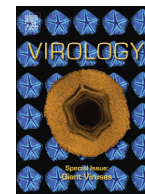




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## How many *Coccolithovirus* genotypes does it take to terminate an *Emiliana huxleyi* bloom?

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### ABSTRACT

Giant viruses are known to be significant mortality agents of phytoplankton, often being implicated in the terminations of large *Emiliana huxleyi* blooms. We have previously shown the high temporal variability of *E. huxleyi*-infecting coccolithoviruses (EhVs) within a Norwegian fjord mesocosm. In the current study we investigated EhV dynamics within a naturally-occurring *E. huxleyi* bloom in the Western English Channel. Using denaturing gradient gel electrophoresis and marker gene sequencing, we uncovered a spatially highly dynamic *Coccolithovirus* population that was associated with a genetically stable *E. huxleyi* population as revealed by the major capsid protein gene (*mcp*) and coccolith morphology motif (CMM), respectively. Coccolithoviruses within the bloom were found to be variable with depth and unique virus populations were detected at different stations sampled indicating a complex network of EhV–host infections. This ultimately will have significant implications to the internal structure and longevity of ecologically important *E. huxleyi* blooms.

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### Introduction

Giant double-stranded DNA viruses of the family *Phycodnaviridae* have long been recognised as significant mortality agents of the globally important coccolithophore, *Emiliana huxleyi* (Bratbak et al., 1996; Wilson et al., 2002b), being classified into the genus *Coccolithovirus* (Schroeder et al., 2002). *E. huxleyi* is considered to be ecologically important, contributing significantly to the total global carbon fixation in the oceans, yet due to this species ability to calcify, its role in the carbon cycle is even more complex (Rost and Riebesell, 2004). *E. huxleyi* is known to form extensive blooms, covering large expanses of water (Holligan et al., 1993). Ecological studies have shown that coccolithophores succeed diatoms in response to stratification and nutrient depletion of surface waters. This initial onset of bloom formation is soon followed by “white-water” which typifies the secondary or dying phase of the bloom (Balch et al., 1991; Holligan et al., 1983). *E. huxleyi*-infecting viruses (EhVs) have often been implicated in the demise of these blooms (Martinez et al., 2007; Wilson et al., 2002b). As mortality agents of

these blooms, EhVs therefore play key roles in local ecosystem dynamics and community succession, as well as biogeochemical cycling (Gobler et al., 1997).

Numerous *Coccolithovirus* isolates have been found to be very similar with regards to their propagation strategy, host range and genomic sequence. For example, the genome of EhV88 has an identical G+C content (40.18%) to EhV86 and they share 100% identity for 231 coding sequences (CDS) (Nissimov et al., 2012). Some also share the same host ranges, including the strains EhV201 and EhV202 which have identical host ranges for the hosts they have been tested against (Martínez Martínez, 2006). However, other isolates have been found to be more different or possess unique properties, such as that of the capsid EhV86 which appears to be surrounded by a lipid envelope and enters host cells via an endocytotic or lipid fusion mechanism (Mackinder et al., 2009). This infection strategy has not yet been identified in other viruses of the family *Phycodnaviridae* and furthermore, 3 EhVs isolates,  $\Phi$ 28,  $\Phi$ 29 and  $\Phi$ 30, have been found to show no sensitivity to ether, suggesting the absence of a lipid envelope (Vaughn et al., 2010). The presence of a lipid envelope in other EhVs has yet to be demonstrated; however, one would expect that those closely related to EhV86 would display this infection mechanism. Other differences have been observed in *Coccolithovirus* genomes with the EhV202 genome only sharing 1 CDS that is

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100% similar to EhV86 and has 83 unique CDSs and encodes only 3 tRNAs (compared to 4–6 in other EhV isolates that have been sequenced) (Nissimov et al., 2012). Other characteristics including the duration of the latent period and burst size are known to vary between EhV isolates (Vaughn et al., 2010). Genotypic diversity of EhVs in the marine environment has been described as high, being typically studied using the major capsid protein gene (*mcp*) as a molecular marker. This marker, first identified by Schroeder et al. (2002), shows sufficient variability between strains for detecting different genotypes in the environment.

