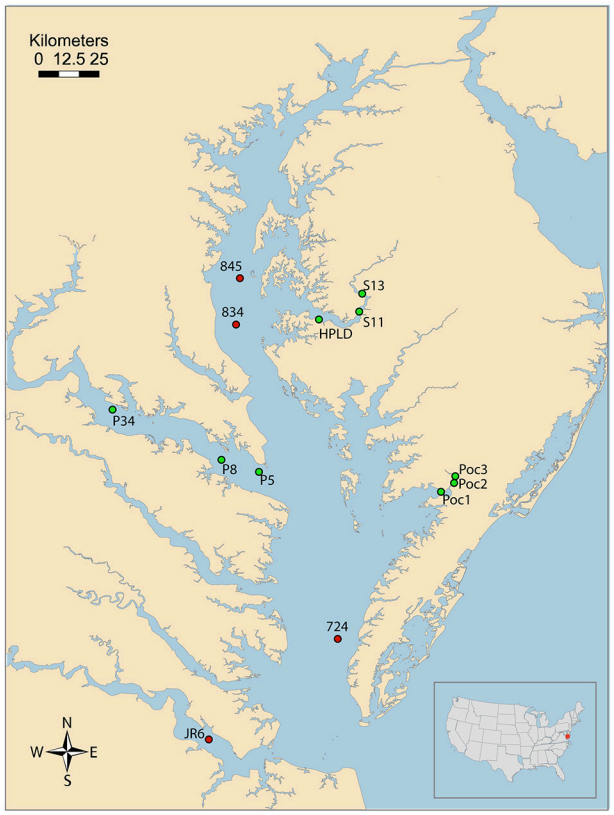

FIGURE 1 | Map of Chesapeake Bay Ecosystem with approximate location of all sampling stations. Stations where dilution experiments were performed are marked with a red circle and experiments where fluorescently labeled cryptophytes were used are marked with a green circle. Map edited from original source: Saxby, T. and Boicourt, K., Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/imagelibrary/).

establishing "ballpark" settings for assessing field populations. However, the acquisition gates used on the flow cytometer did not exactly correspond to these species. The *T. amphioxeia* culture was almost exclusively in gate G3 (97%), while *R. salina* and *S. major* cultures were in gates G1 (19 and 26%) and G2 (81 and 74%).

Genetic Diversity of Cryptophyte Community Within Dilution Experiments

DNA was extracted from filter-collected samples using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's recommendations. Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) gene fragments were PCR amplified from DNA extracts using the cryptophyte plastid-targeting primers L2F (Hoef-Emden, 2005) and crypt_rbcLR2 (Johnson et al., 2016). PCR conditions were: 95°C for 5 min followed by 40 cycles of 95°C for 60 s, 55°C for 60 s, and 72°C for 90 s followed by 72°C for 7 min. PCR products were visualized by agarose gel electrophoresis and later excised and purified from the gels using the Zymoclean Gel DNA Recovery Kit (Zymo Research). Clone libraries were constructed from gel purified fragments using the pGEM-T Easy Vector in the pGEM-T Easy Vector System II cloning kit (Promega Corporation) according to the manufacturer's protocol. A clone library was constructed for each sample and time point used in this study. For each library, ∼45 clones were submitted for Sanger sequencing with a single primer to the W.M. Keck Ecological and Evolutionary Genetics Facility at the MBL as directed. Sequences were edited and assembled into contigs using Sequencher (Gene Codes Corporation). All sequence data were submitted to Genbank (NCBI) under accession numbers MH488130-MH488710.

Grazing on Fluorescently Labeled Cryptophytes

Cultures of the cryptophytes *R. salina*, *Stoeratula major*, and *T. amphioxeia* were grown in F/2-Si media at 15, 10, and 5 PSU salinity, in order to have a range of options for field conditions in Chesapeake Bay. All cultures were grown at 18–20°C and in 14:10 L:D and maintained in log growth phase during field experiments. In order to stain cryptophytes for grazing experiments, a final concentration of 2 μg ml^{-1} proflavine was added to 10–20 ml of cryptophyte culture and cells were allowed to take up the dye for 30 min in darkness. Proflavine is a protein stain that is typically not used for labeling living cells, however, it has been used previously on *Isochrysis galbana* (Dupuy et al., 1999) and marine ciliates (Vincent and Hartmann, 2001) with no short-term mortality. Proflavine was used to stain cryptophyte algae after failed attempts to stain them with Cell-Tracker Green CMFDA (5-chloromethylfluorescein diacetate), a more commonly employed stain (Li et al., 1996). Stained cells were then added to a 15 ml 25 mm diameter glass tower with a 2.0 μm polycarbonate (PC) filter membrane and attached to a side arm flask. Using gentle pressure applied to a hand pump, culture media was slowly removed over 20–30 min, until cells were concentrated down to 2-3 ml. After concentrating the cells, they were washed with F/2-Si media by returning cells to their original volume, and the concentrating and wash step was repeated. When finished, cells were observed live under a dissecting microscope to verify that they were still motile. A small aliquot was then preserved with 1% glutaraldehyde and after 15 min filtered onto a 2 μm PC membrane filter and observed with a Zeiss Axio Scope A1 fluorescence microscope using an excitation BP filter of 450–490 nm in order to verify they were sufficiently stained with proflavine, as well as to count the stock culture. Success of this method depended greatly on how carefully the cells could be concentrated and washed, and the failure rate of the procedure was close to 20%. However, the protocol was considered successful when cryptophyte prey remained motile and all cells were apparently fluorescently stained.

Stained cryptophyte cells were added to Chesapeake Bay or tributary water samples at a final concentration of between ∼1,000 and 10,000 cells ml^{-1}. In most cases these concentrations were substantially higher than *in situ* levels of cryptophytes and likely resulted in estimates of saturated grazing rates. Therefore, these estimates of potential grazing rates are probably akin to

maximum rates (but see discussion). Samples were first screened with a 200 μm mesh in order to remove copepods and other mesozooplankton. Samples (10 ml) were taken and preserved with glutaraldehyde, at time 0, 10, 20, 40, and 120 min, as well as 24 h and stored at 4°C in darkness until filtered (5 ml) onto a PC membrane as described above, and mounted on a glass microscope slide as described previously (Johnson et al., 2013). All species of protist that were observed to have ingested fluorescently labeled cryptophytes (FLC) were counted across all time points and instantaneous ingestion rates (IIR) were determined by taking the slope of ingested FLC cell^{-1} vs. time in hours, and multiply by 24 for prey cells ingested grazer^{-1} day^{-1}. Clearance rates were calculated using the formula $C = IIR/N_{prey}$, where N_{prey} is the concentration of FLC (Rublee and Gallegos, 1989).

