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Coccolithovirus-*Emiliania huxleyi* dynamics: an introduction to the coccolithovirocell

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With 5 figures

Abstract: Coccolithoviruses are large double-stranded DNA viruses that infect the globally ubiquitous coccolithophorid *Emiliania huxleyi,* a marine Haptophyte algae that forms mesoscale blooms. Coccolithoviruses are intrinsically linked to *E. huxleyi* blooms, providing an essential role in their succession dynamics, often responsible for their demise. The type species of the genus *Coccolithovirus* is EhV-86 which, along with all other coccolithovirus isolates to date, have been taxonomically assigned to the fringes of the *Phycodnaviridae,* a family of large DNA viruses that infect algae. Its genome is 407,339 bp and its most notable feature is the presence of a sphingolipid biosynthesis pathway. This and many other features of coccolithovirus genomes provide glimpses to a wider infection strategy that involves unique mechanisms for replication, survival, defence, evolution, dissemination, and communication. A combination of genomic and physiological tools has provided important insights into the infection process of this charismatic virus. The concept of the coccolithovirocell (CLVC) is introduced; the form of infected metabolic life distinct from the uninfected *E. huxleyi* host. It is argued that the coccolithovirocell is the integral cog that sustains the life of globally omnipresent blooms with Gaian equilibrium.

Keywords: *Emiliania huxleyi*, Coccolithovirus; sphingolipid, NCLDV, DMS, Phytoplankton

Introduction and scope of the review

Emiliania huxleyi, a marine Haptophyte, is the most ubiquitous coccolithophore in the oceans with a distribution that spans from the tropics to sub-polar regions. Satellite observations of mesoscale blooms of coccolithophores, dominated by *E. huxleyi* in temperate latitudes (Holligan et al. 1983; Holligan et al. 1993), illustrated what marine geologists had long known, that calcite derived from these vast blooms is an important component of the marine carbon cycle (Balch et al. 2005). *E. huxleyi* greatly impacts marine ecosystems and, in particular, the global carbon and sulphur cycles (Westbroek et al. 1993; Burkill et al. 2002). Blooms of this ubiquitous microalga are known to affect the oceanic carbon pump (Elderfield 2002; Rost & Riebesell 2004) and climate (Charlson et al. 1987). Vast coastal and mid-ocean populations of *E. huxleyi* often disappear, suddenly causing substantial fluxes of calcite to the seabed (Ziveri et al. 2000) and cloud-forming dimethyl sulphide to the atmosphere (Malin 1997; Evans et al. 2007). Ironically, the elaborate calcium carbonate armoury of liths covering the surface of *E. huxleyi* was always thought to prevent virus infection (Fig. 1a). Until about two decades ago, the mechanisms of *E. huxleyi* bloom disintegration were poorly understood, but it is now accepted that viruses are intrinsically linked to bloom crashes (Bratbak et al. 1993; Bratbak et al. 1996; Brussaard et al. 1996; Castberg et al. 2002; Jacquet et al. 2002; Wilson et al. 2002b; Schroeder et al. 2003; Vaughn et al. 2010; Lehahn et al. 2014).

The first suggestion of observation of virus-like particles (VLPs) in *E. huxleyi* was reported by Manton and Leadbeater (Manton & Leadbeater 1974). Although these workers did not show evidence of VLPs in *E. huxleyi*, they discussed that VLPs observed in *Chrysochromulina mantoniae* (plates 65 and 66 (Manton & Leadbeater, 1974)) were apparently similar to those of VLPs commonly found in moribund or dead *Coccolithus huxleyi* cells (not referenced). It was another 15–20 years before similar observations of VLPs up to 200 nm were reported and enumerated in high numbers in dying populations of *E. huxleyi* from samples collected in the North Sea (Brussaard et al. 1996) and Norwegian Fjords off south-west Norway (Bratbak et al. 1993; Bratbak et al. 1995). Bratbak et al. (1996) first reported the isolation of an *E. huxleyi*-specific virus by plaque assay, however they were unable to propagate it further for characterisation.

