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The Possession of Coccoliths Fails to Deter Microzooplankton Grazers

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Phytoplankton play a central role in the regulation of global carbon and nutrient cycles, forming the basis of the marine food webs. A group of biogeochemically important phytoplankton, the coccolithophores, produce calcium carbonate scales that have been hypothesized to deter or reduce grazing by microzooplankton. Here, a meta-analysis of mesocosm-based experiments demonstrates that calcification of the cosmopolitan coccolithophore, *Emiliania huxleyi*, fails to deter microzooplankton grazing. The median grazing to growth ratio for *E. huxleyi* (0.56 ± 0.40) was not significantly different among non-calcified nano- or picoeukaryotes (0.71 ± 0.31 and 0.55 ± 0.34, respectively). Additionally, the environmental concentration of *E. huxleyi* did not drive preferential grazing of non-calcified groups. These results strongly suggest that the possession of coccoliths does not provide *E. huxleyi* effective protection from microzooplankton grazing. Such indiscriminate consumption has implications for the dissolution and fate of $CaCO_3$ in the ocean, and the evolution of coccoliths.

Keywords: coccolithophore, phytoplankton, microzooplankton, biomineralisation, predation, evolution

INTRODUCTION

Coccolithophores are small (2–20 μm), unicellular marine algae which form calcium carbonate ($CaCO_3$) scales (coccoliths) that adorn their cells, making them a key player in the global production of $CaCO_3$ and its export from the upper ocean to deep-sea sediments (Berelson et al., 2007; Broecker and Clark, 2009; Balch, 2018). The coccolithophore *Emiliania huxleyi* (Lohmann) Hay and Mohler, forms large-scale blooms that may span over 250,000 km^2 (Holligan et al., 1993) and is considered one of the most widely distributed and globally abundant algae in the contemporary ocean (Winter et al., 1994; Read et al., 2013). Coccolithophores such as *E. huxleyi* have key roles in several biogeochemical cycles, through the ballasting of organic matter fluxes to the deep-sea (Bach et al., 2016), influencing air-sea CO_2 exchange (Shutler et al., 2013), and atmospheric sulfur production (Simó, 2001). Within coccolithophore blooms, *E. huxleyi* can be responsible for >30% of organic carbon fixation (Poulton et al., 2013; Mayers et al., 2018).

Thus, the factors that mediate the population abundance and distribution of *E. huxleyi* are of fundamental importance to marine biogeochemical cycling and global climate.

Despite numerous studies on coccolithophore ecology in the natural environment, the physiological or ecological function(s) for calcification remain poorly understood. Potential ecophysiological advantages for calcification and coccolith formation can be split into end-product benefits (i.e., precipitated coccoliths), as well as those involved in the process of calcification (Müller, 2019). These mechanisms include protection against calcium poisoning (Müller et al., 2015), to mitigate against stressful light and UV radiation (Xu et al., 2016), and also to reduce host susceptibility to viral infection (Johns et al., 2019). Monteiro et al. (2016) additionally suggested that protection from zooplankton predation could be a significant driver of calcification but acknowledged that additional benefits may exist and that there is little or no direct field data to support these hypotheses.

Microzooplankton (20 – 200 μm) are the primary consumers of small plankton, responsible for consuming up to 70% of daily primary production (Calbet and Landry, 2004; Schmoker et al., 2013). Prey selectivity by microzooplankton is mediated by a variety of factors including prey size, morphology, and chemical composition (Tillmann, 2004). Microzooplankton grazing studies on coccolithophores have revealed disparate results, from reduced grazing rates to enhanced grazer preference for calcified *E. huxleyi* cells (Hansen et al., 1996; Kolb and Strom, 2013; Harvey et al., 2015; Strom et al., 2017). Harvey et al. (2015) found that calcified *E. huxleyi* could slow the growth rate of the dinoflagellate predator, leading to a decline in grazing pressure. Calcified *E. huxleyi* cells typically only comprise 5–40% of total plankton biomass in mixed communities (Poulton et al., 2010, 2013, 2014; Mayers et al., 2018), and hence there is the potential for prey selection of non-calcified phytoplankton cells. Evidence for depression of community-level grazing rates does exist from *E. huxleyi* blooms (Fileman et al., 2002; Olson and Strom, 2002), however, studies that directly enumerate *E. huxleyi* cells rather than community-wide indices (e.g., chlorophyll concentration) find no evidence of a depression in grazing (Holligan et al., 1993; Mayers et al., 2018).

In order to clarify the role of calcification as a protective mechanism against grazing in *E. huxleyi*, we conducted a meta-analysis of 62 experiments designed to measure microzooplankton grazing rates, which were primarily performed during mesocosm experiments in Bergen, Norway over a period of 3 years. This meta-analysis was designed to: (1) examine the magnitude of microzooplankton grazing on *E. huxleyi*; (2) compare grazing rates on *E. huxleyi* with losses from other phytoplankton groups in the same community, including similarly sized (2–20 μm) but non-calcified nanoeukaryotes (haptophytes, cryptophytes, and chlorophytes) and small (<2 μm) non-calcified picoeukaryotes and cyanobacteria (*Synechococcus*); and (3) test for any evidence of selective (or non-selective) grazing on *E. huxleyi* in these mixed communities. Our results indicate significant microzooplankton grazing on *E. huxleyi*, with loss rates that are similar to other co-occurring phytoplankton groups, and no evidence of grazing avoidance of *E. huxleyi*. Our results present the first evidence from field populations that coccoliths do not directly protect against microzooplankton grazing, transforming our understanding of the ecological need for cell calcification and the biogeochemical fate of (ingested) coccolithophore $CaCO_3$.