Statistical Analysis

Differences in growth and grazing rates across different experiments were determined using a one-way ANOVA, and comparisons between treatments were clarified using a Tukey HSD test. All statistics were calculated using the R statistical function "AOV" (R Core Team, 2013).

RESULTS

In Situ Growth and Grazing Rates of Cryptophyte Algae

During a cruise in October 2011 we ran dilution experiments in Chesapeake Bay, and measured dynamics of cryptophyte populations within the experiments using flow cytometry. At each station 2–3 subpopulations of cryptophytes could be discerned using flow cytometry and analyzing forward scatter and orange fluorescence (i.e., phycoerythrin) as well as orange vs. red fluorescence. Growth and grazing rates on these cryptophyte subpopulations varied greatly both within and between stations. Growth rates of *in situ* populations of cryptophytes were higher in the upper Bay (mean: 0.47 d^{-1}) than in the lower Bay and the mouth of the James River Estuary (mean: 0.13 d^{-1}). Grazing rates on cryptophytes were generally high, exceeding combined population growth rates at all stations (**Table 2**). Only 2 of the 12 subpopulations analyzed in these experiments had positive net population growth, G1 at station 845 and G3 at 834. In the lower Bay and James River station, grazing was 3.2–10.3x (mean: 6.5x) that of growth rates, while in the upper Bay it was 0.3–3.6x (mean: 1.9) greater (**Table 2**).

Except for *M. rubrum*-like ciliates, potential grazers were not counted at every station where dilution experiments were performed. The concentration of *M. rubrum* at stations 845, 834, and 724 were 7.7, 23.1, and 3.8 cells ml^{-1}, respectively. Cell counts for microzooplankton groups that may have been potential grazers of cryptophytes were only made for one lower and one upper Chesapeake Bay station, which was similar to the location of stations 724 and 845/834, respectively. In lower Chesapeake Bay, potential grazers included oligotrich ciliates at 3.1 cells ml^{-1}, tintinnid ciliates at 6.6 cells ml^{-1}, and naked heterotrophic dinoflagellates (NHD) at 1.2 cells ml^{-1}. The upper Chesapeake Bay station had 1.9 tintinnids ml^{-1}, 8.1 oligotrichs ml^{-1}, and 26 NHDs ml^{-1}. Counts of tintinnid and oligotrich ciliates only included cells >30 μm.

Impact of Grazing on Cryptophyte Diversity

The impact of grazing on cryptophyte community diversity was assessed from dilution experiments at 4 stations during a cruise in October, by clone library sequencing of cryptophyte 18S rDNA

TABLE 2 | Population-specific apparent growth and grazing rates for cryptophyte algae during dilution experiments in Chesapeake Bay.

Station	Date	Population	Cells ml^{-1}	Growth (d^{-1})	Grazing (d^{-1})	Net
845	10/17/11	G1	564	0.97 (0.32)	0.30 (0.37)	0.67
		G2	1108	0.46 (0.17)	1.39 (0.20)	−0.94
		G3	1005	0.55 (0.22)	1.42 (0.03)	−0.87
		Total	2779	0.60 (0.01)	1.17 (0.28)	−0.57
834	10/17/11	G1	673	−0.18 (0.41)	0.65 (0.52)	−0.82
		G2	418	0.97 (0.15)	1.06 (0.20)	−0.09
		G3	582	0.48 (0.12)	0.35 (0.08)	0.13
		Total	1755	0.34 (0.16)	0.65 (0.08)	−0.31
724	10/19/11	G2	899	0.14 (0.50)	1.42 (0.99)	−1.28
		G3	1937	0.12 (0.35)	0.73 (0.23)	−0.62
		G4	2626	0.39 (0.35)	1.25 (0.10)	−0.86
		Total	5221	0.25 (0.09)	1.09 (0.21)	−0.84
J6	10/19/11	G1	53	−0.07 (0.38)	0.72 (0.52)	−0.79
		G2	315	−0.39 (0.06)	1.76 (0.36)	−2.15
		G3	289	0.47 (0.43)	2.16 (0.84)	−1.69
		Total	656	0.01 (0.20)	1.85 (0.49)	−1.84

Size of cryptophyte cells decreases with increasing population (G) number.

amplicons. The cryptophyte clone libraries were dominated by *Teleaulax* spp. (72%), followed by *Rhodomonas* spp. (17%) and *Hemiselmis* spp. (11%). A *Teleaulax gracilis* phylotype was one of the largest constituents (34.5% of clones) of the cryptophyte communities measured in this study and increased with dilution at all stations (**Figure 2**). A phylotype of *T. amphioxeia* was also a dominant species within the clone libraries (32% of all clones), but only revealed increases in the 20% whole seawater dilution at 3 of the stations, and not in 5% dilution treatments (**Figure 2**). In the upper and mid Bay stations (845, 834), *Rhodomonas* spp. were a major component of clone libraries, but were nearly absent from lower Bay samples. At both the upper and mid-bay stations, a *Rhodomonas* sp. phylotype also revealed increases with dilution, consistent with net growth following the dilution of grazing pressure. No data are available for cryptophyte diversity for the 5% dilution at station 845 because no samples were taken for DNA.