In non-bloom conditions, many different EhV genotypes are known to exist in the water column (Rowe et al., 2011). During the onset of a bloom, EhV communities are found to be highly dynamic; however, as the bloom proceeds, typically one viral genotype is found to dominate and it is generally perceived that this virus strain goes on to cause the termination of the bloom (Martínez Martínez et al., 2007; Sorensen et al., 2009). This virus possibly has a competitive advantage over other EhVs, allowing for it to rapidly increase in numbers and dominate the virus community. As such a high number of different EhVs exist, one would expect virus communities to be changeable over long time scales; however this is not necessarily the case, as illustrated by mesocosm experiments. Two separate mesocosm experiments conducted in a Norwegian Fjord were studied during *E. huxleyi* blooms in 2000 and 2003 using denaturing gradient gel electrophoresis (DGGE) profiling of the *mcp*. Interestingly, the two dominant EhVs during these mesocosm blooms were the same in both years showing the persistence and ultimate success of a particular genotype over a 3 year period (Martínez Martínez et al., 2007). A third bloom, studied 5 years later in 2008, was found to be dominated by a different genotype (Sorensen et al., 2009); all three years were genotyped for the host using the coccolithophore morphology motif (CMM) marker, a sequence present within the 3' UTR of the putative calcification-related *gpa* gene (Schroeder et al., 2005), and were found to be dominated by the same three *E. huxleyi* genotypes. Whilst 3 CMM genotypes dominated these blooms, each CMM genotype is likely to be comprised of several microsatellite genotypes as these markers are considerably more polymorphic (Krueger-Hadfield et al., 2014). The change in the dominant EhV in 2008 may indicate evolution of viral genotypes due to high mutation rates compared to the hosts or alternatively may indicate that a change in environmental conditions may have favoured the newly dominant virus genotype.

EhV diversity and population structure have yet to be fully interrogated within naturally-occurring blooms, on a larger, spatial scale. Martínez Martínez et al. (2012) recently tracked EhVs within a bloom in the North Sea documenting the presence of 3 different assemblages during the course of a 12 day study. In our study we developed on these findings on a spatial scale by looking at both the horizontal and vertical EhV population within a bloom of *E. huxleyi* within the Western English Channel during July 2006. This typically perennial *E. huxleyi* bloom develops during the months of July/August and forms part of the annual succession of phytoplankton in this region (Widdicombe et al., 2010). Here we report for the first time, unlike in mesocosm-based studies, that no dominant EhV genotype was detected during the phase of the bloom. Thereby raising the question whether, on this occasion, a complex cohort of EhVs was responsible for terminating the natural occurring *E. huxleyi* dominated bloom.

## Results

### Characterisation and monitoring of the phytoplankton bloom using satellite imagery

The progression of the phytoplankton bloom in the Western Approaches and English Channel was monitored during summer

2006 using enhanced colour remote sensing data (Fig. 1). The coccolithophore component of the phytoplankton bloom, as defined by a patch of high reflectance (where high densities of coccolithophores are causing a significant back-scattering of light), was apparent within the Western English Channel in early July 2006. The region of reflectance persisted in this region throughout July and into August 2006.

