Dynamics of transparent exopolymer particle production and aggregation during viral infection of the coccolithophore, *Emiliania huxleyi*

Jozef I. Nissimov, Rebecca Vandzura, Christopher T. Johns, Frank Natale, Liti Haramaty and Kay D. Bidle*

Department of Marine and Coastal Sciences, Rutgers University, 71 Dudley Road, New Brunswick, NJ, USA.

Summary

*Emiliania huxleyi* produces calcium carbonate (CaCO₃) coccoliths and transparent exopolymer particles (TEP), sticky, acidic carbohydrates that facilitate aggregation. *E. huxleyi*'s extensive oceanic blooms are often terminated by coccolithoviruses (EhVs) with the transport of cellular debris and associated particulate organic carbon (POC) to depth being facilitated by TEP-bound ‘marine snow’ aggregates. The dynamics of TEP production and particle aggregation in response to EhV infection are poorly understood. Using flow cytometry, spectrophotometry and FlowCam visualization of alcian blue (AB)-stained aggregates, we assessed TEP production and the size spectrum of aggregates for *E. huxleyi* possessing different degrees of calcification and cellular CaCO₃:POC mass ratios, when challenged with two EhVs (EhV207 and EhV99B1). FlowCam imaging also qualitatively assessed the relative amount of AB-stainable TEP (i.e., blue:red ratio of each particle). We show significant increases in TEP during early phase EhV207-infection (~24 h) of calcifying strains and a shift towards large aggregates following EhV99B1-infection. We also observed the formation of large aggregates with low blue:red ratios, suggesting that other exopolymer substances contribute towards aggregation. Our findings show the potential for virus infection and the associated response of their hosts to impact carbon flux dynamics and provide incentive to explore these dynamics in natural populations.

Introduction

Phytoplankton are the basis of marine food webs and are responsible for approximately half of global net primary production (Field et al., 1998). As highly abundant infectious entities in the oceans, marine viruses can cause the demise of phytoplankton blooms (Bratbak et al., 1993; Brussaard et al., 1996a,b; 2008; Wilson et al., 2002; Brussaard, 2004; Lehahn et al., 2014) and drive the release of dissolved and particulate organic matter (DOM and POM), which stimulates microbial activity, facilitates bacterial re-mineralization, enhances nutrient recycling and respiration, as well as short-circuits carbon transport to higher trophic levels (Azam et al., 1983; Fuhrman, 1999; Poorvin et al., 2004; Suttle, 2007; Brussaard et al., 2008). At the same time, enhanced production and release of ‘sticky’ colloidal cellular components, such as transparent exopolymer particles (TEP), by phytoplankton viral infection (Vardi et al., 2012) can cause particle aggregation and enhance carbon export into the deep ocean (Mari et al., 2005; Bidle, 2015; Laber et al., 2018).

An important aspect towards elucidating the relative impact of viruses as agents of attenuation or vertical flux is their interplay with TEP production and aggregate formation. TEP, along with other exopolymer substances, is an important constituent of ‘marine snow’, aiding in the aggregation of organic particles and cellular debris from stress-induced death of phytoplankton (Allredge et al., 1998; Passow, 2002; Bar-Zeev et al., 2013; Bidle, 2015; Nissimov and Bidle, 2017; Thornton and Chen, 2017). Many marine microorganisms produce TEP throughout their life cycle and its production is greatly induced following stress. For instance, it was shown that TEP release is induced in *Emiliania huxleyi* (*E. huxleyi*) cells grown under elevated CO₂ conditions (Engel et al., 2004). TEP production in *E. huxleyi* can also be stimulated by nutrient limitation, particularly in stationary growth phase where cells release acidic polysaccharides that can function as precursors of TEP (Kayano and Shiraiwa 2009; Van Oostende et al., 2013). In other phytoplankton species, such as *Coscinodiscus granii*, elevated levels of TEP are
observed during their exponential growth phase (Fukao et al., 2010).

The cosmopolitan, marine coccolithophore *E. huxleyi* has global importance as a bloom-forming calcifying, photoautotrophic species and plays a crucial role in the carbon biogeochemistry of the oceans. The demise of *E. huxleyi* blooms and the cycling of elements such as C, N and P into dissolved and particulate pools are multifaceted, complex processes that involve nutrient limitation, grazing by zooplankton and infection by large double stranded DNA containing viruses (EhVs) (Bratkab et al., 1993; 1998; Castberg et al., 2002). Given the ability of *E. huxleyi* to photosynthetically fix particulate organic carbon (POC) and to calcify CaCO₃, and that TEP formation is an integral step in carbon export carbon from declining photoautotrophic species and plays a crucial role in the carbon biogeochemistry of the oceans. The demise of *E. huxleyi* strains CCMP374, DHB611 and DHB607 differ in cellular calcification and CaCO₃:POC mass ratios, presenting a dynamic range of cellular constituents relevant to carbon flux. Cellular PIC quotas (pg CaCO₃ cell⁻¹) for CCMP374, DHB611 and DHB607 were 0.36 (±0.01), 8.73 (±1.12) and 18.16 (±0.85) pg cell⁻¹, respectively (Fig. 1), representing a 50-fold range in calcification from ‘naked’ (no coccoliths) to heavily-calciﬁed (many coccoliths) morphotypes. Corresponding CaCO₃:POC mass ratios for these strains ranged from 0.04 (±0.01) to 0.48 (±0.01) (Fig. 1), consistent with previous ratios reported for coccolithophores (Blanco-Ameijeiras et al., 2016) and providing different ‘rain’ ratios relevant to the vertical flux of organic carbon and calcite to the deep ocean (Mekik et al., 2007). The two EhV strains (i.e., EhV207 and EhV99B1) used in this study are phylogenetically distinct and representative of genotypes found in the modern ocean, having been isolated from different geographical locations (Nissimov et al.

and its ability to facilitate particle aggregation (Mari et al., 2007), their findings suggest that EhVs can facilitate aggregation and indirectly contribute to ‘marine snow’ formation. However, this has not been explored specifically in a controlled, laboratory setting.

Traditionally, the operationally deﬁned particulate pool of TEP has been studied by ﬁltration on 0.4 μm pore-size membrane ﬁlters, staining with Alcian Blue (AB), a dye that binds to the acidic polysaccharide constituent of TEP, and subsequent microscopic visualization (Passow and Aldredge, 1994) or extraction (in sulfuric acid) and spectrophotometric measurement against a standard of a known concentration (Passow and Aldredge, 1995; Passow, 2002). Recent advances in FlowCam technology allows direct visualization and characterization (size spectrum, shape, association with cells) of AB-stained TEP particles (Cisternas-Nova et al., 2015; Thuy et al., 2015). To elucidate the interplay between EhV infection, TEP production and particle aggregation, we performed laboratory infection experiments using two phylogenetically distinct coccolithoviruses – EhV207 (Exp1) and EhV99B1 (Exp2) – and three *E. huxleyi* strains that varied in their ability to calcify. We measured *E. huxleyi* cell and virus abundances and TEP concentrations throughout these experiments, and visualized particle aggregation. These experiments provide a foundational understanding into the dynamics of TEP production and aggregation in *E. huxleyi* in response to defined, well-characterized virus infection, without complicating factors of nutrient stress and grazing pressure.