Grazing on Fluorescently Labeled Cryptophytes

In order to estimate potential grazing rates of protistan predators on specific cryptophyte algae, we tracked the ingestion of fluorescently labeled prey added to natural samples in three Chesapeake tributaries (**Tables 3–5**). The mixotrophic ciliate *M. rubrum* was the most persistent grazer of fluorescently labeled (FL) cryptophytes (**Figure 3**), being present in all samples, and sometimes at high concentrations (**Table 3**). Within the Pocomoke River experiments ($n = 14$), only grazing by *M. rubrum* was enumerated, and only *R. salina* was used as prey. The highest ingestion rates for *M. rubrum* were observed for *T. amphioxeia* prey (5.4–18.3 prey pred^{-1} d^{-1}), with grazing on *S. major* and *R. salina* being lower (0.17–6.53 prey pred^{-1} d^{-1}). Grazing by *M. rubrum* on FL *R. salina* and *S. major* was statistically lower than on *T. amphioxeia* [ANOVA, $F_{(2, 19)} = 7.85$, $p = 0.003$; **Figure 4A**]. Potential grazing impact on cryptophyte populations by *M. rubrum* alone, a function of both ingestion rate and abundance, varied across all experiments from consumption of 0.14–25.8% of the population d^{-1}.

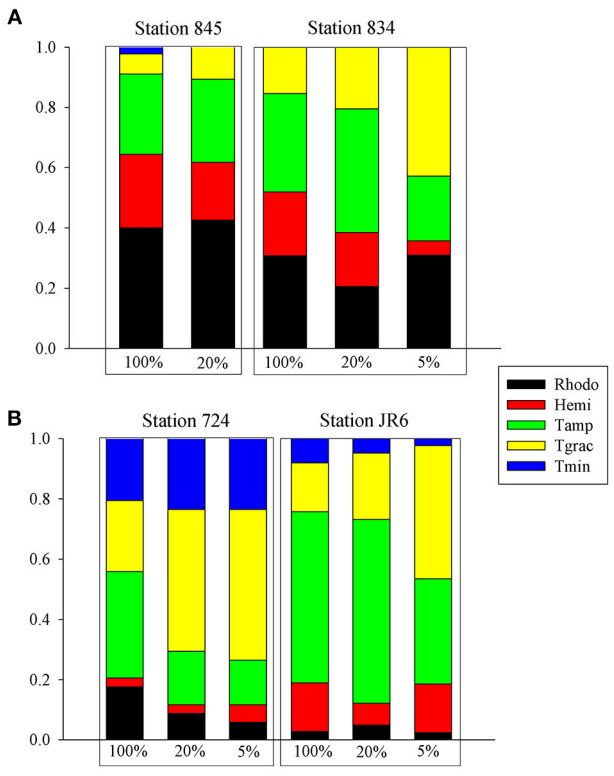

FIGURE 2 | Genetic diversity of cryptophytes in dilution experiments during October 2011 in Chesapeake Bay. Dilution experiment treatment is shown on the x-axis, % clone library composition on the y-axis. **(A)** Results for Stations 845 and 834; **(B)** Results for stations 724 and James River station 6. Rhodo: *Rhodomonas* sp., Hemi: *Hemiselmis* sp., Tamp: *Teleaulax amphioxeia*; Tgrac: *T. gracilis*; Tmin: *T. minuta*.

Grazing on cryptophytes was also documented for various heterotrophic and mixotrophic dinoflagellates. In an experiment within the Potomac River grazing was assessed on all three cryptophyte species, while only *T. amphioxeia* was used in two experiments in the Choptank River. Ingestion rates by dinoflagellates were high, averaging 28 prey pred^{-1} d^{-1} across all dinoflagellate and prey types. Highest grazing rates on cryptophytes were by heterotrophic species (**Table 4**, **Figure 4A**), however, many dinoflagellate species did not ingest cryptophytes during experiments. Generally only small or medium (20–40 μm) naked heterotrophic species and small plastid-containing mixotrophic dinoflagellates were observed to graze cryptophytes. High ingestion rates were measured for both *Prorocentrum cordatum (=P. minimum)* on *T. amphioxeia* prey and for a *Karlodinum veneficum*-like mixotrophic dinoflagellate (referred to hereafter as "*K. veneficum*") on all three species (**Table 4**). Grazing by these mixotrophic dinoflagellates was high in the Choptank River at station S11, which was downriver from a small *P. cordatum* bloom (6,900 cells ml^{-1}) at station S13. No grazing was observed by *P. cordatum* on labeled cryptophytes from within the bloom at S13. For one location we measured grazing on multiple cryptophyte cultures, and therefore could determine if certain prey species are grazed more than others. At station P8 in the Potomac River, no differences were observed for grazing rates on three cryptophyte prey species by the mixotroph "*K. veneficum*" or the heterotrophs *O. marina* and an unidentified naked heterotrophic dinoflagellate, while the heterotrophic *Gyrodinium* sp. had significantly lower grazing on *T. amphioxeia* compared to the other two species [ANOVA, $F_{(2, 3)} = 39.68$, $p = 0.007$; HSD $P < 0.05$; **Figure 4A**]. The overall potential grazing impact on cryptophyte populations by dinoflagellates varied between 0.7 and 15.5% of any one species of labeled cryptophytes d^{-1}.

Since we evaluated relatively small volumes of water for estimating potential ingestion rates, only species that were present at high abundances could be measured. Thus, grazing rates were obtained for only two heterotrophic ciliate species. Both a *Eutintinnus* sp. and an unidentified heterotrophic oligotrich ciliate were found to ingest *T. amphioxeia* at two stations in the Choptank River, and ingestion rates for both ciliate species were high. The heterotrophic oligotrich ciliate had the

TABLE 3 | Grazing rates of *Mesodinium rubrum* (MR)-like ciliates in Chesapeake Bay Tributaries on fluorescently labeled (FL) cryptophyte algae.