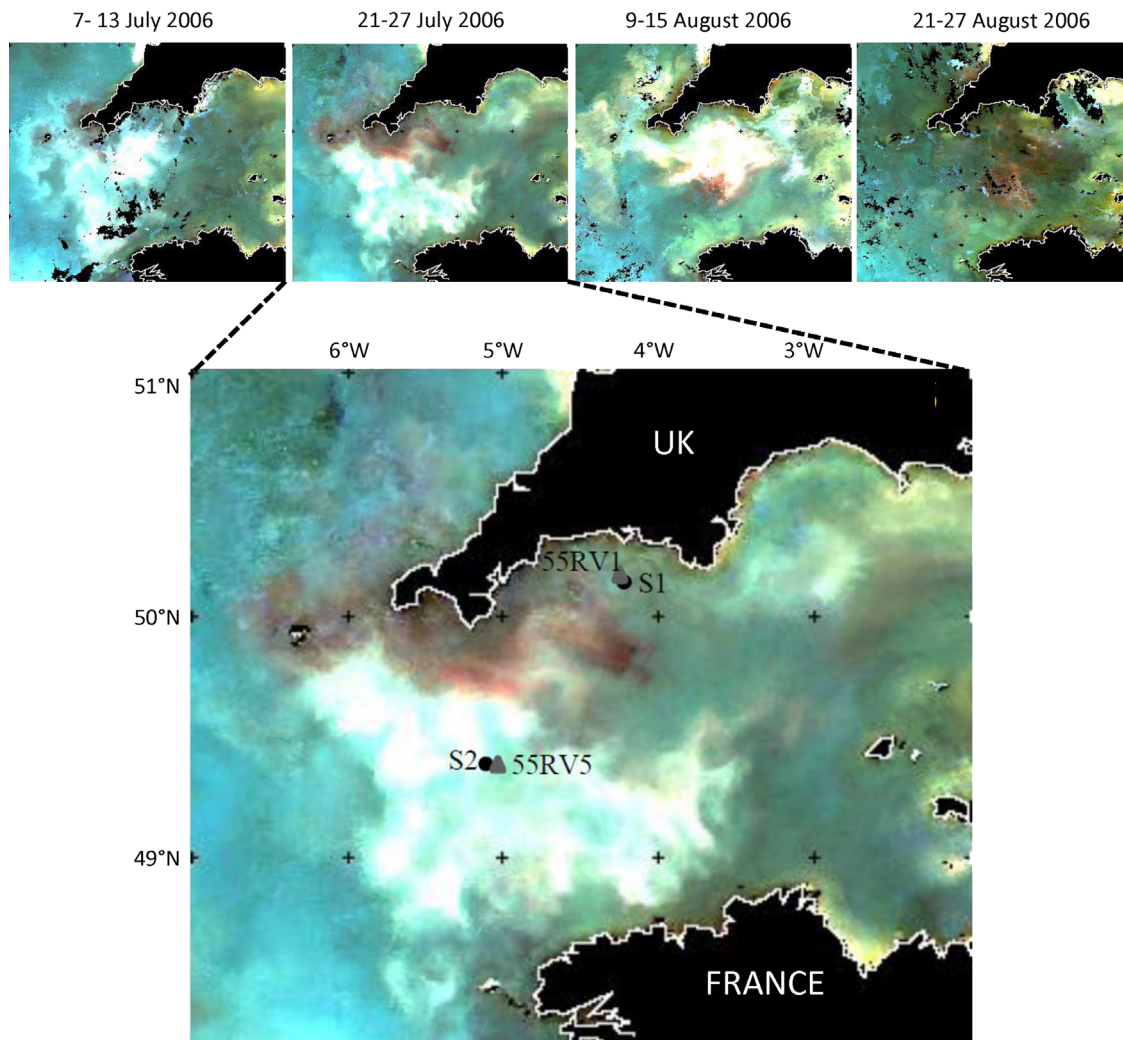
### Water column measurements

The water column was characterised for Chlorophyll *a* (Chl *a*) salinity, temperature and phosphate for CTD (conductivity, temperature, depth) stations 1 and 2 (Fig. 2). Both stations were thermally stratified, with surface waters at 18.8 and 20.2 °C and deeper water temperatures at 11.8 and 13.47 °C respectively (Fig. 2). A thermocline was present at both stations, with station 1 having a less pronounced thermal stratification and a lower SST (sea surface temperature) compared to station 2. Station 1 showed a near constant salinity profile, whereas station 2 had a halocline present at > 15 m depth (Fig. 2). Maximum Chl *a* levels were above the thermocline for both stations, with station 1 having the lowest concentrations across the depth profile (< 0.95 µg l<sup>-1</sup>). At station 2, Chl *a* was highest at 15 m depth of 3.41 µg l<sup>-1</sup>, and decreased with depth. Phosphate levels were lowest at station 1 and showed relatively little change across the depth profile (Fig. 2). A nutricline was present at station 2, with phosphate concentrations highest at 40 m depth.

Flow cytometry showed coccolithophore concentrations to be lowest at station 1 at < 600 cells ml<sup>-1</sup>, indicating this station to be at the edge of the *E. huxleyi* bloom (Fig. 3A). Highest coccolithophore concentrations of up to 2250 cells ml<sup>-1</sup> were detected at station 2 in the upper water column suggesting that this station was situated well within the bloom. *Coccolithovirus* concentrations generally reflected the magnitude of *E. huxleyi* concentrations (Fig. 3B) ranging between  $8.6 \times 10^4$  and  $5.54 \times 10^5$  ml<sup>-1</sup> at station 1. Higher EhV concentrations were detected within station 2, ranging from  $3.5 \times 10^5$  to  $1.1 \times 10^6$  ml<sup>-1</sup>.

### Molecular characterisation of the EhV and *E. huxleyi* population

Denaturing gradient gel electrophoresis (DGGE) analysis was used to examine the extent of *E. huxleyi* genetic diversity within the bloom. Similar migration profiles, with each band corresponding to a single *E. huxleyi* genotype, were identified at each station and also with depth (Fig. 4); however, minor differences in migration profiles were also evident, indicating some variability in the *E. huxleyi* composition between samples. Clone libraries of the *gpa* 3'UTR, which encompassed the CMM marker sequence, were constructed for stations 1 and 2 at 10 m depth of the CTD samples as well as the Continuous Plankton Recorder (CPR) samples 55RV1 and 55RV5. The longest running and most spatially comprehensive programme for monitoring distribution and abundance of planktonic organisms is the CPR survey. The CPR was first used in 1931 with each CPR sample corresponding to 10 nautical miles of towed seawater (Batten et al., 2003). By using the CPR in conjunction with the CTD it was anticipated that additional data would be provided, corresponding to a greater volume/area of water sampled, to validate the structure of the *E. huxleyi* bloom event. A region of 106–115 clones was sequenced for each of the samples, with 25 genotypes amplified from station 1, 15 genotypes from station 2, 18 from 55RV1 and 15 from 55RV5 when the entire *gpa* 3'UTR PCR product was analysed (data not shown). When the CMM was analysed, which is the shorter 32 bp genotyping sequence compared to the longer sequence used for the DGGE, all 4 samples were found to be dominated by the same genotype, corresponding to CMM IV (Fig. 4). Good's coverage estimator was



**Fig. 1.** Weekly MODIS enhanced colour composites for 7–13 July, 21–27 July, 9–15 August and 21–27 August 2006 summarising information regarding the water mass studied in the Western English Channel. The enhanced composite of 21–27 July 2006 details the positions of the CTD stations, stations 1 and 2 (black dots) and CPR samples 55RV1 and 55RV5 (grey triangles). High reflectance (coccolith light scatter) can be observed as the “white water”. The absence of the “white water” in composite dated 21–27 August indicates the disappearance of the free coccoliths from the surface waters. Land masses are masked black.

used to determine the coverage obtained for the CMM clone libraries calculating that  $> 92\%$  of the *E. huxleyi* population had been represented for all samples, indicating that the majority of the diversity had been captured by this analysis.