MATERIALS AND METHODS

Mesocosm Set-Up

Mesocosm experiments were conducted at the Espegrend Marine Biological Station in the Raunefjord near Bergen, Norway (60°22.1′N, 5°28.1′E) (**Table 1**). In 2015, 9 floating KOSMOS (Kiel Off-Shore Mesocosms for Ocean Simulations; Riebesell et al., 2013) were set up and monitored for 50 days, 4 with altered CO_2 concentrations (see Dörner et al., 2020 for further details). Experiments to estimate phytoplankton growth and microzooplankton grazing rates were conducted during days 19 to 31 of the experiment. In 2017, 12 bags were filled from the surrounding fjord, with 4 experimental treatments (3 bags each). These treatments were: replete inorganic nutrients (16:1 N:P as nitrate and phosphate added); shaded (nutrients as in the replete bags but with a shaded screen placed on day 10 of the experiment to reduce surface Photosynthetically Active Radiation (PAR) to 20%); low phosphorus (same nitrate as replete bags and minor phosphorus addition to a 75:1 N:P ratio); and ambient, which had no nutrient manipulation. Following nutrient additions to the relevant bags, all 12 bags were bubbled with ambient air for 2 days before air pumps were removed (see Whalen et al., 2019 for further details). In 2018, 4 bags were filled with unfiltered surrounding fjord water from a depth of ∼5 m and circulation was maintained throughout using airlift pumps (Castberg et al., 2001). Inorganic nutrients (as $NaNO_3$ and K_2HPO_4) were added initially at a 16:1 N:P ratio. Additions were then adjusted based on nutrient concentrations with final total nutrient additions being 20.8 μM nitrate and 1 μM phosphate. Conditions for all mesocosm experiments are detailed in **Table 1**. Experiments to estimate phytoplankton growth and microzooplankton grazing rates were conducted every 2 days for 18 days in both 2015 and 2017, whereas in 2018 experiments were conducted every 2 days until an *E. huxleyi* bloom was evident, at which point frequency shifted to every day.

Dilution Experiments

Phytoplankton growth and microzooplankton grazing rates were estimated using the dilution method (Landry and Hassett, 1982; Landry et al., 1995). The dilution technique measures the growth of a prey population at different dilutions with particle free water relative to an undiluted control over 24 h. The technique assumes that the growth rate is unaffected by dilution, but that the encounter rate between predator and prey is related to the proportion of dilution. The technique also assumes that the predator population is grazing non-selectively and does not always measure the grazer population, or account for trophic cascades (Calbet and Saiz, 2013). A slightly modified version of this method was used, with only one low dilution level (20%) and an undiluted treatment (Morison and Menden-Deuer,

TABLE 1 | Description of experimental treatments, number of dilution experiments, mesocosm nutrient additions and the maximum observed abundance of *E. huxleyi* within mesocosm experiments in 2015, 2017, and 2018.

Experiment/Year	Treatments	No. Dilution experiments	Mesocosm nutrients added (total *N* and *P* in μM)	Max. Abundance *E. huxleyi* (cells mL^{-1})
2015	Ambient	6	0/0	140
	High CO_2	6	0/0	77
2017	Ambient	9	0/0	220
	Replete nutrients	9	16/1	530
	Low phosphorus	9	16/0.21	482
	Shaded	9	16/1	455
2018	Replete nutrients	14	20.8/1	109,061

2015, 2017). Rates calculated using this method are considered accurate compared with those using multiple dilution levels and a linear regression. Furthermore, any non-linear responses in grazing rates have been shown to not bias the reliability of the rate estimates (Morison and Menden-Deuer, 2017). All dilution experiments conducted in 2015, 2017, and 2018 had similar experimental set-ups.

During experiments conducted in 2015, water was collected using a depth-integrated water sampler (IWS, HYDRO-BIOS, Kiel). During 2017, water was collected from ∼1 m depth using a 10 L Niskin bottle, and equal volumes of water were pooled from the triplicate mesocosm bags. In 2018, water was collected using a peristaltic pump at ∼1 m depth. Equal volumes were pooled from the four mesocosm bags. During all years, larger mesozooplankton were removed from the collected seawater by screening all water through 200 μm Nitex mesh into clean carboys. The collected water was then shaded with black plastic and returned to shore. Mixing of the dilutions and partitioning into incubation bottles occurred in a temperature-controlled room, set to ambient water temperature (±2°C) which ranged from 11 to 16°C during all years. Grazer-free diluent (FSW) was prepared by gravity-filtering whole seawater (WSW) through a 0.45 μm inline filter (PALL Acropak™ Supor® membrane capsule) into a clean carboy. This filter pore size was utilized to ensure minimal losses of large and small-sized viruses. To the grazer-free diluent, WSW was added at a proportion of 20%. The 20% dilution and 100% WSW treatments were prepared in single carboys and then siphoned into triplicate 1 L Nalgene™ polycarbonate incubation bottles. To control for nutrient limitation, nutrients (10 μM nitrate and 1 μM phosphate) were added to all dilution bottles, aside from a no nutrient 100% WSW control. The 1-L incubation bottles were incubated for 24 h in an outdoor tank maintained at *in situ* surface temperatures through a flow-through system of ambient seawater. Bottles were allowed to freely float, and the seawater inflow caused gentle agitation throughout the 24 h period. A screen was used to mimic light conditions at ∼1 m depth, and this was verified using a HOBO data logger (Onset).

Phytoplankton Enumeration

Samples for phytoplankton enumeration and subsequent biomass estimation via flow cytometry were treated differently between mesocosm experiments conducted during 2015 and those in 2017 and 2018. In 2015, samples were enumerated using a BD Accuri™ flow cytometer with a C6 auto-sampler, and data was analyzed using the BD Accuri™ C6 software. Water samples for each bottle at the end of 24-h incubations (T24), and in triplicate from initial water during setup (T0) were pre-filtered through a 45-μm mesh and run for 6 to 7 min at a low flow rate (35 μL min^{-1}). Events that triggered the forward scatter threshold value of 80,000 were recorded. The sample introduction probe was washed, and samples agitated between each sample run. All events with red autofluoresence (692 nm) above 3,000 (the background level of fluorescent particles present in de-ionized water and sheath fluid) were considered phytoplankton. Nanoeukaryotes (>2 μm) and picoeukaryotes (<2 μm) were determined based on their forward scatter signal relative to 3 μm calibration beads. Events that fell as picoeukaryotes but displayed orange fluorescence (585 nm) were defined as *Synechococcus* spp. (hereafter referenced as *Synechococcus*) and *E. huxleyi* was determined as events that fell into nanoeukaryotes but displayed an elevated side scatter (SSC) due to the presence of $CaCO_3$ coccoliths. Sphero™ rainbow calibration beads (3 μm, 8-peak) were run as daily quality control. The gating strategy of calcified *E. huxleyi* was confirmed using naked and calcified cultures of strains DHB 607 and CCMP 374 during 2017 and of a natural fjord *E. huxleyi* bloom (∼3,000 cells mL^{-1}) in 2018.