Date	Station	FL-Prey	FL-prey Cells ml^{-1}	MR Cells ml^{-1}	FL-prey: MR (ratio)	MR* counted	IR cells MR-$^{-1}$ d^{-1}	C µl MR^{-1} d^{-1}	GP % pop d^{-1}
POCOMOKE RIVER									
5/20/02	Poc2	*R. salina*	1,550	106	14.6	212	3.28 (1.12)	2.12 (0.72)	22.4
5/20/02	Poc1	*R. salina*	1,226	17.9	68.6	71.5	3.24 (1.02)	2.64 (0.84)	4.72
10/10/01	Poc2	*R. salina*	1,192	75.9	15.8	152	0.47 (0.04)	0.39 (0.04)	2.98
10/10/01	Poc1	*R. salina*	1,036	1551	0.67	163	0.17 (0.03)	0.17 (0.03)	25.8
5/28/02	Poc2	*R. salina*	4,223	27.6	153	110	6.53 (0.35)	1.55 (0.08)	4.26
6/26/02	Poc3	*R. salina*	2,128	99.7	21.3	199	0.98 (0.26)	0.46 (0.12)	4.56
6/26/02	Poc2	*R. salina*	2,062	31.6	65.2	63.3	1.93 (0.58)	0.94 (0.28)	2.97
CHOPTANK RIVER									
5/24/02	13	*R. salina*	3,848	14.2	271	56.9	1.55 (1.58)	0.40 (0.41)	0.57
9/7/02	HPLD	*R. salina*	3,059	5.28	579	26.4	0.79 (1.10)	0.26 (0.36)	0.14
9/7/02	HPLP	*R. salina*	2,417	17.2	140	86.1	1.66 (0.56)	0.69 (0.23)	1.18
9/25/02	HPLD	*R. salina*	1,345	16.6	81.2	82.8	0.39 (0.00)	0.29 (0.00)	0.48
9/25/02	HPLP	*R. salina*	958	12.7	75.6	63.3	0.73 (0.41)	0.77 (0.43)	0.97
9/23/02	HPLD	*R. salina*	730	12.8	56.9	64.1	0.64 (0.21)	0.88 (0.28)	1.12
9/23/02	HPLP	*R. salina*	1,085	20.1	52.6	100	0.68 (0.01)	0.64 (0.01)	1.28
4/24/12	S13	*T. amphioxeia*	10,000	29.3	341.3	87.4	5.4 (0.11)	0.54 (0.01)	1.82
4/24/12	S11	*T. amphioxeia*	10,000	15.8	632.9	47.3	4.61 (0.74)	0.46 (0.07)	0.85
4/25/12	S13	*T. amphioxeia*	10,000	39.0	256.4	57.3	18.3 (2.5)	1.83 (0.25)	8.41
POTOMAC RIVER									
5/1/11	P5	*S, major*	10,000	4.3	2325	22.6	BD	BD	BD
5/1/11	P5	*T. amphioxeia*	10,000	–	–	–	BD	BD	BD
5/1/11	P8	*S, major*	10,000	4.0	2484	10.1	BD	BD	BD
5/1/11	P8	*T. amphioxeia*	10,000	–	–	–	BD	BD	BD
5/1/11	P34	*S, major*	10,000	31	328	147	2.79 (0.82)	0.28 (0.08)	0.83
5/1/11	P34	*T. amphioxeia*	10,000	–	–	–	8.5 (0.12)	0.85 (0.01)	2.59

All numbers are means with standard deviations in parentheses; MR, average number of cells counted during experiment across all samples; IR, ingestion rate; C, Clearance rate; GP, Grazing potential; pop, population (labeled cryptophytes); BD, below detection.*

highest grazing rate of any species observed, at 149 ± 20 prey pred^{-1} d^{-1}. Potential impact on *T. amphioxeia* populations for these ciliates varied from 1.3 to 4.2% of the population d^{-1}.

DISCUSSION

While numerous laboratory studies have demonstrated that cryptophyte algae are consumed at high rates by a variety of heterotrophic and mixotrophic protists (Jeong et al., 2005; Lewitus et al., 2006; Adolf et al., 2008), fewer studies have sought to document their grazing losses *in situ*. Cryptophytes are abundant in coastal marine and estuarine ecosystems (Mallin et al., 1991; Jeong et al., 2013; Johnson et al., 2013), and while they may form blooms (Laza-Martínez, 2012; Šupraha et al., 2014), such events are rare and generally short lived. More typically, cryptophytes form multiple seasonal peaks of sustained abundance, but remain in the planktonic community year round (Mallin et al., 1991; Adolf et al., 2006). Here we provide evidence that *in situ* grazing pressure on cryptophyte algae is high in Chesapeake Bay, and that variability among cryptophyte species in growth rates and susceptibility to grazing may help explain their persistence within the plankton.

In Situ Growth and Grazing Rates

Several previous studies using the dilution technique coupled with either flow cytometry (Paterson et al., 2008) or quantitative pigment analyses (Burkill et al., 1987; McManus and Ederington-Cantrell, 1992; Suzuki et al., 1998; Lie and Wong, 2010) have also measured growth and grazing rates on *in situ* cryptophyte populations. The use of quantitative pigment analysis to monitor *in situ* population dynamics can be problematic due to regulatory changes in cellular pigment levels during experiments (e.g., photoacclimation) as well as incorrect assignment of pigments to taxonomic groups. In the case of the carotenoid alloxanthin, one cannot differentiate between cryptophyte algae *per se* and protists that steal their plastids (e.g., *M. rubrum*), which can be abundant in estuarine and coastal ecosystems (Stoecker et al., 2009). Despite these caveats, such studies are valuable for their potential to estimate group-specific *in situ* population dynamics. Previous studies that have traced the concentration of alloxanthin within dilution experiments suggest that cryptophyte algal populations are dynamic, with high growth and grazing rates. In one such study using mesocosm enclosures within Saanich Inlet, Canada, alloxanthin-based estimates of cryptophyte growth were >1.0 (max 1.7) d^{-1} and grazing >0.5 (max 0.9) d^{-1} during the first 4

TABLE 4 | Grazing rates of dinoflagellates (DINO) in Chesapeake Bay Tributaries on the fluorescently labeled (FL) cryptophytes *Storeatula major*, *Rhodomonas salina*, and *Teleaulax amphioxeia*.