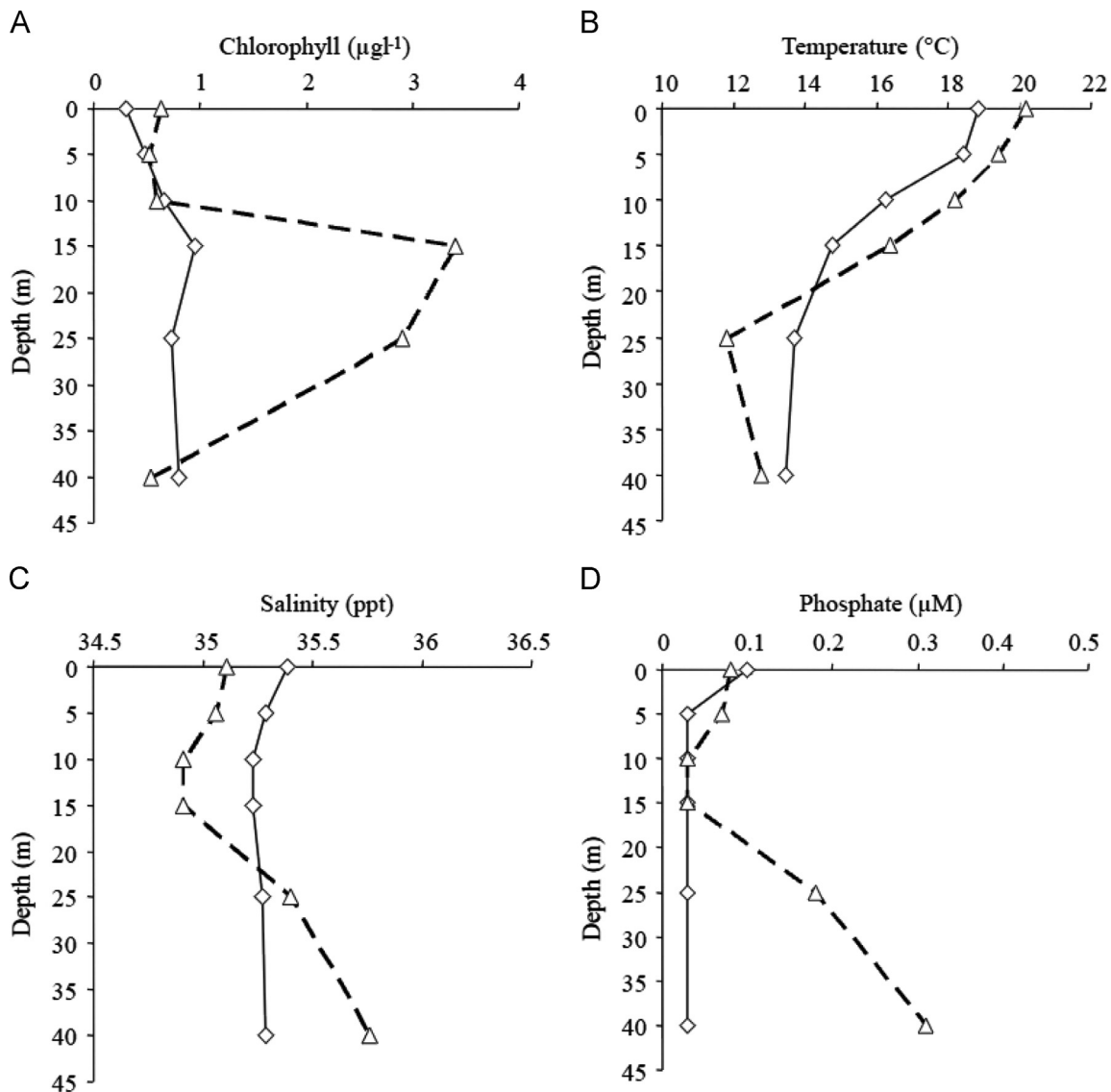
DGGE analysis of the EhV population using *mcp* revealed a highly dynamic virus population with every sample showing variations in DGGE migration profile (Fig. 5). Different profiles were seen between stations and also with depth. The DGGE profile for EhVs was complemented by clone libraries that were constructed for 10 m samples collected from stations 1 and 2. A total of 73 and 70 clones were sequenced for each library and entirely different virus communities were detected in each when the entire 280 bp PCR product was analysed, with no single genotype being shared between the 2 samples (Supporting information S1). Twenty eight different virus genotypes were detected at station 1, with one genotype dominating at 52%, and all of these sequences were unique when compared to isolate sequences in Genbank. 36 different genotypes were detected at station 2 with 2 genotypes dominating the population at 21% and 16%. Two of these genotypes matched sequences previously identified, when a shorter 99 bp sequence was analysed in accordance with those sequences deposited in Genbank (Supporting information S1). OTU58 which comprised 16% of the population at station 2 was identical to EhV

isolates from the English Channel, EhV86, EhV145 and EhV164, as well as sequences from Norway and the North Atlantic. OTU52, which comprised 6% of the population, was identical to OTU19, a sequence previously detected within a bloom in a Norwegian fjord (Martínez Martínez et al., 2007). Good's coverage estimator calculated that only 69% and 57% of the virus population had been represented by the clone library analysis for stations 1 and 2, respectively, suggesting that viral diversity had been under-sampled in these clone libraries.