Parallel breakthroughs in their clonal isolation were reported in 2002 with lytic large double stranded DNA viruses, with genomes approximately 410 kbp, described from the Norwegian Fjords off Bergen, and the English

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Channel off Plymouth (Castberg et al. 2002; Wilson et al. 2002b). At the time they were taxonomically affiliated to the *Phycodnaviridae* (literally translated as "DNA viruses that infect algae") a genetically diverse, yet morphologically similar, family of icosahedral viruses that infect marine or freshwater eukaryotic algae (Wilson et al. 2009); and the genus *Coccolithovirus* was adopted (Cocco: derived from Greek *kokkis*, meaning berry or grain referring to their shape and Lith: from Greek *Lithos*, meaning stone) (Schroeder et al. 2002; Wilson et al. 2011).

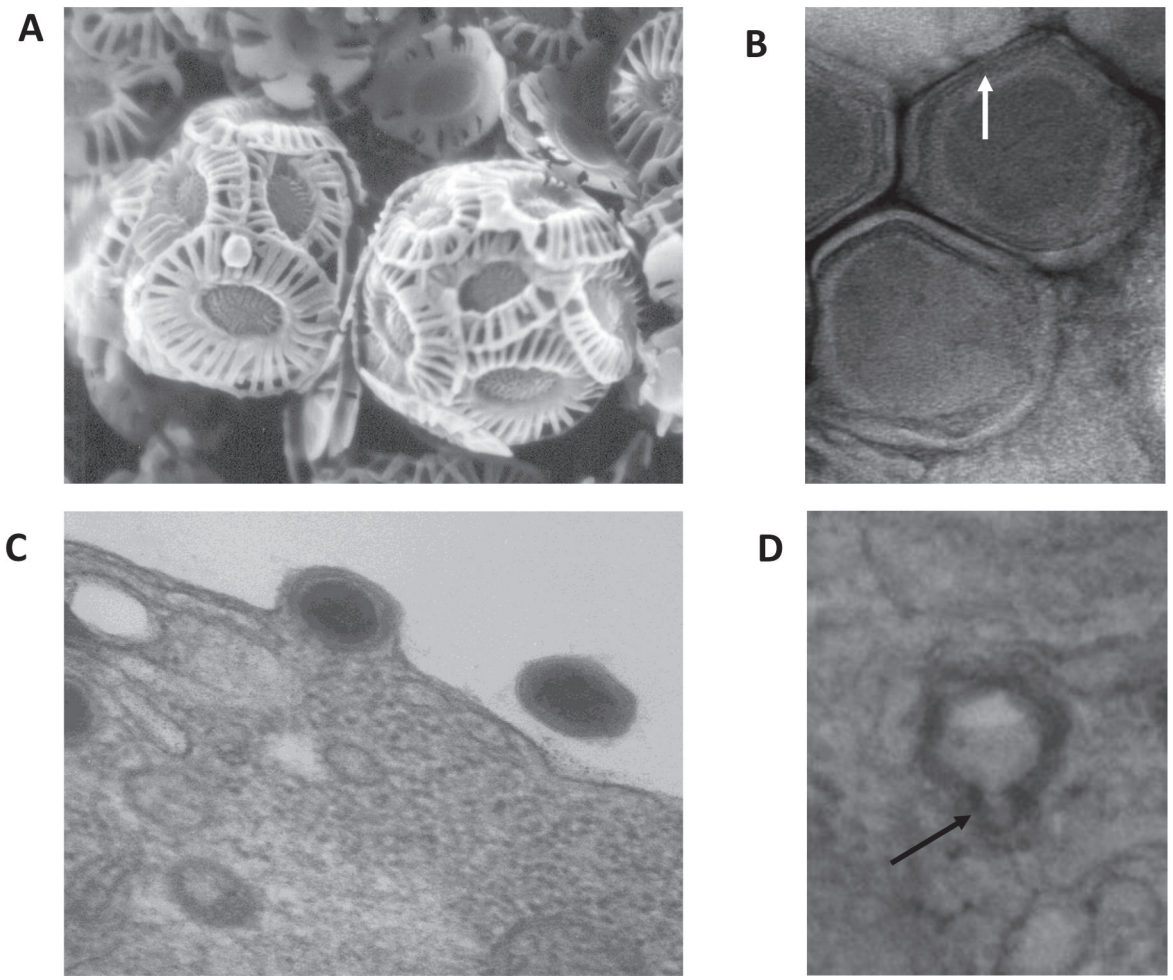

Fig. 1. Electron micrographs of coccolithovirus isolate EhV-86 **A)** This widely used image shows EhV-86 attached to the outside of a coccolith on a collapsed *E. huxleyi* cell. It is regularly erroneously reported as the virus initial attachment to the cell during infection. It is more likely a coincidental artefact of scanning electron microscopy, since we now know that the virus manoeuvres between the coccoliths for initial attachment. **B)** EhV-86 virion (170–190 nm in diameter) showing the putative internal lipid membrane (arrowed) **C)** EhV-86 virions being released into the extracellular space via a budding mechanism at 36 hours post infection. Virions gain an outer lipid host-derived membrane (same as arrowed in B). **D)** Putative tail structure (arrowed) can be observed in EhV-86 in the cytoplasm of infected *E. huxleyi* before release of progeny virions (approx. 3 hours post infection). **C** and **D** Adapted from Mackinder et al. (2009) with permission.