Grazer	Prey	DINO (cells/ml)	FL-prey: DINO (ratio)	DINO* counted	IR cells DINO $^{-1}$ d^{-1}	C µl DINO $^{-1}$ d^{-1}	GP % pop d^{-1}
POTOMAC RIVER, 5/1/11, STATION P8							
Gyro	*S. major*	27.8	360	63.4 (13.7)	36.6 (8.37)	3.66 (0.84)	9.66
	R. salina				58.6 (3.19)	5.86 (0.32)	15.5
	T. amphioxeia				9.9 (2.91)	0.99 (0.29)	2.62
Omar	*S. major*	32.5	308	67.1 (19.3)	6.1 (1.83)	0.61 (0.18)	1.95
	R. salina				3.1 (0.65)	0.31 (0.07)	0.98
	T. amphioxeia				2.2 (2.93)	0.22 (0.29)	0.70
Kvene	*S. major*	12.3	813	30.4 (6.2)	23.1 (4.88)	2.31 (0.48)	2.63
	R. salina				15.0 (3.80)	1.50 (0.38)	1.71
	T. amphioxeia				26.1 (5.97)	2.61 (0.60)	2.97
NHD	*S. major*	7.5	1333	15.5 (6.4)	40.8 (7.26)	4.08 (0.73)	3.59
	R. salina				67.3 (13.6)	6.73 (1.36)	5.93
	T. amphioxeia				67.8 (46.6)	6.78 (4.66)	5.97
CHOPTANK RIVER, 4/24/12, STATION S11							
Pcord	*T. amphioxeia*	30.6	327	103 (18.4)	23.7 (0.1)	2.37 (0.01)	7.23
Kvene	*T. amphioxeia*	9.8	1020	29.5 (2.1)	13.8 (1.0)	1.38 (0.10)	1.35

Prey was added at 10,000 cells ml^{-1}; DINO* is the average dinoflagellate concentration during experiment across all samples; IR, ingestion rate; C, clearance rate; GP, grazing potential; pop, population (labeled cryptophytes); Gyro, Gyrodinium sp.; Omar, Oxyrrhis marina; Kvene, Karlodinium veneficum; NHD, naked heterotrophic dinoflagellate; Pcord, Prorocentrum cordatum.

TABLE 5 | Grazing rates of heterotrophic ciliates in the Choptank River on fluorescently labeled (FL) *Teleaulax amphioxeia*.

Date	Site	Ciliate	Ciliate* (cells/ml)	FL-prey: Ciliate (ratio)	Ciliates counted	IR cells ciliate^{-1} d^{-1}	C µl ciliate^{-1} d^{-1}	GP % pop d^{-1}
4/25/12	S13	Unid. tintinnid	5.3	1875	9.28 (3.45)	26.4 (4.1)	2.64 (0.41)	1.34
4/24/12	S11	*Eutintinnus* sp.	5.0	2000	15.0 (3.65)	67.1 (18.0)	6.7(1.8)	3.35
4/24/12	S11	Oligotrich	2.8	3529	9.50 (1.29)	149 (20.3)	14.9 (2.0)	4.17

Prey was added at 10,000 cells ml^{-1}; Ciliate is the average ciliate concentration during experiment across all samples; IR, ingestion rate; C, clearance rate; GP, grazing potential; pop, population (labeled cryptophytes).*

days of the experiment (Suzuki et al., 2002). In contrast, another study found that microzooplankton within the coastal seas of Eastern Hong Kong selectively grazed alloxanthin-containing populations, with grazing rates 1–45 times that of growth rates (Lie and Wong, 2010). In a Western Australia study that also measured growth and grazing rates using flow cytometry with dilution experiments, the percent of cryptophyte production grazed varied seasonally and over 3 sites between ∼30 and 120% (Paterson et al., 2008).

In Chesapeake Bay, one study found high growth (1.22–2.23 d^{-1}) and grazing (1.08–1.22 d^{-1}) rates based on alloxanthin during spring, becoming lower in August (with net population loss), with both rates becoming negligible during fall (McManus and Ederington-Cantrell, 1992). In our study growth rates of cryptophyte populations measured using flow cytometry during October revealed moderate rates in the upper Bay with net population loss due to grazing, and very low growth rates in the Southern Bay and James River that were greatly exceeded by grazing rates. The James River population had the highest concentrations of *M. rubrum*-like ciliates encountered during the October Chesapeake Bay cruise (Supplemental Table 1). Such high differences between measurements of growth and grazing rates of a phytoplankton population is not uncommon in dilution experiments, and suggests a decoupling of these processes. In an estuarine or productive coastal environment grazing responses may be greater at times, due to higher standing stocks of microzooplankton from greater overall ecosystem productivity and biomass (Calbet and Landry, 2004). Previous studies have shown that cryptophyte populations in Chesapeake Bay peak in fall (McManus and Ederington-Cantrell, 1992), and that blooms of diatoms or cryptophytes appear to alternate within the southern regions (Adolf et al., 2006). Our measurements appear to capture a period of demise for rich cryptophyte populations in these regions, with growth rates being low or negative and decoupled from intense grazing pressure.

The Effects of Grazing on Cryptophyte Community Diversity

Relatively little is known regarding the genetic diversity of cryptophyte algae in marine ecosystems. Several studies