In 2017 and 2018, phytoplankton were enumerated on samples fixed in glutaraldehyde (<1% final concentration). In 2017, each bottle at T24, and in triplicate from T0, 5 mL of sample was taken and screened through a 40-μm mesh (to remove larger organisms and particles) into a cryovial and preserved in glutaraldehyde. Then, 250 μL of this preserved sample was added to a 96-well plate and run on a Guava EasyCyte HT (Millipore) flow cytometer. Samples were differentiated into 4 groups; picoeukaryotes, nanoeukaryotes, *E. huxleyi*, and *Synechococcus.* Picoeukaryotes and nanoeukaryotes were separated based on chlorophyll-*a* fluorescence and SSC signatures (to distinguish size and surface characteristics), with *E. huxleyi* being defined as displaying an elevated SSC relative to nanoeukaryotes due to the presence of $CaCO_3$ coccoliths. Finally, *Synechococcus* was identified based on the presence of phycoerythrin which has orange fluorescence. In 2018, 1 mL of sample was taken into a cryovial and preserved in glutaraldehyde (<1% final concentration). The sample was flash-frozen after 30 minutes to 2 hours at 4°C in liquid nitrogen and stored at −80°C until

analysis. Samples were thawed and analyzed on a FACSCalibur (Becton Dickinson) for 1 to 5 min, based on the number of events triggered per second.

Grazing and growth rates were calculated as in Eq. (4) and (5) of Morison and Menden-Deuer (2017). Grazing (g, d^{-1}) rates were calculated as:

$$g = (kd - k1 + N)/(1 - x)$$

where kd is the average growth rate in the diluted (20% WSW) treatment, and x is the fraction of WSW, k1 + N is the growth rate in 100% WSW with added nutrients (i.e., the growth rate in the absence of nutrient-limited effects). Once grazing rates were calculated, the intrinsic growth rate (μ) was calculated as:

$$\mu = g + k1$$

where k1 is the growth rate without nutrients added (the estimated true net growth rate).

Data Processing and Statistical Analysis

Our data set consists of 496 phytoplankton group-specific rate measurements of different phytoplankton groups. Similar to previous studies that compared a compilation of microzooplankton grazing rates (Calbet and Landry, 2004; Schmoker et al., 2013), negative grazing rates were adjusted to 0.00 d^{-1} (n = 54) and negative growth rates to 0.01 d^{-1} (n = 48) to avoid dividing by negative numbers. Grazing to growth rate ratios (g:μ) were calculated as a proxy for the fraction of production (growth) consumed for each phytoplankton group. The g:μ ratios were arctangent transformed, which reduces the impact of large ratios on averages and makes the data more normally distributed (as in Calbet and Landry, 2004). The arctangent medians and median absolute deviations were then converted back to percent consumed using the tangent function. Levene's tests were used to assess for homogeneity of variance. In all tests, the data was found to not have a normal distribution, and therefore further analysis was done using non-parametric tests. To test for differences between growth and grazing rates, and in g:μ ratios between different phytoplankton groups, pairwise two-sided Kolmogorov-Smirnov tests were conducted using R (v. 3.3.2) (R Core Team, 2015). Model II linear regressions using ranged major axis were carried out in R using the lmodel2 package (Legendre, 2018).

Although this paper has experiments conducted under a variety of different environmental parameters and manipulations, by using large dataset comparisons, we can reduce the influence of potential biases due to environmental differences. Cell abundances from flow cytometry counts were converted to biomass using literature values for organic carbon (Tarran et al., 2006): for *Synechococcus* (8.58 fmol C $cell^{-1}$), picoeukaryotes (36.67 fmol C $cell^{-1}$) and nanoeukaryotes (0.76 pmol C $cell^{-1}$). A conversion factor for the organic component of *E. huxleyi* was used (0.68 pmol C $cell^{-1}$) (Harvey et al., 2015), assuming microzooplankton are not able to assimilate the inorganic carbon (calcite) fraction.

To determine whether a particular phytoplankton group was grazed preferentially, we used the Chesson-Manly (CM) selection index (Manly, 1974; Chesson, 1978, 1983). This index has been used in marine planktonic communities to investigate prey selectivity of copepods (Nejstgaard et al., 1997) and microzooplankton (Evans and Wilson, 2008; Löder et al., 2011). The CM selection index was calculated as:

$$\alpha 1 = \frac{ri - pi}{\sum_{i=1}^{n} ri/pi}$$

where ri is the relative abundance of food in the diet (determined as g) and pi is the relative abundance of food in the environment (determined by flow cytometry). The α is a function of the forage ratio (ri/pi). The CM index assumes neutral grazing is 1/n, where n is the number of food groups present (which for this analysis is four from flow cytometry), a value of 0.25 therefore indicates neutral grazing pressure (i.e., grazing is dependent on abundance in the environment). We were unable to calculate a CM index for experiments where all groups displayed grazing rates which were negative or 0, however, this was only observed within three experiments.

To test for significant differences between values of the CM selection index a two-sided Kolmogorov-Smirnov test was calculated as above.