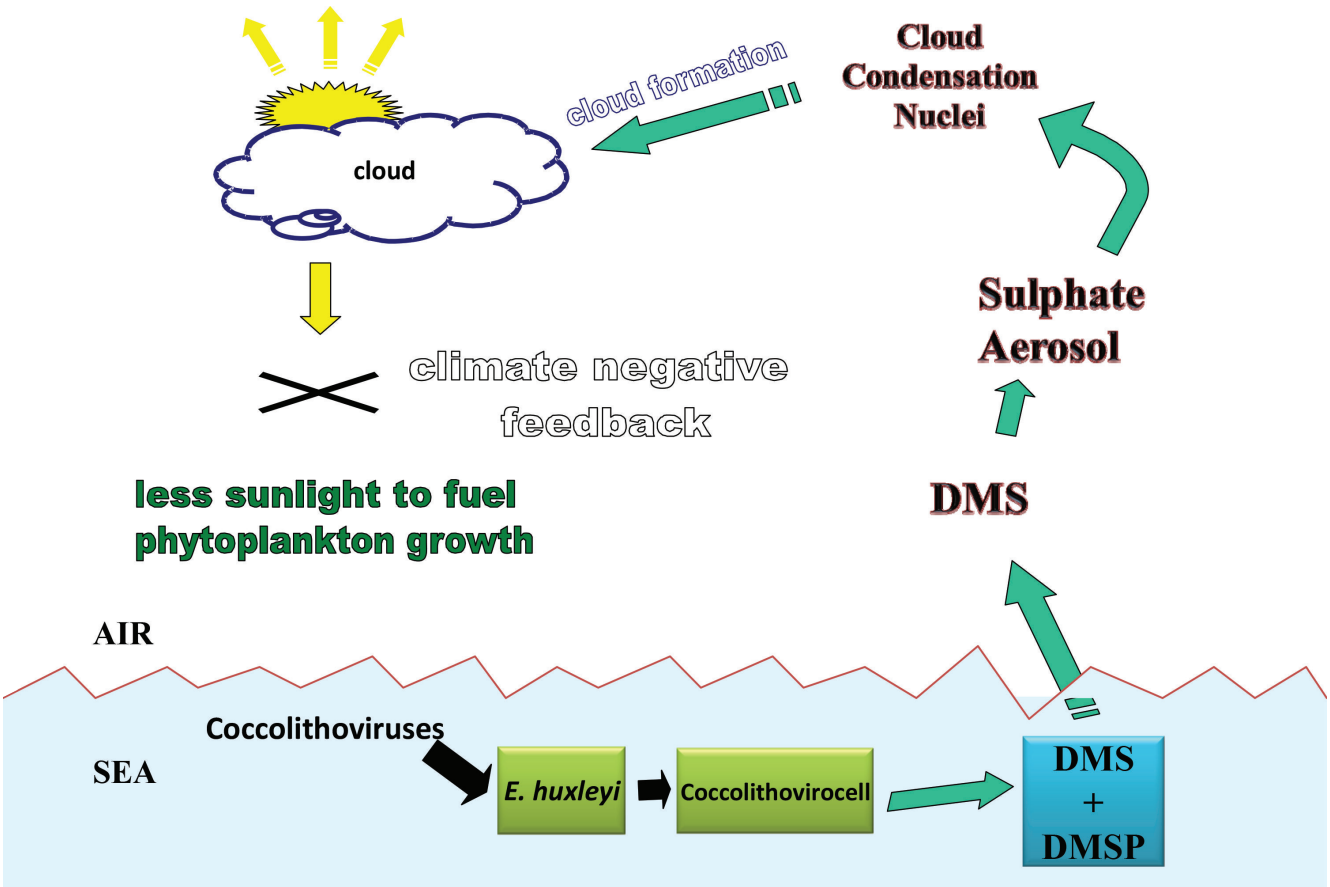

Fig. 2. The Gaia Hypothesis states that the Earth is a self-regulating organism. This may seem plausible when the activity of coccolithoviruses is taken into consideration. They kill continent-sized blooms of their host organism *E. huxleyi* to produce a flux of DMS into the atmosphere which subsequently form clouds and block the vital fuel of phytoplankton growth, sunlight.

All these early observations set the scene for a decade of discovery in what is a truly unique genus of viruses; certainly in terms of their size, which at the time of their discovery were the largest known virus genomes (though this has been usurped many times over following the discovery of the so-called giant viruses (La Scola et al. 2003; Claverie et al. 2006)); and also what was assumed as their vicarious role in global biogeochemical cycling. Research on the coccolithoviruses was underpinned by a desire to determine their ecological role in what was seen as Red Queen dynamics with *E. huxleyi* (Van Valen 1973), a common metaphor for a co-evolutionary arms race. This would take two generic methodological directions, molecular (genomic) and physiological; both clearly interlinked and driven by environmental selection pressures at a global scale. Certainly, proponents of the Gaia (Lovelock & Margulis 1974) and CLAW (Charlson et al. 1987) hypotheses would find a clear mechanistic roles for coccolithoviruses in a global self-regulatory ecosystem (Fig. 2). It has been said that the illuminated region is only a small part of the 3.7 km mean depth of the ocean, yet it houses several of the great engines of planetary control (Tett, 1990). Absorption of light