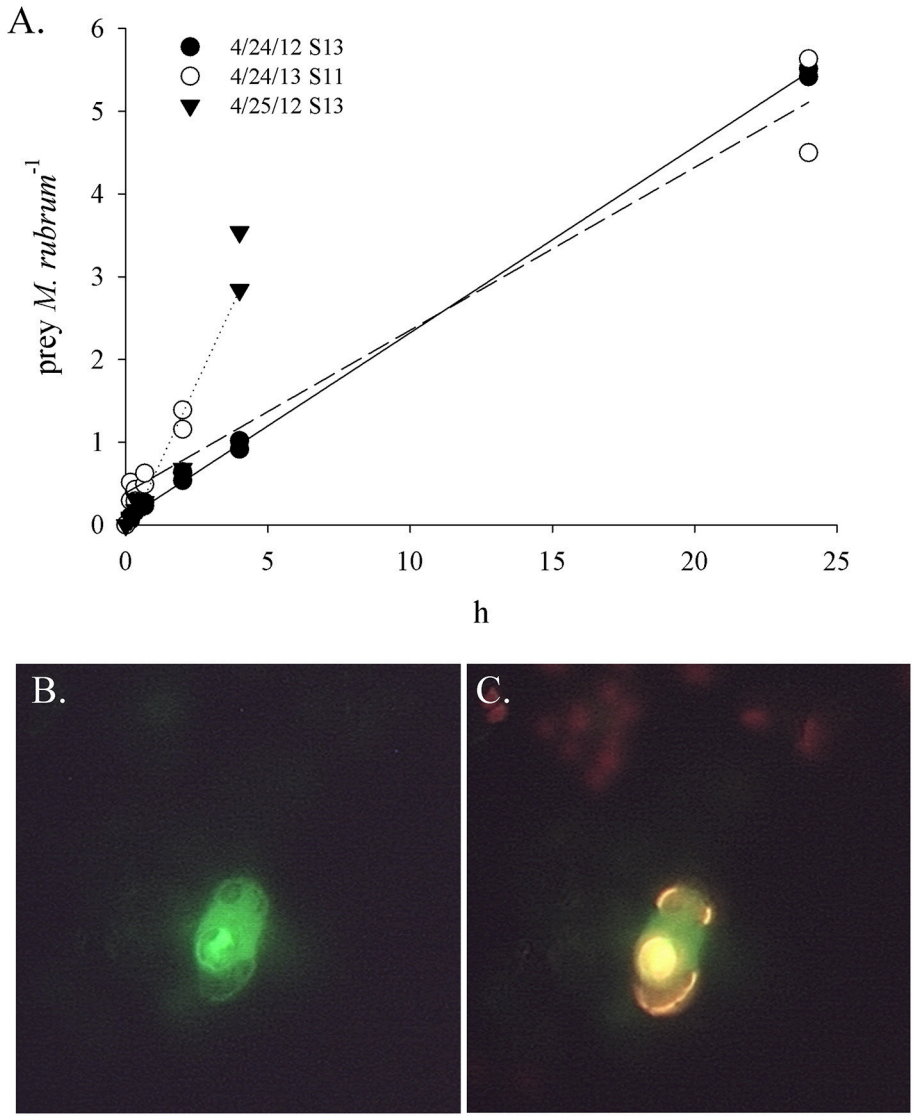

FIGURE 3 | Ingestion of fluorescently labeled cryptophytes (FLC) by *Mesodinium rubrum* in the Choptank River. **(A)** Examples of ingested FL *Teleaulax amphioxeia* over time. Regression equations for each experiment are as follows: 4/24/12 station 13, $y = 0.225x + 0.072$, $R^2 = 0.999$; 4/24/12 station 11, $y = 0.567x + 0.160$, $R^2 = 0.908$; 4/25/12 station 13, $y = 0.748x + 0.156$, $R^2 = 0.888$; **(B)** An *M. rubrum* cell with an ingested FL *Rhodomonas salina* under green emission (BP 515–565 nm); **(C)** Same *M. rubrum* cell as in **(B)** with an ingested FL *R. salina* under a dichromatic long pass emission (>515 nm).

using microscopy or molecular approaches have found that *Teleaulax*/*Plagioselmis*/*Geminigera* (TPG) and *Hemiselmis* cryptophytes are abundant in marine ecosystems (Hill et al., 1992; Cerino and Zingone, 2007; Metfies et al., 2010; Johnson et al., 2016; Luo et al., 2016). However, these studies are somewhat limited in scope due to their regional specificity. While the study of Johnson et al. (2016) included a broad geographic sampling for cryptophyte genetic diversity, many of the samples were from red tides of the ciliate *M. rubrum* and therefore were likely skewed toward *Teleaulax*-dominated communities. While the present study doesn't provide a comprehensive assessment of cryptophyte diversity in Chesapeake Bay, it reveals relative changes in genetic diversity of their communities when grazers are diluted. In this study *Teleaulax* cryptophytes were dominant in the Southern Bay and James River, comprised about 50% of the population in the middle Bay (834), and were between 35 and 40% in the upper Bay (845). During every experiment, a *T. gracilis* phylotype increased in response to dilution, suggesting that grazing pressure on this species is high. However, interpreting the observed dynamics of other taxa in these experiments was more complicated. In experiments at stations 834 and JR6, we observed increases in *T. amphioxeia* only at 20% dilution, but a decline in the proportion of this phylotype within the clone library at 5%. This result suggests that as grazing pressure is diluted, species with higher growth rates are better able to capitalize and grow (e.g., *T. gracilis*). At station

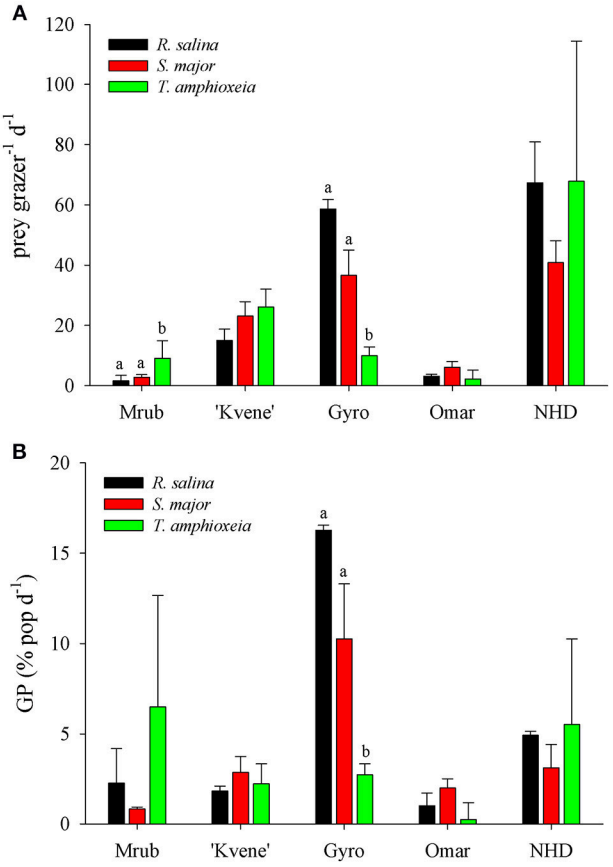

FIGURE 4 | Grazing by phagotrophic protists on three species of cryptophytes. **(A)** Ingestion rates (IR) of *Mesodinium rubrum* (Mrub), a *Karlodinium veneficum*-like dinoflagellate ("Kvene"), a *Gyrodinium* sp. (Gyro), *Oxyrrhis marina* (Omar), and an unidentified naked heterotrophic dinoflagellate (NHD) on *Rhodomonas salina*, *Storeatula major* and *Teleaulax amphioxeia*. **(B)** Grazing potential (GP) on cryptophyte populations. Same information as in **(A)**. Significant differences between IR and GP on different prey indicated by letters.