**Results and Discussion**

**Features of host and virus strains**

*Emiliania huxleyi* strains CCMP374, DHB611 and DHB607 differ in cellular calcification and CaCO₃:POC mass ratios, presenting a dynamic range of cellular constituents relevant to carbon flux. Cellular PIC quotas (pg CaCO₃ cell⁻¹) for CCMP374, DHB611 and DHB607 were 0.36 (±0.01), 8.73 (±1.12) and 18.16 (±0.85) pg cell⁻¹, respectively (Fig. 1), representing a 50-fold range in calcification from ‘naked’ (no coccoliths) to heavily-calciﬁed (many coccoliths) morphotypes. Corresponding CaCO₃:POC mass ratios for these strains ranged from 0.04 (±0.01) to 0.48 (±0.01) (Fig. 1), consistent with previous ratios reported for coccolithophores (Blanco-Ameijeiras et al., 2016) and providing different ‘rain’ ratios relevant to the vertical flux of organic carbon and calcite to the deep ocean (Mekik et al., 2007). The two EhV strains (i.e., EhV207 and EhV99B1) used in this study are phylogenetically distinct and representative of genotypes found in the modern ocean, having been isolated from different geographical locations (Nissimov et al.

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Growth and infection dynamics of Emiliania huxleyi

Specific growth rates ($\mu$) of uninfected *E. huxleyi* were generally similar among strains over 24–72 h for uninfected treatments in experiments conducted with EhV207 (e.g., Exp1; Supporting Information Fig. S1-A), with maximum growth rates of 0.85 ($\pm$0.29)–1.55 ($\pm$0.12) d$^{-1}$. In contrast, the $\mu$ of uninfected *E. huxleyi* in experiments conducted with EhV99B1 (e.g., Exp2; Supporting Information Fig. S1-C) were similar among strains at 24, 72 and 96 h, with a range between 0.24 ($\pm$0.02) and 0.63 ($\pm$0.13) d$^{-1}$. Although there were differences in the specific growth rates of uninfected controls between the two experiments, the growth rates in both experiments are well within those previously reported for healthy, non-nutrient limited *E. huxleyi* grown at 18°C (Fielding, 2013).

Control cultures likely did not experience nutrient limitation. Previous experiments set up in the exact same manner and for which NO$_3^-$ and PO$_4^{3-}$ concentrations were measured found respective concentrations of 679 and 20.7 μM after 7 days of growth.

In Exp1, prominent cell lysis was observed for EhV207-infected CCMP374 and DHB611 cells at 24–48 h post infection (hpi) (Fig. 2A and B; Supporting Information Fig. S1B). Cell lysis was delayed for EhV207-infected CCMP607 to 72–96 hpi (Fig. 2C; Supporting Information Fig. S1B) and there were on average 18-fold more cells at the end of the experiment at 96 hpi than in infected CCMP374 and DHB611 (Fig. 2A–C). The slower decline of EhV207-infected DHB607 cells was accompanied by a lower virus production (Fig. 2A–C) and virus burst sizes (Supporting Information Fig. S2), indicating that *E. huxleyi* DHB607 was less susceptible to infection by EhV207.

In Exp2, cell lysis was observed for all three EhV99B1-infected *E. huxleyi* strains at 48–72 hpi (Fig. 2D–F), demonstrating different infection dynamics than those observed for EhV207. EhV99B1 had a longer latency period (i.e., the time it took for population decline to occur and to observe new virus particle production) even when infecting *E. huxleyi* strains such as DHB607 and DHB611, which share its geographical origin of isolation. Here, the steadier decline in cell abundances was observed for EhV99B1-infected CCMP374, in which there were on average seven-fold more *E. huxleyi* cells at the end of the experiment at 96 hpi than in EhV99B1-infected DHB607 and DHB611 (Fig. 2D–F). This correlated with a lower mortality rate for infected CCMP374 than for infected DHB607 and DHB611 treatments (Supporting Information Fig. S1D). The slower decline of infected CCMP374 cells did not manifest in a lower production of viral particles (Fig. 2D), evident by statistically indistinguishable average burst sizes for the three *E. huxleyi* strains (Supporting Information Fig. S2). EhV99B1 clearly had a reduced ability to infect and kill CCMP374; viruses were produced, yet cell lysis was hindered.

**TEP production and bacterial abundance**

While the levels of TEP in both EhV207-infected and uninfected treatments (e.g., Exp1) increased 24 hpi for all *E. huxleyi* strains (Fig. 3A–C), significant differences between EhV207-infected and virus-free controls were only seen in DHB611 and DHB607, where infection resulted in a pronounced, early production of TEP prior to the detection of cell lysis (Fig. 3B and C). TEP concentrations were 2- and 3.1-fold higher 24 hpi for infected DHB611 and DHB607 respectively, compared to
uninfected controls. TEP concentration subsequently decreased and was higher in uninfected controls, remaining so until the end of the experiment (Fig. 3B and C). Similarly, TEP was higher in uninfected than in infected CCMP374 treatments 48–96 hpi (Fig. 3A).

TEP dynamics were very different for experiments conducted with EhV99B1. A large increase in TEP production at 24 hpi was not observed for any of the *E. huxleyi* strains (Fig. 3D–F). Instead, TEP gradually increased in both uninfected and EhV99B1-infected treatments throughout the experiment (Fig. 3D–F), and for the most part there were no significant differences in TEP up to 72 hpi. An exception was CCMP374, for which there was a 1.76-fold higher TEP concentration 24 hpi in the uninfected controls compared to infected treatments (Fig. 3D). At 72 hpi, TEP concentration was 1.57, 1.9- and 1.57-fold higher in the uninfected CCMP374, DHB611 and DHB607 treatments, respectively, in relation to the EhV99B1-infected treatments (Fig. 3D–F). At
the end of the experiment (96 hpi), TEP levels were 1.64- and 1.18-fold higher in uninfected CCMP374 and DHB611 respectively than their respective infected treatments (Fig. 3D and E).

Recognizing the importance of bacteria in the interpretation of TEP dynamics, we monitored the bacterial abundance in our closed system. Although stock cultures were rendered nearly axenic through several rounds of antibiotic treatment prior to the infection experiments (Supporting Information Fig. S3), bacterial populations did emerge over the 96 h experimental time course in both experiments, with notable differences in the bacterial abundance (BA) between infected and uninfected treatments (Fig. 3; Supporting Information Fig. S4). Increases in BA are common with virus lysis of phytoplankton cells, fueled by heterotrophic utilization of leaked organic matter from virus-lysed cells (Wilhelm and Suttle, 1999; Suttle, 2005). Bacteria have also been shown themselves to contribute to TEP production, colonize TEP particles, utilize it as a carbon source, and stimulate aggregation (Passow, 2002; Gärdes et al., 2011).

All infected treatments (with both EhV207 and EhV99B1) had higher BAs after 96 h compared to their respective uninfected controls (Fig. 3; Supporting Information Fig. S4). BAs were similar among infected and uninfected treatments up through 24 hpi in Exp1 (Supporting Information Fig. S4A), after which they increased in infected treatments. In Exp1, the highest
BA dynamics. Nonetheless, sources in this closed system likely contributed to the range. We acknowledge that other POC and DOC (divided by total TEP carbon) in our system would be to resident bacteria, calculated BGEs (bacterial carbon divided by Xanthan Gum equivalents) could have supported the observed BA dynamics, given they corresponded with sharp decreases in TEP by 48 hpi in EhV207-infected DHB611 and DHB607 treatments. Using published values of average cellular carbon content of bacteria (i.e., 150 fg carbon cell⁻¹; Fagerbakke et al., 1996; Zimmerman et al., 2014) and bacterial growth efficiency (BGE; i.e., 20%; Bjørnsen, 1986), we calculated the proportion of TEP-associated carbon that could have supported the observed bacterial growth (including associated respiration) after 48 and 96 h; these time periods respectively represented the initial drop in TEP and the cumulative values at the end of the experimental time course. Between 7% and 17% of the BAs and activity could be supported by TEP-associated carbon after 48 hpi (Supporting Information Fig. S5); it could support 84%–115% of BA and activity at the end of the experiment.