RESULTS

Plankton Growth and Microzooplankton Grazing Rates

Dilution experiments were conducted during mesocosm experiments over three years to determine phytoplankton-group specific growth and microzooplankton grazing rates. By utilizing flow cytometry, we were able to distinguish specific rates for similarly sized phytoplankton groups (picoeukaryotes, nanoeukaryotes, *Synechococcus* spp., and *E. huxleyi*). The *E. huxleyi* group contained only cells with the presence of calcified scales. Non-calcified *E. huxleyi* may have also been present, but they are non-distinguishable from other similarly sized nanoeukaryotes. This experimental design allowed us to determine if certain features, such as size or the presence of coccoliths can affect microzooplankton grazing rates.

The highest median growth rates ($\pm$ median absolute deviation) were observed for picoeukaryotes (0.39 $\pm$ 0.34 d^{-1}) and *E. huxleyi* (0.25 $\pm$ 0.24 d^{-1}), while *Synechococcus* and nanoeukaryotes appeared to grow at lower rates (0.17 $\pm$ 0.16 d^{-1}, 0.14 $\pm$ 0.13 d^{-1}, respectively (**Figure 1**). Growth rates were not statistically different between *E. huxleyi*, picoeukaryotes, and nanoeukaryotes [using a pairwise two-sided Kolmogorov-Smirnov test (KS, **Supplementary Table 1**)]. However, significant differences in growth rates were observed between *E. huxleyi* and *Synechococcus* (KS, p = 0.02) and picoeukaryotes and *Synechococcus* (KS, $p < 0.01$). Grazing rates were highest on nanoeukaryotes and *E. huxleyi* (0.25 $\pm$ 0.25 and 0.26 $\pm$ 0.26 d^{-1}, respectively), closely followed by picoeukaryotes (0.22 $\pm$ 0.13 d^{-1}) (**Figure 1**). The lowest grazing rates were exerted on *Synechococcus* (0.07 $\pm$ 0.07 d^{-1}). Similar to growth rates, no significant differences (KS) in grazing rates were observed between *E. huxleyi*, picoeukaryotes,

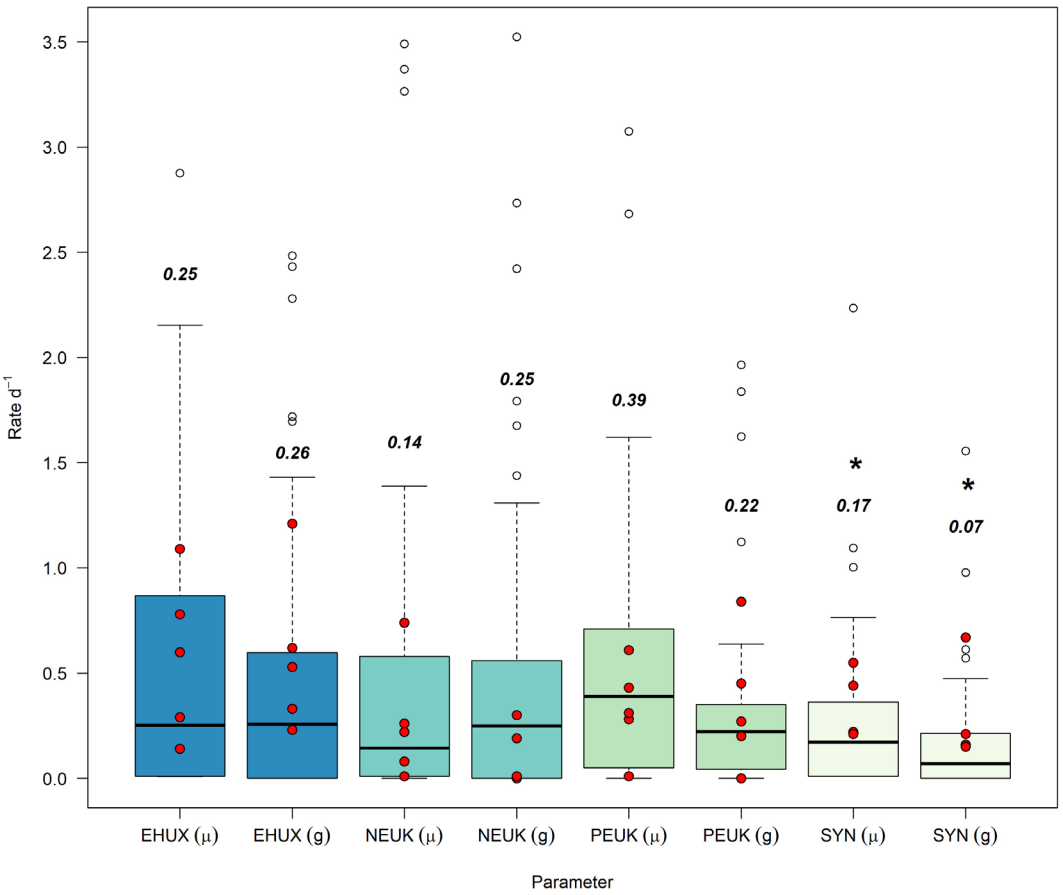

FIGURE 1 | Growth (μ) and grazing (g) rates (d^{-1}) from all mesocosm experiments, separated by analysis groups. Bold text represents the median values, unfilled points are outliers that are still included in data analysis, and asterisks (*) are groups that have rate distributions that are significantly different from *E. huxleyi*. Red overlaid dots are from non-mesocosm field experiments from Mayers et al. (2018) in the Celtic Sea (Northwest European Shelf). EHUX, *E. huxleyi*; NEUK, nanoeukaryotes; PEUK, picoeukaryotes; SYN, *Synechococcus* spp.

and nanoeukaryotes (**Supplementary Table 2**). Grazing on *Synechococcus* was significantly lower than on any other group (KS, $p < 0.01$). These mesocosm-derived growth and grazing rates compared well to similar measurements from a spring cruise in the Celtic Sea (Mayers et al., 2018), with ∼75% of field observations fitting within interquartile ranges (**Figure 1**).