845 a phylotype of *Rhodomonas* sp. increased with dilution, suggesting that it too had high grazing pressure and *in situ* growth rates. *Rhodomonas* tends to be larger than many other marine cryptophyte species (Johnson, 2015) and station 845 was the only sample where the largest population of cryptophytes measured by flow cytometry had the highest growth rate (**Table 2**). Our observations of changes in cryptophyte diversity in response to protistan predation is not surprising, as selection or preference for certain species has been observed among various mixotrophs. Acquired phototrophic predators such as *M. rubrum* (Park et al., 2007; Hansen et al., 2012; Peltomaa and Johnson, 2017), *M. chamaeleon* (Moeller and Johnson, 2017), and the dinoflagellate *Nusuttodinium aeruginosum* (Onuma and Horiguchi, 2016) have all been shown to select certain cryptophyte species as prey and/or selectively retain plastids from certain species. Selective grazing on cryptophyte species by constitutive mixotrophic protists, i.e., that possess their own plastids (Mitra et al., 2016), is more rare, but has been demonstrated in the dinoflagellate *P. cordatum* (Johnson, 2015). Other mixotrophic dinoflagellates, such as *K. veneficum*, are known to graze on a variety of cryptophytes (Adolf et al., 2008). Less is known regarding selective grazing by heterotrophic protists on cryptophytes.

Grazing on Fluorescently Labeled Cryptophytes

The majority of our grazing experiments using fluorescently labeled prey were conducted using 10,000 cells ml^{-1}, which is at or near saturating levels of cryptophyte prey for most protist grazers. Saturation of ingestion rate varies with predator type and concentration, but in general dinoflagellates have lower saturation levels then ciliates, occurring in the mixotrophic *Prorocentrum donghaiense* around 5,000 cells ml^{-1} (Jeong et al., 2005) and in a small heterotrophic *Gyrodinium* sp. at around 2,000 cells ml^{-1} (Jakobsen and Hansen, 1997) when feeding on cryptophytes. For the ciliate *M. rubrum* saturation of ingestion rate when feeding on *T. amphioxeia* occurred in a Danish strain (variant F) between 3,000 and 7,000 cells ml^{-1} (Smith and Hansen, 2007), while in a Chesapeake Bay strain (Variant G) it occurred between 5,000 and 7,000 cells ml^{-1} (Peltomaa and Johnson, 2017). In contrast ingestion rate of the medium sized mixotrophic oligotrich ciliate, *Strombidium rassoulzadegani*, saturated around 15,000 cells ml^{-1} when fed *Rhodomonas lens* (Schoener and McManus, 2012). While many of our experiments using *R. salina* prey to measure grazing by *M. rubrum* were probably below concentrations (i.e., 730–4,223 cells ml^{-1}) that would have saturated grazing using *T. amphioxeia* as prey, its functional response to *Rhodomonas* spp. has not been determined.

Another important consideration when adding labeled prey to natural assemblages is the concentration of similar species preexisting within the community. Unfortunately we do not have cell count data for cryptophytes within these samples prior to adding labeled prey, but the mean and range concentrations of cryptophytes observed in Chesapeake Bay are 1,432 and 15,720 cells ml^{-1}, respectively (Johnson et al., 2013).

Previous studies have measured high potential grazing rates by mixotrophic dinoflagellates on cryptophyte algae added to natural samples in Chesapeake Bay (Li et al., 1996), and many cultured mixotrophic dinoflagellates have also been shown to have high grazing rates for cryptophytes (Jeong et al., 2005; Adolf et al., 2008). In Chesapeake Bay, both *K. veneficum* and *P. cordatum* have been observed to possess phycoerythrin-containing food vacuoles (Li et al., 1996, 2000, 2001; Stoecker et al., 1997). Predation on cryptophyte populations in Chesapeake Bay is thought to play an important role in bloom initiation of *K. veneficum* due to the stimulation of its growth rate by mixotrophy (Adolf et al., 2008). Mixotrophic grazing by a *P. cordatum* culture isolated from the James River region of Chesapeake Bay was induced under N and P limitation and was almost exclusively limited to ingestion of the cryptophyte *T. amphioxeia* (Johnson, 2015). Consistent with these previous studies, here we found that both "*K. veneficum*" and *P. cordatum* ingested *T. amphioxeia* prey at high rates while "*K. veneficum*" also readily ingested *R. salina* and *S. major*.

In our samples, heterotrophic dinoflagellates were the greatest consumers of labeled cryptophyte prey, due to both their high abundance and ingestion rates (**Figure 4B**). Small (∼20–25 μm) naked heterotrophic dinoflagellates (e.g., *Amphidinium* sp., *Gyrodinium, Oxyrrhis, Pfiesteria*) are known to graze voraciously upon cryptophyte algae (Jakobsen and Hansen, 1997; Strom et al., 1998; Eriksen et al., 2002; Jeong et al., 2010), in addition to other prey. In this study, small naked heterotrophic dinoflagellates, consistent in size and shape with *Gyrodinium* sp., were observed to consume large quantities of *R. salina* and *S. major*, but not *T. amphioxeia*. A microcosm study of natural communities from the Southern Ocean rich in cryptophyte algae also found that cryptophytes are consumed at high rates by heterotrophic *Gyrodinium*-like dinoflagellates (Bjørnsen and Kuparinen, 1991). While we found *O. marina*-like cells consumed fluorescently labeled cryptophytes, their rates were lower than co-occurring mixotrophic and other heterotrophic dinoflagellates. Previous studies on cultures of *O. marina* suggest that it prefers to ingest slightly larger prey than cryptophytes, such as raphidophytes and dinoflagellates (Jeong et al., 2003, 2010).