## Discussion

Satellite imagery indicated that the bloom was within the secondary phase at the time of sample collection as high reflectance was detected at this time and the weeks following. This reflectance is caused by the coccoliths that have been shed from dead/dying cells creating a backscattering of light. *E. huxleyi* numbers, as enumerated by AFC also reflected those typically encountered during bloom events (Balch et al., 1991; Holligan et al., 1983). The DGGE profile for *E. huxleyi* showed a genetically stable population; however, genetic variability was readily detected between depths/stations.





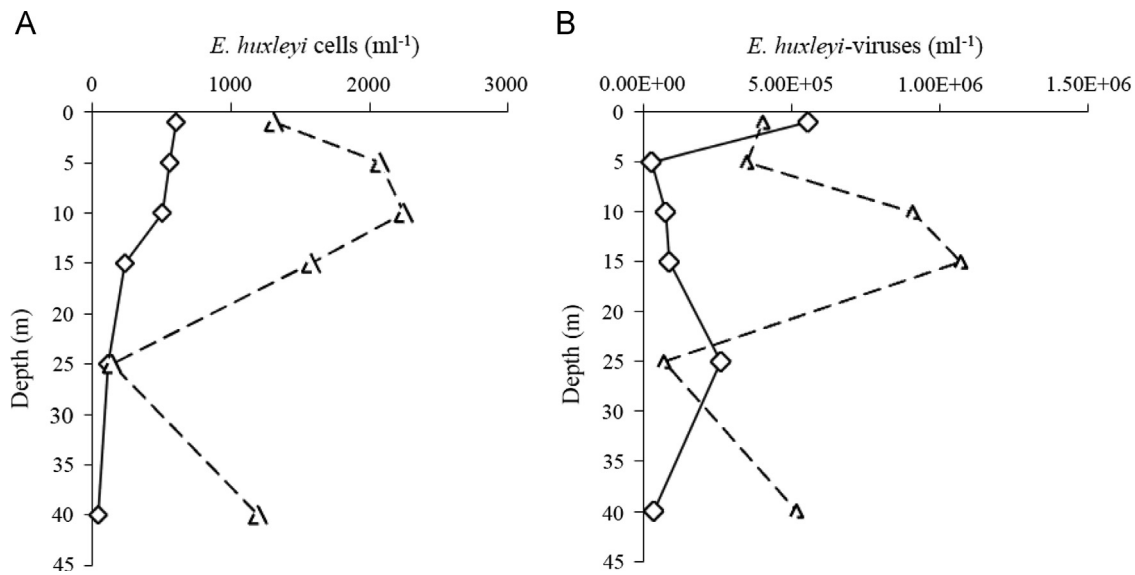
**Fig. 2.** Vertical distribution of chlorophyll (A), temperature (B), salinity (C) and phosphate (D) within two CTD stations sampled during the phytoplankton bloom in summer 2006 in the Western English Channel where  $\diamond$  is station 1 and  $\triangle$  is station 2.

The *E. huxleyi* population composition revealed that CMM IV was dominant in all four clone libraries, regardless of the sampling method employed i.e. CTD vs CPR. In particular the CPR samples, which correspond to a greater spatial scale of sampling, showed the dominance of CMM IV genotype across the bloom with it considered to have been ubiquitous both horizontally and vertically. Single gene markers do have limited use in population-related studies; however, microsatellite analysis of the samples also confirmed the presence of dominant alleles (Krueger-Hadfield et al., 2014). The dominant CMM IV genotype will be comprised of several microsatellite genotypes and these alleles are likely to have some sort of adaptive advantage over others which could possibly include faster growth rates, lower nutrient requirements, resistance to predation or to viral lysis, although this cannot yet be proven. However, CMM IV has been found to occur and dominate in a wide range of environmental niches, from cold to warm and often changeable environments (Krueger-Hadfield et al., 2014).