We did not empirically measure BGEs in our system. Rather, our carbon budget calculations used an average BGE of 20% for demonstration purposes and likely contributed to carbon use values in excess of 100%. Indeed, there is a wide range of BGEs for bacteria in natural aquatic systems (< 5%–60%; del Giorgio and Cole, 1998) and a general lack of insight into what regulates it. Assuming the removal of TEP carbon was solely due to resident bacteria, calculated BGEs (bacterial carbon divided by total TEP carbon) in our system would be between 3.5% and 23.1%, consistent with the above range. We acknowledge that other POC and DOC sources in this closed system likely contributed to the observed bacteria population dynamics. Nonetheless, these calculations provide some interpretive context for the observed bacterial growth.

BAs were similar among all treatments and E. huxleyi strains between 0 and 48 hpi in Exp2 with EhV99B1 (Supporting Information Fig. S4B). Despite the lower TEP production in infected cells in this experiment (Fig. 3D–F), BAs were still high at 96 hpi (Supporting Information Fig. S4B). The highest BA at 96 hpi was for EhV99B1-infected DHB611, which correlated in this experiment with the highest fold increase in bacteria (5.1 ± 1.64) between infected and control treatments (Fig. 3E). The BA in EhV99B1-infected CCMP374 and DHB607 cultures was similar but still much higher than in their respective uninfected controls (Fig. 3D and F). The uninfected treatments had the lowest BA and were similar across the different E. huxleyi strains (Fig. 3D–F; Supporting Information Fig. S4B).

Notably, the enhanced bacterial production in EhV99B1-infected treatments at 96 hpi were associated with cell lysis and virus production (Fig. 2D–F; Supporting Information Figs. S1 and S2) in the absence of appreciable TEP (Fig. 2D–F). This suggests that EhV99B1 infection elicited a different response than that of EhV207, perhaps triggering the production of alternate extracellular metabolites. It has been previously reported that bacteria can utilize Coomassie stainable particles (CSPs) as a source of carbon and nitrogen (Long and Azam, 1996; Cisternas-Novoa et al., 2015), as an alternative to TEP. Indeed, dissolved organic matter in the ocean consists of diverse high and low molecular weight compounds available to bacteria (Amon and Benner, 1996). It is currently unknown if EhV infection induces CSP production and whether the shift between TEP and CSP is influenced by the type of infecting virus and the consequent response of the different E. huxleyi morphotypes.

**TEP composition and aggregation of Emiliania huxleyi**

FlowCam imaging directly characterized the number, size and blue:red ratio of Alcian Blue (AB)-stained, polysaccharide containing aggregates for infected and uninfected E. huxleyi cells (Figs. 4 and 5; Supporting Information Fig. S6).

Visually, the stained aggregates of EhV207-infected and uninfected CCMP374 and DHB611 treatments appeared blue, whereas some aggregates of uninfected DHB607 treatments did not (Fig. 4A). This suggests that, in the absence of EhV207 infection, E. huxleyi-derived metabolites other than TEP contributed towards aggregation. Proteinaceous CSPs, can also facilitate the formation of aggregates and ‘marine snow’ (Long and Azam, 1996; Cisternas-Novoa et al., 2015; Thornton and Chen, 2017). Quantification of the average blue to average red ratio (blue:red) of large aggregates (Fig. 4B–D), revealed that aggregates derived from EhV207-infected CCMP374, DHB611 and DHB607 cells all had a higher blue:red ratios at the end of the experiment compared to those aggregates observed for uninfected treatments,
consistent with the hypothesis that infection facilitates TEP production that can contribute to aggregation. However, regression analysis showed no dependence of the aggregate size across *E. huxleyi* strains to the blue:red ratio of the imaged aggregates (Supporting Information Fig. S7), thus indicating that more AB stainable polysaccharides does not necessarily translate into cells and debris aggregating into larger particles.

In comparison, no visual compositional differences were observed between EhV99B1-infected and uninfected *E. huxleyi* cells via FlowCam imaging (Fig. 5A). While all representative aggregates of CCMP374, DHB611 and DHB607 appeared blue (Fig. 5A), the only *E. huxleyi* strain in which the average blue:red ratio was higher in infected treatments was in CCMP374 (Fig. 5B). The average blue:red ratio was lower in infected treatments for both DHB611 and DHB607, (Fig. 5C and D), consistent with EhV99B1-induced aggregates for these strains being associated to a lesser degree with AB-stained polysaccharides than their respective uninfected controls. Given that large aggregates formed with lower blue:red ratios for these infected strains and that there was no dependence of aggregate size to the amount of AB stainable polysaccharides (Supporting Information Fig. S8), it supports the potential involvement of other unknown exopolymers in aggregation.

Differences in the size range and number of aggregates were observed among the *E. huxleyi* strains (Figs. 6 and 7). There was a general reduction in the number of aggregates on EhV207-infection, suggesting that virus infection and associated TEP production did not stimulate aggregation during the 96 h experiment period. CCMP374 and DHB607 both had aggregate sizes ranging from 7 to 21 μm in diameter (Fig. 6); whereas the
biggest aggregates observed for DHB611 were 16 \textmu m (Fig. 6). The number of aggregates in all three size bins (7–11, 12–16 and 17–21 \textmu m) was significantly reduced in EhV207-infected DHB607 relative to uninfected controls, whereas the only significant reduction in aggregate number for EhV207-infected CCMP374 and DHB611 cells was in the 7–11 \textmu m size range (Fig. 6). This observed reduction after 96 hpi may have been due to the rapid utilization and remineralization of TEP (and associated particles) produced at 24 hpi by bacteria over the 96 h time course. It is worth noting that this large peak in TEP was not accompanied with a shift in larger particle sizes (Supporting Information Fig. S9).

There was also a decrease in the total number of 7–11 \textmu m-sized aggregates in all three EhV99B1-infected E. huxleyi strains relative to their controls (Fig. 7). A decrease in the number of aggregates in the 12–16 \textmu m size range was also seen in EhV99B1-infected DHB607 and DHB611 treatments (Fig. 7). While there was no difference in the number of > 12 \textmu m sized aggregates between EhV99B1-infected and uninfected CCMP374 and DHB611 cells, there were significantly more aggregates in those > 17 \textmu m in size in EhV99B1-infected DHB607 relative to uninfected controls (Fig. 7). EhV99B1-infection of DHB607 clearly stimulated the aggregation of cells towards larger particles after 96 h.

Ecological implications for EhV-induced TEP production and aggregation

Given sinking rates in the field vary exponentially with aggregate size (Alldredge and Gotschalk, 1988), larger aggregates will sink faster than smaller, more buoyant ones. With an estimated density of 0.70–0.84 g cm$^{-3}$, TEP and other exopolymer derivatives may contribute to ‘ascending particles’ (Azetsu-Scott and Passow, 2004) and enhance retention of
particulate carbon in the upper ocean. At the same time, the association of sticky TEP with dense $(2.8 \text{ g cm}^{-3})\text{CaCO}_3$-containing coccoliths (and associated cellular organic carbon) will produce descending particles with high sinking rates. It follows that the biogeochemical impact of EhV-induced TEP production will depend on the nature of the particles formed, influenced by the specific $E.\text{huxleyi}$-EhV interactions, and as proposed here and elsewhere (Carrias et al., 2002; Gärdes et al., 2011; Van Oostende et al., 2013).

EhV207-infection was generally accompanied by a reduced ability to infect heavily calcified $E.\text{huxleyi}$ DHB607, along with a reduction in the number of aggregated TEP-containing particles in all $E.\text{huxleyi}$ strains. The early TEP production at 24 hpi prior to cell lysis (Figs. 2B and C and 3B and C) did not result in larger aggregated particle sizes (Supporting Information Fig. S9) but appeared to stimulate the growth of the resident bacterial communities in this closed system, with the total TEP carbon pool $(\text{in fg C ml}^{-1})$ being adequate to support the collective biomass and respiration of bacterial cells after 96 hpi. The decrease in total TEP concentrations post 24 h in infected calcifying $E.\text{huxleyi}$ could also be explained by either a shift into a more dissolved pool of TEP $(<0.4 \mu\text{m pore-size})$ or a compositional transformation (perhaps facilitated by microbial remineralization) into material that is not stained with AB, both of which would have escaped detection by our methods. The stability of TEP requires abiotic factors such as the presence of aggregates.