The highest cell-specific grazing rates that we observed were by large heterotrophic ciliates, including two tintinnid species and an oligotrich. While the grazing rate of the oligotrich ciliate was extremely high, it was comparable to previous rates measured for other large species grazing on cryptophytes (Müller and Schlegel, 1999). In contrast, maximum ingestion rates of *S. rassoulzadegani* on *R. lens* were ∼10 cells ciliate^{-1} d^{-1}, (Schoener and McManus, 2012). While this was an order of magnitude lower than our observed rate for an unidentified oligotrich species (**Table 5**), the species that we observed was larger than *S. rassoulzadegani* (not shown). The mixotrophic oligotrich ciliate *Laboea strobila* is known to ingest and steal plastids from cryptophyte prey (Stoecker et al., 1988), and natural populations of the ciliate have been reported to have predominantly cryptophyte plastids (McManus and Fuhrman, 1986).

Acquired phototrophs, or non-constitutive mixotrophs (NCMs), which steal plastids from algal prey, are one of the main consumers of cryptophyte algae in marine ecosystems (Stoecker et al., 2009). In particular *M. rubrum* and *M. major* are known to selectively graze cryptophytes from the *Teleaulax/Plagioselmis/Geminigera* (TPG) group (Park et al., 2007; Myung et al., 2011; Hansen et al., 2012; Peltomaa and Johnson, 2017). In the Columbian River Estuary, *M. rubrum/major* have been observed to rapidly ingest large quantities of cryptophytes by "gathering" prey cells in their feeding tentacles and possibly their cirri as well as cytoplasmic protrusions from their oral region (Peterson et al., 2013). This novel observation needs to be validated under laboratory conditions in order to determine the mechanism of this unusual feeding behavior. Garcia-Cuetos et al. (2012) observed a "medusa" form of *M. major* that had cytoplasmic projections from its oral regions, and a similar phenotype has been observed in blooms from the Southampton estuary (Crawford, 1993), however feeding events were not observed. We observed the highest grazing rates by *Mesodinium* spp. on *T. amphioxeia*, however, moderate grazing rates were also observed on *R. salina* and *S. major* in several experiments (**Table 3**). While previous studies have demonstrated that *M. rubrum* will ingest non-*Teleaulax/Geminigera/Plagioselmis* (TGP) cryptophytes (Myung et al., 2011; Hansen et al., 2012), these species have not been shown to support sustained growth. One recent study that used an *M. rubrum* isolate from the James River in Chesapeake Bay, found that the ciliate ingested and sequestered plastids from *T. acuta* and *T. amphioxeia* equally, but found no evidence for ingesting *S. major* (Peltomaa and Johnson, 2017). This result contrasts with our observations of *in situ* populations of *M. rubrum* ingesting fluorescently labeled *S. major*. Our average grazing rates measured for *M. rubrum* on *T. amphioxeia* in this study (9.3 prey MR^{-1} d^{-1}) were consistent with rates measured previously for a culture from the James River (10–13 prey MR^{-1} d^{-1}) (Peltomaa and Johnson, 2017). In comparison, the average grazing rates on *R. salina* and *S. major* for *M. rubrum* in Chesapeake Bay were about an order of magnitude lower, both only 1.65 prey MR^{-1} d^{-1}. While *M. rubrum* had lower ingestion rates on non-*Teleaulax* cryptophytes, our experiments demonstrated that it could still have a profound effect on cryptophyte populations (**Table 3**, **Figure 4B**). In two cases, the potential impact on cryptophyte populations was >20% of the population d^{-1}, caused in one case by elevated *M. rubrum* concentrations and ingestion rates, and in another case by low ingestion rates but very high ciliate concentrations (**Table 3**). These data suggest that when *M. rubrum* is abundant, they may clear cryptophyte populations, even if they are not their preferred TGP prey. Causes of variation observed here in grazing rates by *M. rubrum* on fluorescently labeled cryptophytes obviously depend upon the prey species offered, however other factors such as feeding history, the variant(s) of *M. rubrum* present, and the makeup and abundance of *in situ* cryptophyte populations are other possible sources.

CONCLUSIONS

Our findings demonstrate that cryptophyte populations in Chesapeake Bay are heterogeneous in their species composition, cell size, and growth rates, and experience high *in situ* grazing pressure. During October 2011 in Chesapeake Bay, cryptophyte populations had high grazing pressure due to protistan predators throughout the system, but the impact was particularly severe in the southern regions where growth rates were very low. Our experiments using fluorescently labeled cryptophyte prey, which were conducted primarily during spring, demonstrated high species-specific grazing rates for various dinoflagellates and ciliates that sometimes varied with prey species. The combined potential impact of various grazers on fluorescently labeled cryptophytes ranged between 19 and 50% of the population d^{-1}, which was lower than our community loss rates estimated from dilution experiments for cryptophyte populations (44–313% d^{-1}). However, direct comparisons between methods are problematic since the experiments were run in different seasons and locations and conducted over different time scales. Further, not all grazers of fluorescently labeled cryptophytes were enumerated, since many predators were at concentrations that were too low to include. Future experiments

with fluorescently labeled cryptophytes could be improved by processing larger fixed sample volumes for cell counts. In summary, our findings are the first to demonstrate species-specific selection of cryptophyte prey by natural communities of protist grazers. Observed changes in cryptophyte diversity in dilution experiments combined with high ingestion rates on labeled *T. amphioxeia*, suggest that *Teleaulax* cryptophytes generally dominate this ecosystem, possess high growth rates, and are heavily grazed. These results help to explain why certain mixotrophs that select *Teleaulax* as prey, such as *M. rubrum* and *P. cordatum*, are able to maintain high abundance within coastal ecosystems. Further, since heterotrophic protists in our labeled cryptophyte experiments showed either no preference for *T. amphioxeia* or grazed them at lower rates, competition with these mixotrophic predators for cryptophyte prey may be somewhat alleviated, thus facilitating overlapping niches for phagotrophic protists.

AUTHOR CONTRIBUTIONS

MJ designed and performed experiments, analyzed data, and wrote the manuscript. DB and MF helped with experiments, sample analysis, and in preparing the manuscript. EB helped with experiments and in preparing the manuscript. DS helped in designing and performing experiments, analyzing data, and in preparing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00241/full#supplementary-material

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