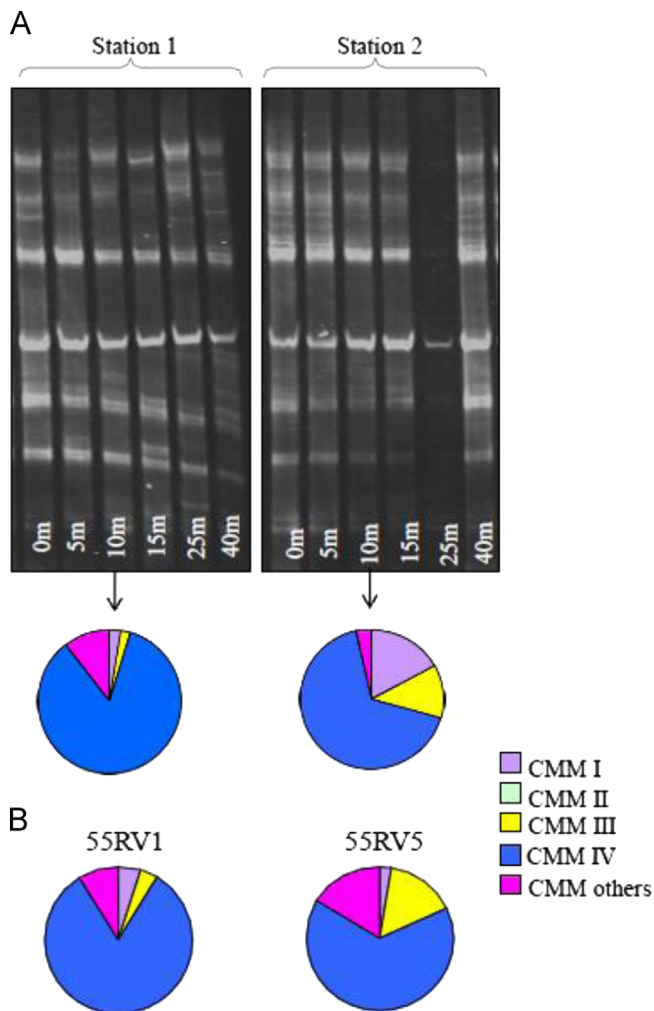
Extensive *Coccolithovirus* diversity was detected within the Western English Channel *E. huxleyi* bloom. High EhV diversity has previously been reported by Rowe et al. (2011) where they detected 35 unique MCP sequences in a transect of the North Atlantic; however, the sequences detected in this study were

detected during non-bloom conditions in the open ocean with the authors ascribing the high diversity to the nature of the environment sampled, i.e. the open ocean. Such a high number of EhV genotypes detected within a bloom, as seen in the present study (64 genotypes), have not previously been reported and this suggests that extensive combinations of virus host infections are at play within a bloom event.

Sequence analysis showed the 2 stations to have a completely different EhV population structure and all the sequences within station 1 were unique, revealing a completely different viral population here. Due to the nature of the sampling, station 2 was sampled after station 1, so it is important to acknowledge that the variations seen here between stations may partially be a reflection of temporal variability as well as spatial. Sorensen et al. (2009) saw changes in the EhV population in a mesocosm bloom within 2 h, so a time lag between stations is indeed likely to show differences; however, the variability detected with depth must represent spatial differences. Such definitive differences in EhV populations in different areas of the bloom are unlikely to be purely a function of temporal, i.e. progression of infection, as one would expect some overlap of latent periods and therefore some genotypes would be common between stations. Cell counts and

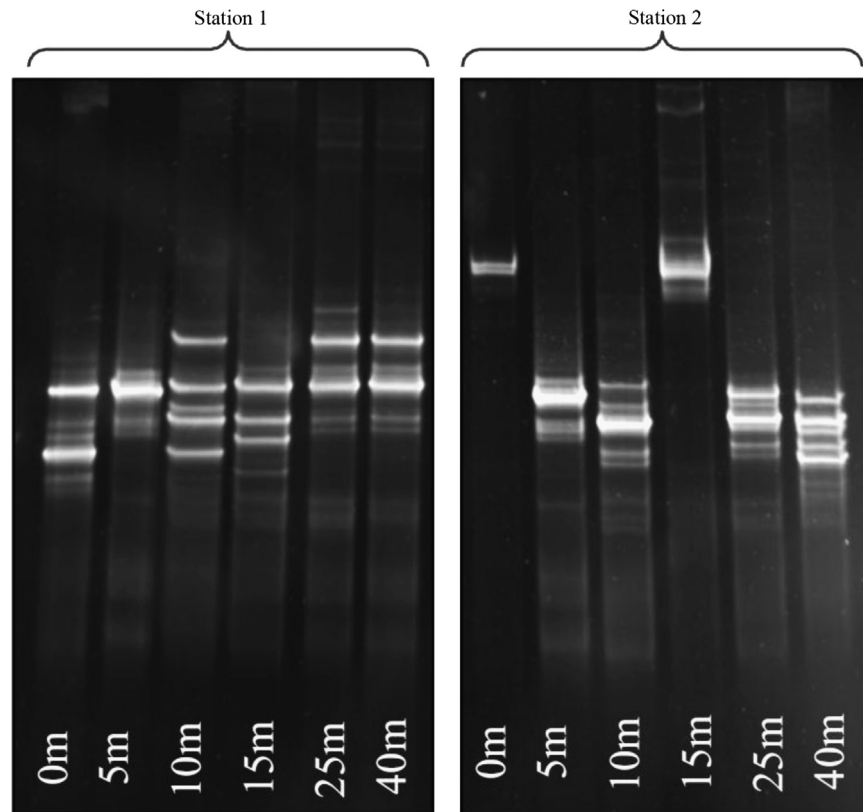


**Fig. 3.** Vertical distribution of coccolithophores and *Coccolithovirus* within the two CTD stations sampled during the phytoplankton bloom in summer 2006 in the Western English Channel where ◇ is station 1 and △ is station 2.