**Fig. 6.** Size group patterns of imaged aggregates for EhV207-infected $E.\text{huxleyi}$ strains 96 hours post infection. Aggregates were binned into arbitrary size ranges based on their area based diameter (ABD) after imaging with FlowCam. Size ranges are indicated below each panel. Bars represent the average number of aggregates $(n = 3, \pm SD)$ within each size range at the end of the experiment for uninfected control (white) and EhV207-infected (grey) cultures. The presence of arrows indicate statistically significant $(p < 0.05)$ respective shifts in aggregate number between the two corresponding treatments (control and infected). ND (not detected), indicates that particles in the size range of 17–21 $\mu\text{m}$ were not detected in $E.\text{huxleyi}$ DHB611 cultures.
of Ca\(^{2+}\) ions (Alldredge et al. 1993; Meng and Liu, 2016). It is unknown if the availability of calcium ions during infection of \(E.\ huxleyi\) changes significantly to impact the stability of TEP. Likewise, the cellular mechanisms behind TEP production and transformation are not well understood. Some viruses (specifically bacteriophages) are indeed known to encode for polysaccharide degrading enzymes (Sklar and Joerger, 2001; Sutherland et al., 2004). The vast majority of genes in EhVs encode for hypothetical proteins with no assigned function (Nissimov et al., 2017), so it is unknown if EhV-encoded polysaccharide-degrading enzymes play roles in TEP dynamics.

EhV99B1-infection, conversely, was characterized by a more ‘general’ ability to infect both ‘naked’ and calcified \(E.\ huxleyi\) cells, without significantly stimulating TEP production. Nonetheless, EhV99B1 infection of heavily calcified DHB607 resulted in a shift towards larger aggregates (Fig. 7), with lower average blue:red ratios, likely through the induction of and facilitation by other exopolymer substances. Based on these observations, which incorporate calculations of the concomitant growth, respiration and interactions of cohabitating bacteria (Supporting Information Fig. S5), infection of natural, calcified \(E.\ huxleyi\) populations by EhV99B1-like viruses may result in enhanced aggregation and sinking of PIC (and associated POC). Similar mechanisms may have indeed facilitated the very high vertical fluxes of PIC and POC observed during EhV infection of natural calcified \(E.\ huxleyi\) populations in the North Atlantic (Lehahn et al., 2014; Collins et al., 2015; Laber et al., 2018), for which EhV99B1-like viruses were observed to be the dominant, cell-associated genotypes and for which sinking material was enriched in infected cells (Laber et al., 2018). Future characterization of the buoyant density of resulting TEP-bound particles for ‘naked’ and calcified \(E.\ huxleyi\) cells challenged with EhVs is important to further understand the impact of EhVs on vertical carbon fluxes.

Although we did not investigate grazing dynamics in this study, it is likely that the number and size distribution of aggregated particles during EhV-infection will also influence their relative consumption by micro- and macrozooplankton grazers. Zooplankton generally consume particles less than one-tenth of their own body size.

Fig. 7. Size group patterns of imaged aggregates for EhV99B1-infected \(E.\ huxleyi\) strains 96 h post infection. Size ranges of aggregates are indicated below each panel and the bars show the average number of aggregates (\(n = 3, \pm \) SD) within each size range at the end of the experiment for uninfected control (white) and EhV99B1-infected (grey) cultures. The presence of arrows indicate statistically significant (\(p < 0.05\)) respective shifts in aggregate number between the two corresponding treatments (control and infected).
Conclusions

Our findings reveal a strong, yet variable interplay between *E. huxleyi*, EhVs, TEP and particle aggregation. The observed differences in TEP production and cell aggregation are influenced by inherent differences of the infecting viruses and the associated specific host responses to infection. Clearly, TEP production and aggregation in *E. huxleyi* can be impacted by virus infection. The observed variability in virus and host responses with regard to TEP production and aggregation were noteworthy as they can certainly impact the ecosystem outcome. While it is difficult to extrapolate our lab-based results to natural populations, our work shows the potential for EhV infection to trigger aggregation and serve as mechanistic context to help explain observed TEP production, aggregation, and high PIC and POC fluxes of EhV-infected blooms in the North Atlantic (Lehahn et al., 2014; Collins et al., 2015; Laber et al., 2018). As such, future field studies in natural blooms of *E. huxleyi* should involve detailed investigations of TEP production, the buoyant densities of TEP-associated particles, the relative involvement of bacteria in its production and/or utilization, the fate and composition of aggregates throughout the water column, and the role of TEP as a removal mechanism of viruses.

Experimental procedures

*Emiliania huxleyi* strains, incubation conditions and antibiotic treatment

*Emiliania huxleyi* strain CCMP374 (noncalcifying) was originally isolated from the Gulf of Maine in 1990, and was obtained from the National Center for Marine Algae and Microbiota (https://ncma.bigelow.org). *E. huxleyi* DHB611 (mildly-calcifying) and DHB607 (heavily-calcifying) were isolated from Raunfjorden near Bergen (Norway) by Daria Hinz during a mesocosm experiment in 2008 (Vardi et al., 2012; Kimmance et al., 2014) and obtained from the Plymouth Culture Collection (https://www.mba.ac.uk/culture-collection/). These *E. huxleyi* strains were chosen on the basis of their morphological characteristics (i.e., a dynamic range of calcification and mass CaCO3:POC ratios, as confirmed by direct PIC and POC measurements; Fig. 1).

These strains were cultivated in a nutrient rich f/2 media without silicate (f/2-Si; Guillard and Ryther, 1962; Guillard, 1975), at 18°C, and a 14:10 h light:dark cycle (light intensity of 168–210 μmol of photons m−2 s−1). Prior to infection experiments, three rounds of subculturing and treatment with an antibiotic cocktail (Supporting Information Table S1) were performed to reduce the levels of bacteria associated with the three *E. huxleyi* strains in culture. The extent of bacterial reduction was monitored by InFlux Mariner 209S flow cytometer (Supporting Information Fig. S3). The third round of antibiotic treated *E. huxleyi* (Supporting Information Table S2) was used to set up antibiotic-free stock cultures in f/2-Si, which were subsequently used as the primary stocks for infection experiments.

EhV strains and lysate stocks

Two *Emiliania huxleyi* virus strains (i.e., EhVs) were used in this study for two separate infection experiments: *Emiliania huxleyi* virus 207 (e.g., EhV207 in Exp1), isolated previously in 2001 from the English Channel (Nissimov et al., 2012a); and *Emiliania huxleyi* virus 99B1 (e.g., EhV99B1 in Exp2), isolated in 1999 from a Norwegian Fjord (Pagarete et al., 2013). The viruses are phylogenetically related but differ in a number of putative genes encoded on their respective genomes (Nissimov et al., 2017).