energy by *E. huxleyi* is one of these engines, and coccolithoviruses can be considered as their lubricants, without which, the system would arguably cease to function. This review will summarize research of coccolithoviruses in the context of their ecological role; it will focus on the development of a molecular and physiological toolbox that has allowed us to garner functional information to start developing new theories on the global importance of these tiny giants and will introduce the concept of the coccolithovirocell (CLVC).

Anatomy of a tiny giant

Isolation of coccolithoviruses is relatively straight forward where 0.2 µm-filtered seawater from a dying *E. huxleyi* bloom is added directly to an exponentially growing culture

of the host (note many coccolithoviruses will be caught on a 0.2 μm filter, but the concentration of lytic viruses in these scenarios is typically so high, enough get through to kill the culture). If lytic viruses are present, host *E. huxleyi* culture crash is typically observed within 3–5 days. Enrichments have also been used to increase chances of virus adsorption to hosts (Vaughn et al. 2010). To ensure clonal isolation it is best to use plaque assay purification (Schroeder et al. 2002; Wilson et al. 2002b; Vaughn et al. 2010), although dilution to extinction techniques have also been used (Castberg et al. 2002). In a unique exploitation of biological concentration processes, Frada et al. (2014) isolated coccolithoviruses from the microbiome of copepods that had been feeding on infected *E. huxleyi* in a natural bloom; the infected cells were naturally concentrated in the copepod guts, reportedly enhancing the ability to isolate viruses.