**Fig. 4.** (A) DGGE gel image and clone library population composition of *E. huxleyi*-amplified PCR products of *gpa* from the Western English Channel bloom, for each CTD stations at 6 different depths. The DGGE for each CTD stations is depicted and representative pie charts corresponding to the CMM clone libraries constructed for stations 1 and 2, 10 m depth are depicted beneath their corresponding DGGE profile. (B) Pie charts for CPR samples 55RV1 and 55RV5 corresponding to the clone libraries of CMM.

satellite imagery also suggest that the water was sampled during the secondary stage of the bloom. Detached coccoliths have been found to be attributable for the greatest amount of backscatter light, as detected by satellites (Balch et al., 1991; Holligan et al., 1993) and coccoliths are known to rapidly detach during stationary phase cultures (Balch et al., 1992). Therefore the areas of “white water”, as defined by the satellite imagery, are where free coccoliths are most abundant and therefore represent older parts of the bloom (Balch et al., 1991). It is at this stage of the bloom in mesocosm experiments that EhV diversity typically stabilises, if indeed they exert control over the bloom (Martínez Martínez et al., 2007). The water column sampled was not homogenous and graduations in salinity and temperature were evident with increasing depth as well as clear differences between stations. As the EhV population differences cannot be ascribed to unique host population niches within the bloom it is therefore hypothesised that small scale environmental heterogeneity within the water column is accounting for some of the variation in the EhV composition. High levels of inter-clonal variation have been reported within phytoplankton species, including *E. huxleyi* (reviewed by Wood and Leatham (1992)), with *E. huxleyi* clones showing variations in salinity tolerance and temperature-dependent growth rates. The environmental differences observed across the bloom potentially will affect the physiological state of the *E. huxleyi* hosts present which conceivably could make them more or less susceptible to infection, hence having a knock-on effect to the structure of the EhV population. Certainly, the lower depths (25 and 40 m) at station 1, which are more similar with respect to temperature and salinity, share a more similar EhV DGGE profile and the stability across this 15 m may be related to the more stable environment here which may be a response of the *E. huxleyi* population or indeed the EhV population itself. This hypothesis is in agreement with the studies of Martínez Martínez et al. (2012) and also that of Coolen (2011) who both recognised environmental factors, albeit on a more substantial scale, influenced the EhV composition in the environments they studied. It is important to note however that Martínez Martínez et al. (2012) found EhV strains in a North Sea bloom to be present from 5–50 m depth despite changes in the physical environment, including temperature (Wilson et al., 2002a), so the marked differences here are intriguing. It is possible that the persistence of EhVs throughout the water column in the North Sea bloom could actually be EhVs present within dead or sinking cells and the EhVs within the



**Fig. 5.** DGGE gel image of *Coccolithovirus*-amplified PCR products of *mcp* from the Western English Channel bloom, for both stations at 6 different depths.

secondary phase of the bloom in the Western English Channel are actually actively infecting EhVs.

Clearly complex virus interactions are at play within *E. huxleyi* bloom populations and EhV populations appear to be structured, not only by the presence of particular hosts but their external environment also appears to be an important factor for the persistence and potentially the infectivity/infection cycle of EhVs. Such dynamics of the coccolithoviruses may potentially influence whether a bloom is eventually terminated by viruses or not. If environmental heterogeneity, large or small, has such an impact on the EhV composition, if the environment does not favour an EhV that has traits that are more likely to make it successful, e.g. large burst size and short latent period, then the bloom may continue to persist for a longer duration. Other factors, such as grazers or competition between species, may then become more important in bloom termination. Indeed, the North Sea bloom demise was eventually attributed to grazing (Archer et al., 2001; Martínez Martínez et al., 2012) and it may be that the environmental changes i.e. the influx of warmer water, that re-structured the EhV population, contributed to this. Further studies investigating temperature effects and pressure effects, for example, on isolates and natural communities are warranted to help unravel the importance of these factors on virus-host interactions and, as such, their impact on these ecologically important phytoplankton blooms.

## Materials and methods

### Sampling

Seawater samples were collected during an *Emiliania huxleyi* bloom in the Western English Channel (Fig. 1). Sample stations were selected by using images derived from the ocean colour

sensor, Moderate Resolution Imaging Spectroradiometer (MODIS). Stations 1 and 2 were sampled using 10 l niskin bottles mounted on a 19+ Seabird CTD cast that measured temperature and salinity, collecting from 0 m, 5 m, 10 m, 15 m, 25 m and 40 m depths and samples. Due to sampling logistics, samples were collected consecutively with stations 1 and 2 collected at 10:12 and 20:30 on 26/07/2006 respectively. Water samples were stored in dark until filtration in the laboratory immediately following the field sampling i.e. 27/07/06 (a.m.). The bloom was also sampled using a CPR tow according to Batten et al. (2003). CPR samples 55RV5 and 55RV1 were collected at 23:06 on 26/07/2006 and 04:23 on 27/07/2006 respectively, with each CPR sample corresponding to 10 nautical miles of tow. CPR samples were analysed microscopically according to Batten et al. (2003) and were then stored at room temperature in dark until DNA extraction.