The two virus strains used in the experiments were propagated on a monthly basis by first infecting exponentially growing *E. huxleyi* CCMP374, DHB611 and DHB607 cultures (~5 × 10^8 cell ml−1) and then filtering the lysates with 0.45 μm pore-size syringe filters to remove debris. We then quantified the number of viral particles in these fresh virus lysate stocks by flow cytometry (see below) and stored them in the dark at 4°C until used for subsequent infection experiments.
Determination of particulate organic and inorganic carbon cell quotas

The calcification state and CaCO₃:POC mass ratios of the three *E. huxleyi* strains were determined before performing infection experiments. Cellular PIC and POC quotas for exponentially growing cells (~5 x 10⁵ cell ml⁻¹) in f/2-Si (Fig. 1) were determined using a previously described method by Harvey and colleagues (2015). Briefly, cells were vacuum filtered (~60 μm) onto precombusted (~400°C; 4 h) GF/F filters using precombusted glassware and dried at 60°C. Triplicate filters from each culture were placed into a glass desiccator with concentrated HCl fumes for 24 h to dissolve PIC. The other set of triplicate filters was untreated and represented total carbon (TC). Both sets of triplicate filters (acidified [x3] and non-acidified [x3]) were trimmed, packed into tin boats and combusted in a CNS Elemental Analyzer (Carlo Erba NA 1500). PIC was determined by taking the difference between TC and acidified samples and multiplying by the number of cells ml⁻¹. CaCO₃:POC mass ratios were calculated by dividing cellular PIC quotas by cellular POC quotas.

**Set-up and sampling of infection experiments**

Two experiments were performed in triplicate: one with EhV207 (Exp1) and one with EhV99B1 (Exp2). Experiments used triplicate cultures of the aforementioned *E. huxleyi* strains, each containing 50 ml of f/2-Si media. Based on the total number of EhV particles detected by flow cytometry (FC) in a freshly made primary EhV lysate stock (see above), viruses were added to exponentially growing cultures at a virus:cell ratio of 5:1, consistent with previous *E. huxleyi*- EhV infection dynamics experiments and published literature (Fulton et al., 2014). Virus free controls consisted of adding the same volume of f/2-Si into exponentially growing cultures. To reduce potential effects of added nutrients to the controls, the addition of the f/2-Si media to the 50 ml control triplicates was kept to a minimum, and did not exceed 250 μl. *E. huxleyi* cell and virus abundances and total concentration of TEP were monitored daily until culture clearance over a 96 h period; a time period during which EhV infection has been shown to be complete (Vardi et al., 2009; Fulton et al., 2014; Rose et al., 2014; Nissimov et al., 2016). Aggregate formation was examined 24 and 96 hpi via FlowCam imaging (see below).

**Emiliania huxleyi, virus and bacteria abundance measurements**

*E. huxleyi* cell abundance measurements were performed using an Accuri C6 flow cytometer (BD Biosciences), where 200 μl of the thawed samples were distributed into 96 well plates and counted using chlorophyll red fluorescence (692 nm) vs. side scatter (SSC). Each sample was analysed for 2 min with a flow rate of 14 μl min⁻¹. To ensure the accuracy of cell counts at this flow rate, we performed a comparative analysis of Accucount Beads (sizes of 2, 3.17, 5.34 and 7.28 μm) on the Accuri C6 and InFlux Mariner 209S flow cytometers. Our analysis revealed little variation in the number of beads counted by the two instruments at the respective sizes (i.e., 8.6%, 4.4%, 5.1% and 4.7% difference) with an average of 5.4 x 10⁵, 7.6 x 10⁵, 7.5 x 10⁵ and 7.4 x 10⁵ beads ml⁻¹ respectively.

The total number of viruses in the primary virus lysate stocks and throughout the experiments was counted using SYBR Gold stain and an InFlux Mariner 209S flow cytometer (FC) based on the protocol developed by Brussaard and colleagues (2010). Briefly, a 1 ml subsample of each virus stock or infected culture was fixed with glutaraldehyde (final concentration of 0.5%), incubated in the dark at 4°C for 15–30 min, snap frozen in liquid nitrogen (LN) and stored at −80°C until processed. Samples were thawed at room temperature and diluted 50-fold in Tris-EDTA (TE) buffer (1M TRIS pH-8, 0.5M EDTA and MilliQ) in flow cytometry tubes. SYBR Gold was diluted into the TE buffer at a 20 000:1 ratio of the commercial stock. The subsamples were agitated and incubated at 80°C for 10 min, followed by 5 min at room temperature in the dark. Each sample was analysed for 1 min on the
FC with a variable flow rate in μl min⁻¹ and then the number of events per μl was counted. The final virus numbers ml⁻¹ calculations took into account the different flow rates for each set of measurements.

Bacterial abundance was also quantified throughout the experiments, in a similar manner to the virus abundance measurements, with the only difference being the incubation step, which was at room temperature instead of 80°C. This minimized the effects of the heating step on the bacterial integrity, which could lead to an underestimation of the total number of bacteria counted. Flow cytometry data were analysed with FlowJo version 7.6.5.

**Spectrophotometric analysis of transparent exopolymer particles**

The total TEP production per ml⁻¹ was determined by bulk analysis employing filtration and spectrophotometric quantification (optical density; Passow and Alldredge, 1994; Vardi et al., 2012). A 0.02% Alcian Blue (AB 8GX, SIGMA A5268) working solution was made weekly (diluted from a stock of 1% AB in 3% glacial Acetic Acid), passed through 0.2 μm pore-size syringe filters prior to use to remove particulate debris and stored at 4°C. Subsamples (3 ml) of each experimental treatment were filtered daily in triplicates onto 0.4 μm polycarbonate filters (Millipore) under a low and constant vacuum pressure of 80% sulfuric acid (ACROS 42322-025) with gentle agitation (2–3 times) for 2 h. The filters were then removed and absorbance of the extracted, AB-stained TEP, was measured at 787 nm using the Molecular Devices SpectraMax M3 plate reader. Optical density (OD) values were corrected for f/2-Si media blanks and plotted against a Xanthan Gum (XG) standard curve of known concentrations (see below).

**TEP carbon calibration and calculations**

Xanthan Gum (XG; Sigma G1253) was used as a reference polysaccharide to express TEP values as XG equivalents per liter (XG eq l⁻¹). A known mass of XG per filter was stained with AB, extracted and the OD was measured as described (Passow and Alldredge, 1995; Bittar et al., 2018). Briefly, XG stock solution (75 mg l⁻¹ in acetaete buffer) was diluted to 3.75, 9.375, 18.75, 37.5, 56.25 and 75 μg XG ml⁻¹. Triplicate 1 ml volumes of each concentration were stained by adding 0.5 ml Alcian Blue (0.02% in 0.06% acetic acid) followed by immediate filtration onto a 0.45 μm pore-size polycarbonate filter. Each filter was rinsed with ultrapure water, extracted in 80% Sulfuric acid and measured spectrophotometrically as described above for TEP. The final calibration curve (Supporting Information Fig. S10) consisted of repeated measurements of five batches of Alcian Blue to account for technical variability in batches of stain and staining procedure. The average f value was 49.8.

Estimations of TEP carbon usage by resident bacterial populations in our closed system were calculated using the measured bacterial abundances (Supporting Information Fig. S4), along with published values on the cellular carbon content of bacteria (150 f. C cell⁻¹; Fagerbakke et al., 1996; Zimmerman et al., 2014) and bacterial growth yield (20%; Bjørnsen, 1986). The latter represents the proportion of carbon incorporated into bacterial production with the balance going to respiration.

**FlowCam visualization of aggregates**

The formation and size spectrum of aggregates were visualized and quantified at 24 hpi and at the end of each experiment (i.e., 96 hpi) for infected and virus free control treatments for the three *E. huxleyi* strains using a FlowCam and VisualSpreadsheet® software (version 4.0.27), using a modified method to those described by Cisternas-Novoa and colleagues (2015) and Thuy and colleagues (2015). Briefly, subsamples (500 μl) were stained with 200 μl of 0.02% AB. An additional 20 μl of acetic acid (glacial, ACROS 42322-025) was added to lower the pH of the AB stained samples to pH 2.5, which is the required for adequate staining of both sulfated and carboxylated polysaccharides (Passow and Alldredge, 1995). The samples were then incubated at room temperature for 10 min and applied to the FlowCam sample intake. A10X objective lens was used in the auto-image mode setting (20–30 frames per second) with the instrument threshold set to image particles that ranged from 7 to 100 μm with a minimum distance between particles of 1 μm. The collection of particles was restricted to those > 7 μm in diameter based on the known size range of *E. huxleyi* cells (i.e., 3–7 μm) and an upper cutoff of 100 μm based on the size of the flow cell used. This cutoff allowed us to operationally define an imaged particle > 7 μm as an aggregate. The flow rate was adjusted to 0.1–0.08 ml min⁻¹ to prevent duplicate images and auto-image mode was set to stop after 100 μl of sample had been imaged.