Although growth and grazing rates within the different phytoplankton groups varied widely we found a significant positive relationship between growth and mortality rates for all the phytoplankton groups combined (Model II linear equation, $y = -0.01 + 0.83x$, $p < 0.001$, $n = 248$, $r^2 = 0.59$) (**Supplementary Figure 1**). The same trend was also observed for individual groups (**Supplementary Figure 2**), with nanoeukaryotes and *Synechococcus* spp. showing the most significant relationships (r^2 = 0.71 and 0.72, respectively), and *E. huxleyi* and picoeukaryotes displaying the least (r^2 = 0.55 and 0.46, respectively). For all groups *p*-values were <0.01.

Consumption of Plankton Production

Using the ratio of grazing (*g*) to growth (μ) allows us to look at the proportion of daily growth (and production) consumed by microzooplankton. The median *g*:μ was highest for nanoeukaryotes (0.71 ± 0.31), indicating the central tendency of the dataset is that ∼70% of daily nanoeukaryote production could be consumed by microzooplankton grazers (**Figure 2**). The median ratio was slightly lower for *E. huxleyi* (0.56 ± 0.40) and picoeukaryotes (0.55 ± 0.34), while *Synechococcus* (0.35 ± 0.33) had the lowest median of the group. There were no significant differences (KS) in the distributions of *g*:μ between *E. huxleyi*, nanoeukaryotes, and picoeukaryotes, though the distribution of *g*:μ for *Synechoccocus* was significantly different ($p < 0.05$) from all the other groups (**Table 2**).

Selective or Non-selective Grazing

In order to understand how *E. huxleyi* abundance may influence grazing dynamics, the proportion of *E. huxleyi* in the population, relative to the total number of nano- and pico-sized cells was compared to the proportion of *E. huxleyi* that was consumed by microzooplankton. Deviations from the 1:1 would indicate either grazing selectivity (higher proportion consumed), or grazing avoidance (smaller proportion consumed). The majority of data points fit close to the 1:1 line (**Figure 3A**),

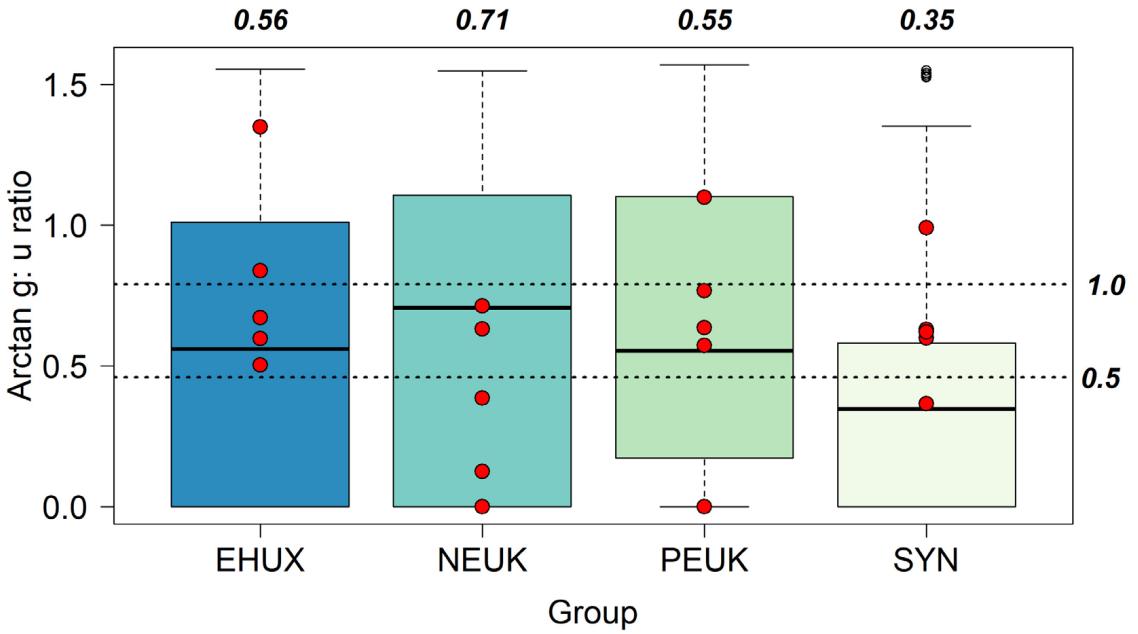

FIGURE 2 | Box plots of arctangent grazing: growth ratios (g:μ) of different phytoplankton groups. Unfilled points represent outliers of the data set but are still included in data analysis. Dashed lines labeled as 1.0 and 0.5 represent 100 and 50% of daily growth consumed by grazers, respectively, of tangent (×) values. Bold italicized values are the median values converted back from arctangent values using the tangent (×) function. Red points are from non-mesocosm field experiments from Mayers et al. (2018) in the Celtic Sea (Northwest European Shelf).

TABLE 2 | *p*-values from a two-sided Kolmogorov-Smirnov test of *g:*μ ratios between different phytoplankton groups.

	E. huxleyi	**Nanoeukaryote**	**Picoeukaryote**	***Synechococcus* spp.**
E. huxleyi	-	0.68	0.28	**0.02**
Nanoeukaryote		-	0.53	**< 0.01**
Picoeukaryote			-	**0.03**
Synechococcus spp.				-

Bold values indicate significant differences (p < 0.05).

indicating that the consumption of *E. huxleyi* is directly related to its proportion within the environment. We observe a low (r^2 = 0.14) but significant (p < 0.01) positive linear relationship between these two variables. Conversely, between the proportion of *E. huxleyi* and the proportion of nanoeukaryotes in microzooplankton diets exhibit a low (r^2 = 0.24) but significant (p < 0.01) negative relationship, which suggests that when *E. huxleyi* dominates the community they comprise the majority of the consumption in this size group. A similar analysis for picoeukaryote and *Synechococcus* spp. composition of microzooplankton diets determined much weaker relationships (r^2 = 0.03 and 0.09, respectively) with the proportion of *E. huxleyi*. For picoeukaryotes this was also observed to be non-significant (p = 0.11) (**Supplementary Figure 3**).