### Remote sensing

Satellite imagery was provided by the NERC Earth Observation Data Acquisition and Analysis Service (NEODAAS) and was subsequently processed for enhanced colour composites.

### Analytical flow cytometry (AFC)

All flow cytometry was performed on a FACScan flow cytometer (Beckton Dickinson, California, USA) with FACSflow as the sheath fluid and the standard filter set up on the CTD samples only. *E. huxleyi* numbers were determined according to Olson et al. (1993). Samples for enumeration of virus-like-particles and bacteria were fixed with glutaraldehyde at a final concentration of 0.5% for 30 min at 7 °C before being snap frozen and stored at –80 °C. Enumeration of virus-like particles was completed on thawed samples using the nucleic acid stain SYBR green according to the methods of Brussaard (2004).

## DNA extraction

21 water samples collected at all depths for stations 1 and 2 were vacuum filtered through 0.45 µm PALL Gelman filters and were subsequently stored at –20 °C until further processing on 22/09/2008. DNA was then extracted from these filters using the DNeasy blood and tissue DNA extraction kit (Qiagen) according to Sorensen et al. (2009). DNA was extracted from the CPR samples 55RV1 and 55RV5 according to Ripley et al. (2008)

## Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)

PCR/DGGE analysis of the CTD stations was carried out according to the protocol for *E. huxleyi* and *E. huxleyi* viruses (EhV) detailed in Schroeder et al. (2003) using primers specific to the calcium binding protein gene (*gpa*) for *E. huxleyi* and the major capsid protein (*mcp*) gene in EhV. PCR products from each depth and station were run on a 30–50% denaturing gel according to Schroeder et al. (2003) to visualise the respective population structures.

## Clone library construction and sequencing

Diversity of *E. huxleyi* and EhV communities was evaluated in four samples using a ~100 bp sequence of the *gpa* gene or ~280 bp region of the *mcp* gene: station 1; 10 m depth, station 2; 10 m depth, 55RV1 and 55RV5 (EhV analysis only carried out for stations 1 and 2). PCR was undertaken using the *gpa*-primers CBP\_F4 (5'-AGT CTC TCG ACG CTG CCT G-3') and CBP\_R4 (5'-CTA GCA CCA GTC TTT GGA CTT TGC-3') for *E. huxleyi* and major capsid protein primers MCP\_F1 (5' GTC TTC GTA CCA GAA GCA CTC GCT-3') and MCP\_R1 (5'-ACG CCT CGG TGT ACG CAC CCT CA-3') for EhVs. Reactions were carried out in 50 µl volumes containing 2 µl DNA template, 20 pmol Forward primer, 20 pmol Reverse Primer, 1 × *Taq* reaction buffer, 1.25 mM MgCl<sub>2</sub>, 0.0625 mM each deoxy-nucleotide triphosphate and 1 unit *Taq* polymerase (Promega). Reactions were carried out in a thermocycler (MJ research) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 40 cycles for *gpa* and 35 cycles for *mcp* of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension step at 72 °C for 5 min. PCR reactions were verified by gel electrophoresis on a 2% (w/v) agarose gel in 1 × TAE buffer and were subsequently viewed on a UV transluminator (BioRad). Bands corresponding to the *gpa* PCR product for *E. huxleyi* or the *mcp* PCR product for EhV were excised from the gel using a sterile scalpel and were purified from the agarose using the Qiaex II gel extraction kit (Qiagen) according to the manufacturer's instructions. Clone libraries were constructed using these purified PCR products using the TA cloning kit (Invitrogen) according to the manufacturer's instructions, with one clone library being constructed for each sample. Inserts were screened for using M13 PCR and positive PCR products were incubated with ExoSAP-IT (USB corporation) before being sequenced using the ABI Big Dye terminator cycle sequencing ready reaction kit version 3.1 (Applied Biosystems) at Gene-service, Cambridge, UK. Any novel sequences generated were deposited in Genbank under accession numbers GQ404381 to GQ404428 for *E. huxleyi* and GU936205 to GU936205 for EhV.

## Sequence data analysis

Sequences for each clone were manually verified and aligned using BioEdit ([www.bioedit.com](http://www.bioedit.com)). A sequence that varied by one or more nucleotides was considered to be a different genotype. Coverage of the clone libraries was estimated using Good's coverage estimator (Good, 1953) using the formula  $[1 - (n/N)] \times 100$ ,

where  $n$  is the number of sequences occurring once and  $N$  is the total number of sequences analysed.

## Acknowledgments

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2014.07.017>.

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