To account for any AB precipitation that may have naturally occurred in our seawater-based media due to a direct staining of seawater-based samples and the addition of
the glacial acetic acid, we ran our experimental samples alongside AB-stained, f/2-Si blanks (which included the addition of 20 μl of acetic acid) and quantified the number and size of the imaged particles. This analysis revealed an average of 18 (±5.6) imaged particles across the different days and treatments, with an average size range of 8 μm (±2.3). The low number of small-sized imaged particles in these blanks provided confidence that the chemical additions during staining did not significantly impact the total number or size of imaged AB-stained particles (most of the imaged particles in experiments at this size range were in the hundreds to thousands). We acknowledge, as previously reported elsewhere (Passow and Alldredge, 1995; Cisternas-Novoa et al., 2015), that our described FlowCAM method is qualitative. Both the presence of salts in seawater and the addition of acidic solutions to cell suspensions can enhance the presence of AB-stained particles. Here, we merge our TEP visualization via FlowCAM with traditional, quantitative TEP measurements to relate its production with particle dynamics. We suggest that further refinements in the technique are warranted moving forward to constrain and/or minimize the possible release of TEP from chemical treatment of cells and its interaction with salts.

The number of counted aggregates and their measured size (expressed as an ‘area-based diameter’; ABD) were calibrated by running 1 ml (n = 3) of COUNT-CAL™ polystyrene calibration beads (ThermoFisher Scientific; cat # CC20-PK; diameter = 20 ± 0.25 μm; 3000 beads ml⁻¹). Measurements of these calibration beads (n = 3, ± SD) yielded an average concentration of 2172 (±112) per ml⁻¹ and average area based diameter (ABD) of 21.7 (±0.13). Based on this calibration, reported aggregate numbers use a correction factor of 1.25. ABD values of the measured COUNT-CAL beads were within 3.5% of their actual size so no correction factor was applied.

Files were analysed manually after the completion of the experiments for quality control and identification of duplicate images. Imaged aggregates were arbitrarily grouped into five particle size ranges that spanned 5 μm in diameter (i.e., 7–11, 12–16, 17–21, 22–26 and 27–31 μm) and a FlowCam image analysis setting that calculates particle diameter based on the measured area of the visualized particle (FlowCam Manual, 2011). Contrary to previous methods that used imaging software that splits images to red, blue and green channels and used the red channels to identify TEP (Engel, 2009), we used the automated FlowCam generated blue:red ratios of the imaged aggregates to quantify compositional characteristics of imaged aggregates, with higher ratios corresponding to a higher proportion of AB-stained polysaccharides (Supporting Information Fig. S6).

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References

Alldredge, A.L., and Gotschalk, C. (1988) In situ settling behavior of marine snow. Limnol Ocean 33: 339–335.

Alldredge, A.L., Passow, U., and Logan, B.E. (1993) The abundance and significance of a class of large, transparent organic particles in the ocean. Deep Sea Res I 40: 1131–1140.

Alldredge, A.L., Passow, U., and Haddock, H.D. (1998) The characteristics and transparent exopolymer particle (TEP) content of marine snow formed from thecate dinoflagellates. J Plankton Res 20: 393–406.

Allen, M.J., Schroeder, D.C., Holden, M.T.G., and Wilson, W.H. (2006) Evolutionary history of the Coccolithoviridae. Mol Biol Evol 23: 86–92.

Amon, R.M.W., and Benner, R. (1996) Bacterial utilization of different size classes of dissolved organic matter. Limnol Oceanogr 41: 41–51.

Azam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L.A., and Thingstad, F. (1983) The ecological role of water-column microbes in the sea. Mar Ecol Prog Ser 10: 257–263.

Azetsu-Scott, K., and Passow, U. (2004) Ascending marine particles: significance of transparent exopolymer particles (TEP) in the upper ocean. Limnol Oceanogr 49: 741–748.

Bar-Zeev, E., Avishay, I., Bidle, K.D., and Berman-Frank, I. (2013) Programmed cell death in the marine cyanobacterium Trichodesmium mediates carbon and nitrogen export. ISME J 7: 2340–2348.

Bidle, K.D. (2015) The molecular ecophysiology of programmed cell death in marine phytoplankton. Ann Rev Mar Sci 7: 341–375.

Bidle, K.D., and Vardi, A. (2011) A chemical arms race at sea mediates algal host-virus interactions. Curr Opin Microbiol 14: 449–457.

Bittar, T.B., Passow, U., Haramaty, L., Bidle, K.D., and Harvey, E.L. (2018) An updated method for the calibration of transparent exopolymer particle (TEP) measurements. Limnol Oceanogr Methods; doi:10.1002/lom3.10268.

Bjornsen, P.K. (1986) Bacterioplankton growth-yield in continuous seawater cultures. Mar Ecol Ser 30: 191–196.

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Laber, C.P., Hunter, J.E., Carvalho, A.F., Collins, J.R., Hunter, E.J., Schieler, B., et al. (2018) Coccolithovirus facilitation of carbon export in the North Atlantic. *Nat Microbiol* 3: 537–547.

Lehahn, Y., Koren, I., Schatz, D., Frada, M., Sheyn, U., Boss, E., et al. (2014) Decoupling physical from biological processes to assess the impact of viruses on a mesoscale algal bloom. *Curr Biol* 24: 2041–2046.

Litchman, E., Ohman, M.D., and Kiorboe, T. (2013) Trait-based approaches to zooplankton communities. *J Plank Res* 35: 473–484.

Long, R.A., and Azam, F. (1996) Abundant protein-containing particles in the sea. *Aquat Microb Ecol* 10: 213–221.

Mackinder, L.C.M., Worthy, C.A., Biggi, G., Hall, M., Ryan, K.P., Varsani, A., et al. (2009) A unicellular algal virus, *Emiliania huxleyi* virus 86, exploits an animal-like infection strategy. *J Gen Virol* 90: 2306–2316.

Mari, X., Rassoulzadegan, F., Brussaard, C.P.D., and Wassmann, P. (2005) Dynamics of transparent exopolymeric particles (TEP) production by *Phaeocystis globosa* under N- or P-limitation: a controlling factor of the retention/export balance. *Harmful Algae* 4: 895–914.

Mari, X., Kerros, M.E., and Weinbauer, M.G. (2007) Virus attachment to transparent exopolymeric particles along trophic gradients in the southwestern lagoon of New Caledonia. *Appl Environ Microbiol* 73: 5245–5252.

Martínez Martínez, J., Schroeder, D.C., Larsen, A., Bratbak, G., and Wilson, W.H. (2007) Molecular dynamics of *Emiliania huxleyi* and co-occurring viruses during two separate mesocosm studies. *Appl Environ Microbiol* 73: 554–562.

Martínez Martínez, J., Schroeder, D.C., and Wilson, W.H. (2012) Dynamics and genotypic composition of *Emiliania huxleyi* and their co-occurring viruses during a coccolithophore bloom in the North Sea. *FEMS Microbiol Ecol* 81: 315–323.

Mekik, F., Loubere, P., and Richard, M. (2007) Rain ratio variation in the Tropical Ocean: tests with surface sediments in the eastern equatorial Pacific. *Deep Sea Res Part II Top Stud Oceanogr* 54: 706–721.