Though *E. huxleyi* losses from microzooplankton grazing appear dependent on *E. huxleyi* relative abundance, there appears to be no negative affect on grazing rate when *E. huxleyi* abundance is high (i.e., we observe no reduced digestion). To further examine prey selection, we calculated the CM selection index α (see section "Materials and Methods"), which compares the proportion of food group grazed to the proportion of that food group observed in the environment (community). Values of α range from 0 (complete avoidance) to 1 (complete preference) with a value of 0.25 for our case (based on four potential prey groups) indicative of neutral grazing (i.e., grazing is directly proportional to relative abundance in the environment). Median values of α were highest for nanoeukaryotes (0.26 ± 0.21), similar for *E. huxleyi* and picoeukaryotes (0.24 ± 0.24 and 0.21 ± 0.15, respectively) and lowest for *Synechococcus* spp. (0.08 ± 0.08) (**Figure 4**). Significantly different distributions of α values were only observed between *Synechococcus* and all other phytoplankton groups (p < 0.001; **Table 3**). As median values for α were remarkably close to 0.25 for *E. huxleyi*, nanoeukaryotes, and picoeukaryotes, we can conclude that no preferential grazing occurred, and grazing losses were simply a function of their relative abundance in the environment. Results were not significantly different between mesocosm treatments for the selectivity index, apart from two exceptions. The first, between *E. huxleyi* and picoeukaryotes in ambient conditions, was only

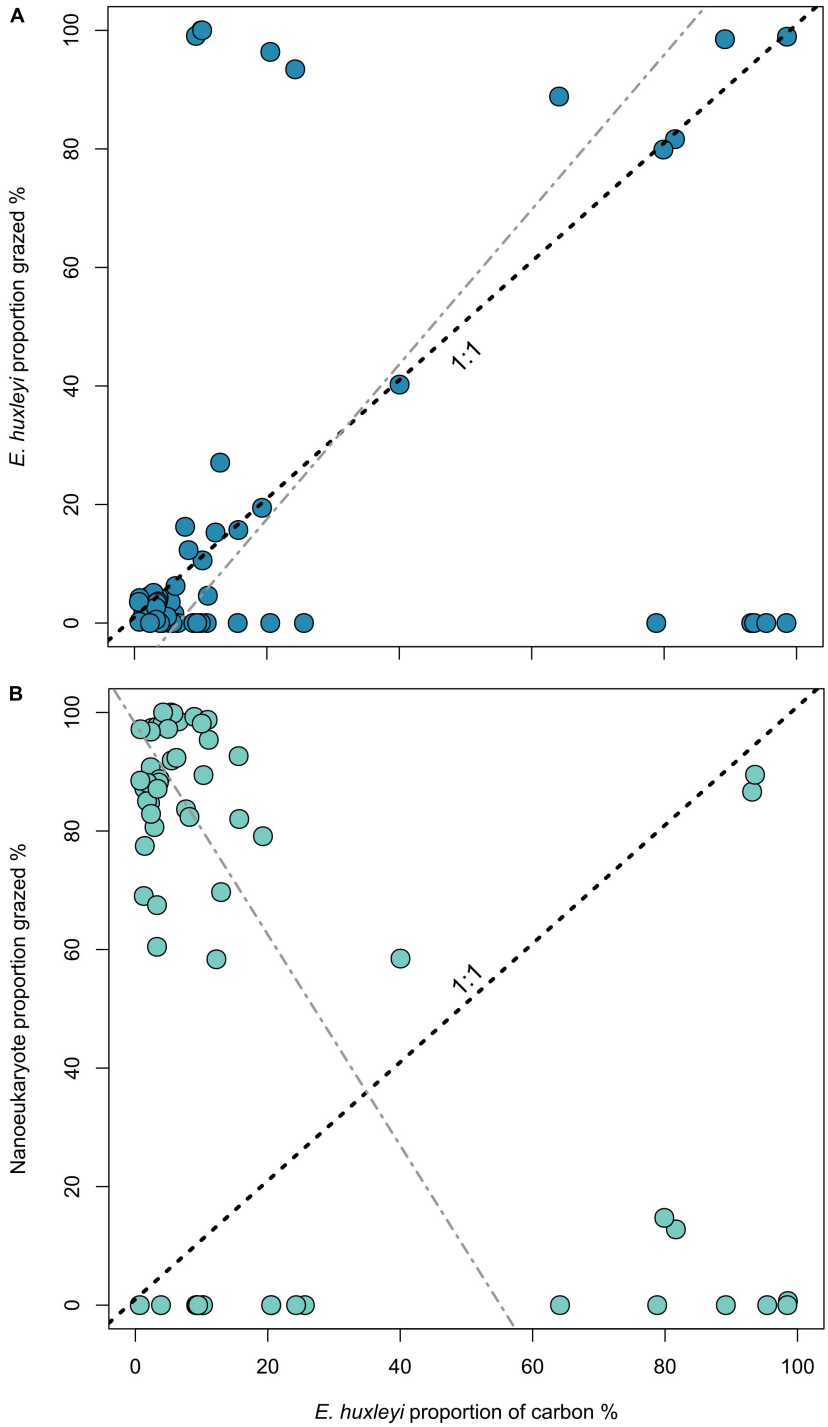

FIGURE 3 | Proportion of *E. huxleyi* within the community (estimated from flow cytometry counts) against the proportion of *E. huxleyi* carbon consumed **(A)** and nanoeukaryote carbon consumed **(B)**. Dashed line indicates the 1:1 line and gray line displays the Model II regression by reduced major axis. For **(A)** line equation is $y = -8.57 + 1.31x$, $r^2 = 0.14$, and for **(B)** $y = 98.09 - 1.78x$, $r^2 = 0.24$, for both $p < 0.01$.

marginally significant ($p = 0.05$). The second, between *E. huxleyi* and nanoeukaryotes within high CO_2 conditions ($p = 0.03$), which was only based on six samples, the lowest sample size in our analysis.

DISCUSSION

Through consumption of phytoplankton, microzooplankton have the potential to drive shifts in plankton population dynamics

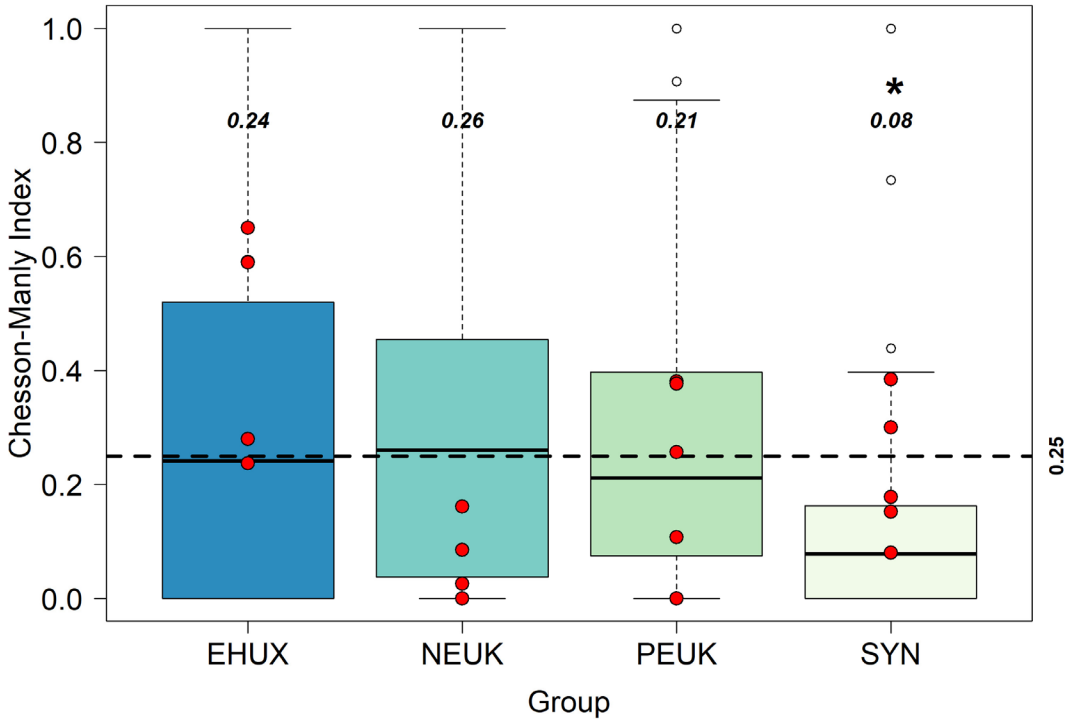

FIGURE 4 | Box plots of the Chesson-Manly index for preferential grazing. The dashed horizontal line is the 0.25 value, which is the line of "neutral grazing preference" (Manly, 1974). Red points are field samples from Mayers et al. (2018). Unfilled data points represent outliers of the data set, values in bold italics are the median values for each phytoplankton group, * symbol indicates significantly different from *E. huxleyi*.

TABLE 3 | *p*-values from a two-sided Kolmogorov-Smirnov test of Chesson-Manly index values between different phytoplankton groups.

	E. huxleyi	**Nanoeukaryote**	**Picoeukaryote**	***Synechococcus* spp.**
E. huxleyi		0.26	0.26	**0.03**
Nanoeukaryote			0.65	**<0.01**
Picoeukaryote				**<0.01**
Synechococcus spp.				

Bold values indicate significant differences ($p < 0.05$).

as well as influence trophic cascades and nutrient cycling (Schmoker et al., 2013; Steinberg and Landry, 2017). The intensity of microzooplankton predation is based on a myriad of factors, including the ability to capture prey and prey palatability (Montagnes et al., 2008). The successful consumption of plankton is often made difficult for microzooplankton by chemical, behavioral, or morphological defenses (Tillmann, 2004). Indeed, for coccolithophores, coccoliths have been previously proposed to be such a morphological defense (Young, 1994; Jaya et al., 2016; Monteiro et al., 2016). However, the data presented here indicate that microzooplankton grazers readily consume *E. huxleyi* no differently than similarly sized, non-calcified pico- or nanoeukaryotes.

Similarities in the fraction of production lost to grazing among the phytoplankton groups examined, suggests that *E. huxleyi* presence does not decrease community-level growth or grazing rates. In fact, the CM index (α) suggests that microzooplankton grazing losses of different phytoplankton groups are a function of their abundance in the environment, with no group-specific selective grazing occurring (apart from *Synechococcus*). Additionally, no trends in growth rate, grazing losses, or prey selectivity based on the *in situ* density of *E. huxleyi* cells was observed, although grazer growth was not monitored in these experiments. This non-selectivity was observed irrespective of experimental condition, as the field-based dilution experiments utilized in the analyses were conducted under differential nutrient, CO_2, and light conditions. In addition, these trends in grazing dynamics are observed despite the inherent variability in the composition and abundance of microzooplankton among the different mesocosm experiments sampled. Overall, these results argue against calcification and coccolith formation in *E. huxleyi* as protective traits against microzooplankton predation.