Meng, S., and Liu, Y. (2016) New insights into transparent exopolymer particles (TEP) formation from precursor materials at various Na(+/)Ca(2+)/ ratios. *Sci Rep* 6: 19747.

Morison, F., and Menden-Deuer, S. (2015) Early spring phytoplankton dynamics in the subpolar North Atlantic: the influence of protistan herbivory. *Limnol Oceanograph* 60: 1298–1313.

Nissimov, J.I., and Bidle, K.D.B. (2017) Stress, death, and the biological glue of sinking matter. *J Phycol* 53: 241–244.

Nissimov, J.I., Worthy, C.A., Rooks, P., Napier, J.A., Kimrnance, S.A., Henn, M.R., et al. (2011a) Draft genome sequence of the Coccolithovirus *Emiliania huxleyi* virus 203. *J Virol* 85: 13468–13469.

Nissimov, J.I., Worthy, C.A., Rooks, P., Napier, J.A., Kimrnance, S.A., Henn, M.R., et al. (2011b) Draft genome sequence of the coccolithovirus EhV-94. *Stand Genomic Sci* 5: 1–11.

Nissimov, J.I., Worthy, C.A., Rooks, P., Napier, J.A., Kimrnance, S.A., Henn, M.R., et al. (2012a) Draft genome sequence of four coccolithoviruses: *Emiliania huxleyi* virus EhV-88, EhV-201, EhV-207, and EhV-208. *J Virol* 86: 2896–2897.

Nissimov, J.I., Worthy, C.A., Rooks, P., Napier, J.A., Kimmnance, S.A., Henn, M.R., et al. (2012b) Draft genome sequence of the coccolithovirus *Emiliania huxleyi* virus 202. *J Virol* 86: 2380–2381.

Nissimov, J.I., Jones, M., Napier, J.A., Munn, C.B., Kimrnance, S.A., and Allen, M.J. (2013) Functional inferences of environmental coccolithovirus biodiversity. *Virol Sin* 28: 291–302.

Nissimov, J.I., Napier, J.A., Kimrnance, S.A., and Allen, M.J. (2014) Permanent draft genomes of four new coccolithoviruses: EhV-18, EhV-145, EhV-156 and EhV-164. *Mar Genomics* 15: 7–8.

Nissimov, J.I., Napier, J.A., Allen, M.J., and Kimrnance, S.A. (2016) Intragenus competition between coccolithoviruses: an insight on how a select few can come to dominate many. *Environ Microbiol* 18: 133–145.

Nissimov, J.I., Pagarete, A., Ma, F., Cody, S., Dunigan, D. D., Kimrnance, S.A., et al. (2017) Coccolithoviruses: a review of cross-kingdom genomic thievery and metabolic thuggery. *Viruses* 9: E52. doi:10.3390/v9030052.

Pagarete, A., Allen, M.J., Wilson, W.H., Kimrnance, S.A., and de Vargas, C. (2009) Host-virus shift of the siphingolipid pathway along an *Emiliania huxleyi* bloom: survival of the fattest. *Environ Microbiol* 11: 2840–2848.

Pagarete, A., Lanzén, A., Puntervoll, P., Sandaa, R.A., Larsen, A., Larsen, J.B., et al. (2013) Genomic sequence and analysis of EhV-99B1, a new coccolithovirus from the Norwegian fjords. *Intervirology* 56: 60–66.

Pagarete, A., Kusonmano, K., Petersen, K., Kimrnance, S. A., Martínez Martínez, J., Wilson, W.H., et al. (2014) Dip in the gene pool: Metagenomic survey of natural coccolithovirus communities. *Virology* 466–467: 129–137.

Passow, U. (2002) Transparent exopolymer particles (TEP) in aquatic environments. *Prog Oceanogr* 55: 287–333.

Passow, U., and Aldredge, A.L. (1994) Distribution, size and bacterial colonization of transparent exopolymer particles (TEP) in the ocean. *Mar Ecol Prog Ser* 113: 185–198.

Passow, U., and Aldredge, A.L. (1995) A dye-binding assay for the spectrophotometric measurement of transparent exopolymer particles (TEP). *Limnol Oceanogr* 40: 1326–1335.

Poorvin, L., Rinta-Kanto, J.M., Hutchins, D.A., and Wilhelm, S. W. (2004) Viral release of iron and its bioavailability to marine plankton. *Limnol Oceanogr* 49: 1734–1741.

Rose, S.L., Fulton, J.M., Brown, C.M., Natale, F., Van Mooy, B.S.A., and Bidle, K.D. (2014) Isolation and characterization of lipid rafts in *Emiliania huxleyi*: a role for membrane microdomains in host-virus interactions. *Environ Microbiol* 16: 1150–1166.

Rosenwasser, S., Mausz, M.A., Schatz, D., Sheyn, U., Malitsky, S., Aharoni, A., et al. (2014) Rewiring host lipid metabolism by large viruses determines the fate of *Emiliania huxleyi*, a bloom-forming alga in the Ocean. *Plant Cell* 26: 2689–2707.

Rowe, J.M., Fabre, M.-F., Gobena, D., Wilson, W.H., and Wilhelm, S.W. (2011) Application of the major capsid protein as a marker of the phylogenetic diversity of *Emiliania huxleyi* viruses. *FEMS Microbiol Ecol* 76: 373–380.
Schatz, D., Shemi, A., Rosenwasser, S., Sabanay, H., Wolf, S.G., Ben-Dor, S., et al. (2014) Hijacking of an autophagy-like process is critical for the life cycle of a DNA virus infecting oceanic algal blooms. New Phytologist 204: 854–863.

Schoeder, D.C., Oke, J., Malin, G., and Wilson, W.H. (2002) Coccolithovirus (Phycodnaviridae): characterisation of a new large dsDNA algal virus that infects Emiliania huxleyi. Arch Virol 147: 1685–1698.

Sklar, I.B., and Joerger, R.D. (2001) Attempts to utilize bacteriophage to combat Salmonella enterica serovar enteritidis infection in chickens. J Food Saf 21: 15–29.

Sutherland, I.W., Hughes, K.A., Skillman, L.C., and Tait, K. (2004) The interaction of phage and biofilms. FEMS Microbiol Lett 232: 1–6.

Suttle, C.A. (2005) Viruses in the sea. Nature 437: 356–361.

Suttle, C.A. (2007) Marine viruses-major players in the global ecosystem. Nat Rev Microbiol 5: 801–812.

Thornton, D.C.O., and Chen, J. (2017) Exopolymer production as a function of cell permeability and death in a dia- tom (Thalassiosira weissflogii) and a cyanobacterium (Synechococcus elongatus). J Phycol 53: 245–260.

Thuy, N.T., Lin, J.C., Juang, Y., and Huang, C. (2015) Temporal variation and interaction of full size spectrum alcian blue stainable materials and water quality parameters in a reservoir. Chemosphere 131: 139–148.

Van Oostende, N., Moerdijk-Poortvliet, T.C.W., Boschker, H. T.S., Vyverman, W., and Sabbe, K. (2013) Release of dissolved carbohydrates by Emiliania huxleyi and formation of transparent exopolymer particles depend on algal life cycle and bacterial activity. Environ Microbiol 15: 1514–1531.

Vardi, A., Van Mooy, B.A.S., Fredricks, H.F., Popendorf, K. J., Ossolinski, J.E., Haramaty, L., et al. (2009) Viral glycosphingolipids induce lytic infection and cell death in marine phytoplankton. Science 326: 861–865.

Vardi, A., Haramaty, L., Van Mooy, B.A.S., Fredricks, H.F., Kimmance, S., Larsen, A., et al. (2012) Host-virus dynamics and subcellular controls of cell fate in a natural coccolithophore population. Proc Natl Acad Sci U S A 109: 19327–19332.