Strong microzooplankton control of *E. huxleyi* populations in the field, as evidenced by our results, brings into question the role of grazing in the formation of large-scale *E. huxleyi* blooms. Although community level metrics (chlorophyll-*a*) have

estimated a reduction in grazing pressure during *E. huxleyi* blooms (Fileman et al., 2002; Olson and Strom, 2002), by directly enumerating *E. huxleyi* this effect is not observed. Additionally, it is not seen in studies using specific counting methods (Holligan et al., 1993; Mayers et al., 2018), supporting our findings that grazing is dependent on environmental abundance. Phytoplankton prey can employ a suite of predation avoidance mechanisms, such as morphological armament (e.g., coccoliths, silicified frustules and setae) or chemical-based avoidance (Kolb and Strom, 2013) that have been hypothesized to disrupt "normal" predator-prey dynamics and allow bloom formation (Irigoien et al., 2005). The data presented here suggest that coccoliths do not provide protection against ingestion, yet there are many other steps, including digestion and assimilation that are necessary for a "successful" grazing event (Montagnes et al., 2008). For example, Harvey et al. (2015) found a negative impact on microzooplankton growth upon consumption of calcified *E. huxleyi*, which would ultimately result in a decrease in grazing and an increase in *E. huxleyi* population growth. Further, *E. huxleyi* has a high level of genetic plasticity (Read et al., 2013), with variability in cell $CaCO_3$ quotas having bloom-level impacts (Poulton et al., 2013), and strain-specificity in grazing interactions (Harvey et al., 2015; Strom et al., 2017). Therefore, we cannot discount that coccoliths may provide a protective function during grazing interactions outside of ingestion or that grazer selectivity against particular *E. huxleyi* strains or morphotypes (or ecotypes) could occur in the natural environment. Grazing selectivity at other points of the grazing interaction process could result in the suppression of microzooplankton growth, potentially aiding in the success of *E.* huxleyi bloom formation through reduced grazing pressure. However, we find no evidence of this supression in the data considered here or within field samples during a spring bloom in the Celtic Sea (Mayers et al., 2018).

Microzooplankton are not the only mortality factor of photosynthesizing plankton, larger mesozooplankton (e.g., copepods) can also exert strong grazing control on plankton populations, including microzooplankton. Previous experiments and field observations have demonstrated that copepods will ingest *E. huxleyi* cells and defecate coccolith-rich fecal material, though whether this is density-dependent, selective, non-selective, or has any negative influence on copepod physiology is unclear (Sikes and Wilbur, 1982; Harris, 1994; Nejstgaard et al., 1997, 2008). In our experiments, mesozooplankton were deliberately excluded in order to capture grazing pressure only from the microzooplankton. Previous mesocosm experiments with copepod addition have observed increases in photosynthetic nanoflagellate biomass (Zöllner et al., 2009; Pree et al., 2016), which may have included *E. huxleyi* (though they were not enumerated in these studies). This growth implies that mesozooplankton may actually release *E. huxleyi* populations from microzooplankton predation control.

The large *E. huxleyi* virus (EhV) is also an important mortality factor for *E. huxleyi* (e.g., Wilson et al., 2002). Strains with decreased calcification displayed higher rates of infection, even though highly calcified cells may have higher virus absorption coefficients (Johns et al., 2019), providing evidence that coccoliths may act to reduce virus-host interactions. Infected *E. huxleyi* cells in culture have been observed to be grazed preferentially compared with non-infected cells (Evans and Wilson, 2008). Thus, even if coccoliths do not protect against direct predation, overall *E. huxleyi* success is governed by a myriad of interactive mortality factors that either promote or suppress bloom formation.

Given no grazing selectivity against calcified cell ingestion, the fate of $CaCO_3$ produced by *E. huxleyi* and then grazed by microzooplankton is currently unclear. The gut pH of microzooplankton food vacuoles is assumed to be $\sim$ 3 to 5 pH units in order to ensure enzymes associated with digestion work optimally (Nagata and Kirchman, 1992; Gonzalez et al., 1993). When the food vacuole pH is not buffered by high $CaCO_3$ concentrations, microzooplankton grazing may drive a high proportion of $CaCO_3$ production being dissolved within microzooplankton food vacuoles in the surface ocean. Previous studies highlighting relatively rapid (3–18 days) turnover of upper ocean $CaCO_3$ stocks, both regionally (Balch et al., 1992; Poulton et al., 2006, 2007, 2013), and globally (Balch et al., 2005, 2007; Hopkins et al., 2019), as well as regional comparisons of $CaCO_3$ production and export (Thomalla et al., 2008), are all supportive of this hypothesis. However, there is also evidence which suggests that grazer digestion would be influenced by a high influx of $CaCO_3$. White et al. (2018) found that the gut of a copepod becomes increasingly buffered upon ingestion of *E. huxleyi*, limiting dissolution, though, it has not been established if similar dynamics occur for microzooplankton. Further investigation into the biogeochemical ramifications of $CaCO_3$ ingestion and digestion by microzooplankton is necessary to allow better parameterization of models of oceanic carbon fluxes.

The question of why coccolithophores calcify continues to be of ecological and evolutionary importance and interest. Monteiro et al. (2016) suggest that up to 40% of the photosynthetic energy budget in coccolithophore cells is required to support cellular calcification and coccolith extrusion, which is a significant metabolic cost for a unicellular organism for which we cannot easily identify the benefit. Further, it was suggested that the benefits of calcification may vary regionally (Monteiro et al., 2016), complicating the process of identifying the mechanisms driving this process. Yet, despite this complexity, the results presented here demonstrate that microzooplankton grazing losses for *E. huxleyi* are not significantly different from similar-sized, but non-calcified plankton. Hence, calcification and coccolith formation within natural mixed communities does not appear to be a direct adaptive trait to deter microzooplankton ingesting *E. huxleyi*. However, our findings are specific to the bloom-forming coccolithophore *E. huxleyi*. Adaptive benefits of calcification can be species specific (Müller, 2019), and our results may thus not translate to other coccolithophore species. The inability of microzooplankton to discriminate against calcified cells provides a significant piece in the mechanistic framework for understanding coccolithophore bloom formation and persistence, the cycling of $CaCO_3$ in the ocean, and the evolution of coccoliths.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are included in the article/**Supplementary Material** and will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KM, KB, KT, BS, SG, SW, GT, UR, AL, AV, and EH assisted with the set-up of mesocosm experiments and conducting dilution experiments in 2016, 2017, and 2018. KM, AP, DM, and EH were responsible for the design of the manuscript and the data analysis. All authors contributed toward the writing of 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.2020.569896/full#supplementary-material

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