Vermont, A., Martinez Martinez, J., Waller, J.D., Gilc, I.G., Leavitt, A.H., Floge, S.A., et al. (2016) Virus infection of Emiliania huxleyi deters grazing by the copepod Acartia tonsa. J Plankton Res 38: 1194–1205.

Wilhelm, S.W., and Suttle, C.A. (1999) Viruses and nutrient cycles in the sea. Bioscience 49: 781–788.

Wilson, W.H., Taran, G.A., Schroeder, D., Cox, M., Oke, J., and Malin, G. (2002) Isolation of viruses responsible for the demise of an Emiliania huxleyi bloom in the English Channel. J Mar Biol Assoc United Kingdom 82: 369–377.

Wilson, W.H., Schroeder, D.C., Allen, M.J., Holden, M.T.G., Parkhill, J., Barrett, B.G., et al. (2005) Complete genome sequence and lytic phase transcription profile of a Coccolithovirus. Science 309: 1090–1092.

Wilson, W.H., Van Etten, J.L., and Allen, M.J. (2009) The Phycodnaviridae: the story of how tiny bugs rule the world. Curr Top Microbiol Immunol 328: 1–42.

Zimmerman, A.E., Allison, S.D., and Martiny, A.C. (2014) Phylogenetic constraints on elemental stoichiometry and resource allocation in heterotrophic marine bacteria. Environ Microbiol 16: 1398–1410.

Ziv, C., Malitsky, S., Othman, A., Ben-Dor, S., Wei, Y., Zheng, S., et al. (2016) Viral serine palmitoyltransferase induces metabolic switch in sphingolipid biosynthesis and is required for infection of a marine alga. Proc Natl Acad Sci U S A 113: E1907–E1916.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Growth rates of uninfected and infected E. huxleyi cultures respectively. The average ($n = 3, \pm SD$) specific growth rates ($\mu$; d$^{-1}$) and net growth rates ($\mu_{net}$; d$^{-1}$) were calculated daily over a 96 h period for experiments conducted with EhV207 (A&B) and EhV99B1 (C&D).

Fig. S2. Burst size of infected E. huxleyi strains. (A and B) The average ($n = 3, \pm SD$) burst sizes (i.e., number of viruses produced per lysed cell) of EhV207, (dark grey bars) and EhV99B1, (light grey bars) infected E. huxleyi strains.

Fig. S3. Flow cytometry scatter plots of non-axenic and antibiotic-treated E. huxleyi cultures. The top row of scatter plots represent the original stock cultures prior to the addition of antibiotics; the bottom row of plots represent E. huxleyi CCMP374, DHB611 and DHB607 stock cultures after three rounds of antibiotic treatments; (see Supporting Information Table S1). In each plot the x axis represents the side scatter and the y axis represents the green fluorescence (SYBR gold staining). Square boxes (gates) in the middle of the plots designate respective bacterial populations, whereas the top right corner shows the E. huxleyi cells (both indicated by arrows). The total events counted by the InFlux cytometer are indicated next to each gate. The bacterial and E. huxleyi cell abundances ml$^{-1}$ in CCMP374, DHB611 and DHB607 prior and after antibiotic treatment are shown in Supporting Information Table S2.

Fig. S4. Bacterial abundances in infected and virus free E. huxleyi cultures. The average ($n = 3, \pm SD$) bacterial abundances associated with infected (dashed lines) and virus free control (solid lines) cultures of E. huxleyi CCMP374 (black lines), DHB611 (grey lines) and DHB607 (blue lines) in Exp1 (A) and Exp2 (B).

Fig. S5. Calculated usage of TEP-associated carbon by resident bacteria in E. huxleyi DHB611 and DHB607 experiments with EhV207. A and B. The calculated, volume-based carbon availability from bulk TEP (using XG equivalents data derived from Fig. 2) and (C and D) volume-based carbon utilization by resident bacteria (encompassing both bacterial growth and respiration for which abundance data derives from Fig. 2 and Supporting Information Fig. S4) between 24–48 h and 24–96 h. Calculations of carbon usage by resident bacterial populations used published values for the cellular carbon content of bacteria (i.e., 150 f. C cell$^{-1}$; Fagerbakke et al., 1996; Zimmerman et al., 2014) and bacterial growth efficiency (BGE; 20%; Bjørnsen, 1986), the latter representing the proportion of carbon incorporated into bacterial production with the balance going to respiration. The 24–48 h time window corresponds with the initial drop in bulk
TEP in the experimental system (see Fig. 2), the removal of which could not be accounted for by bacterial activity alone. Using the aforementioned carbon content and BGE values, this activity only accounted for 7%–27% of the observed carbon removal. All (103%–141%) or nearly all (84%) of the observed TEP-associated carbon removal could be accounted for by bacterial activity after 96 h, using the same values. Note that we did not empirically measure BGE in our system. Rather, our carbon budget calculations used an average BGE of 20%, which resulted in some values in excess of 100%. There is a wide range of BGEs for bacteria in natural aquatic systems (< 5%–60%; del Giorgio and Cole, 1998) and a general lack of insight into what regulates it. Assuming removal of TEP carbon (A and B) was due to resident bacteria, calculated BGEs would be between 3.5% and 23.1%, consistent with the above range.

Fig. S6. Representative FlowCam images of Alcian Blue-stained aggregates from uninfected E. huxleyi DHB607. The FlowCam allows for visualization of cell aggregates and an assessment of the association of E. huxleyi cells with AB-stainable TEP. The round, phase bright circles in the two images with ID #2659 and #4249 are cells, whereas the light blue colour surrounding the cells in image #2659 is AB-stained TEP (indicated by arrows). In this representative snapshot of a control, uninfected DHB607 replicate at 96 hours, the ratio of their average blue:red differs, indicating that aggregates of similar size can have different amounts of TEP associated with them.

Fig. S7. Linear regression for average blue:red ratios and size (taken as ABD; 8–21 μm) of visualized aggregates in experiments with EhV207. A higher ratio indicates a greater proportion of AB stained polysaccharides. ABD shown on the x axis is the area based diameter of an imaged aggregate.

Fig. S8. Linear regression for average blue:red ratios and size (taken as ABD; 17–31 μm) for visualized aggregates in experiments conducted with EhV99B1. A higher ratio indicates a greater proportion of AB stained polysaccharides.

Table S1. Antibiotic cocktail for the treatment of E. huxleyi cultures. The antibiotic cocktail was used to minimize the bacterial populations in our stock E. huxleyi cultures prior to the infection experiments. Several rounds of antibiotic treatment were employed. The last round was analysed by flow cytometry (see Supporting Information Fig. S3) and represent the primary E. huxleyi CCMP374, DHB611 and DHB607 stocks from which new cultures for subsequent infection experiments were set up (see Experimental Procedures).

Fig. S9. Patterns in size-grouped, imaged aggregates for the three E. huxleyi strains, 24 h post infection by EhV207 and EhV99B1. Aggregates were binned into arbitrary size ranges based on their area based diameter (ABD) after imaging with FlowCam. Size ranges are indicated below each panel. Bars represent the average number of aggregates (n = 3, ± SD) within each size range at the end of the experiment for uninfected control (white) and infected (grey) cultures. Arrows indicate statistically significant (p < 0.05) respective shifts in aggregate number between the two treatments (controls and infected).

Fig. S10. Xanthan Gum calibration curve. Relationship between known concentrations of the polysaccharide xanthan gum (XG) and the optical density (OD) of AB-stained and extracted TEP (Passow, 2002; Vardi et al., 2012). Data points represent average readings (±SD) from five different batches of AB with linear regression applied. Regression was used for samples in this study to convert TEP production to XG equivalents based on a protocol by Bittar et al. (2018).