Once you have a source of infectious coccolithoviruses, successful lysis of host cultures is typically obvious from cell clearance, and viruses can be easily enumerated by flow cytometry following SYBR Green I staining, by virtue of a relatively high side-scatter signal compared to other viruses (Marie et al. 1999; Brussaard et al. 2000; Jacquet et al. 2002; Wilson et al. 2002a; Wilson et al. 2002b; Brussaard 2004; Mojica et al. 2014). The virions have icosohedral symmetry, they range from 150–200 nm in diameter and they are tailless until they are internalized during infection (Fig. 1d). Their capsid is surrounded by a lipid envelope (Fig. 1b, c) comprised largely of glycosphingolipids, but also lesser amounts of polar glycerolipids (Vardi et al. 2009; Fulton et al. 2014); and they enter the *E. huxleyi* host via either an endocytotic or an envelope fusion mechanism (Fig. 3) (Mackinder et al. 2009). Coccolithovirus internalization and virion breakdown takes place within the host on a timescale of seconds. Between 3–4 h post-infection, virus progeny are released *via* a budding mechanism (Fig. 1c) during which virions become enveloped with host plasma membrane. This propagation strategy is different from any other algal viruses studied to date, indeed, coccolithoviruses appear to have an entry/exit strategy more analogous to animal-like Nucleocytoplasmic Large DNA Viruses (NCLDVs) (Mackinder et al. 2009). Virus particles continue to be released in a chronic style for a further 24–48 hours before the host cells finally disintegrate. One isolate from the Gulf of Maine (not phylogenetically characterised), had a much faster lysis period with host lysis occurring within 5 hours post infection (Vaughn et al. 2010). Typically, high burst sizes in the region of 500–1000 virions per infected cell are observed and are thought to be facilitated by autophagy (Schatz et al. 2014); a process usually associated with defence against cellular stress. In host range studies, all coccolithoviruses isolated only infect a narrow range of *E. huxleyi* strains, no other microalgae tested are susceptible. It is noteworthy that *E. huxleyi* strains that contain high DMSP lyase activity (Steinke et al. 1998) are not susceptible virus infection and has led to suggestions that metabolites in the DMS production pathway have an antiviral effect (Evans et al. 2006b).

Despite the relative ease of isolation, there are actually very few coccolithovirus isolates in circulation and largely derive from the English Channel (Schroeder et al. 2002; Wilson et al. 2002b); South Western Norwegian Fjords (off Bergen) (Castberg et al. 2002); Gulf of Maine (Vaughn et al. 2010); and some isolates from the North Atlantic (Rowe et al. 2011; Frada et al. 2014). Diagnostic markers designed on the coccolithovirus-specific: Major Capsid Protein (MCP) gene (Schroeder et al. 2002; Larsen et al. 2008); Serine Palmitoyltransferase (SPT) gene (Pagarete et al. 2009; Nissimov et al. 2013); and phosphoglycerate mutase (PGM) gene (Coolen 2011) are all able to amplify coccolithovirus sequences from a wide range of oceanographic provinces suggesting a wide temporal (as much as 7000 years in the Black Sea (Coolen 2011)) and spatial diversity of coccolithoviruses throughout the Atlantic region (Schroeder et al. 2003; Martínez Martínez et al. 2007; Larsen et al. 2008; Rowe et al. 2011; Martínez Martínez et al. 2012; Nissimov et al. 2013; Frada et al. 2014; Highfield et al. 2014). Host-virus interaction analyses using tools such as denaturing gradient gel electrophoresis (DGGE) as well as metagenomic analyses, have reported a significant decrease in coccolithovirus diversity during the progression of bloom events followed in experimental mesocosms (Schroeder et al. 2003; Martínez Martínez et al. 2007; Pagarete et al. 2014); however, significant changes in diversity can even occur over time scales as short as a few hours (Sorensen et al. 2009). From an initial high diversity, a few dominant ecotypes eventually dominate as the bloom develops. In open ocean situations, often with low concentrations of host *E. huxleyi* cells, coccolithovirus diversity appears to remain high (Rowe et al. 2011; Nissimov et al. 2013; Highfield et al. 2014), perhaps driven by stable co-existence equilibrium (Thyrhaug et al. 2003). Though, if you conduct temporal analysis of a naturally occurring bloom, it is possible to detect fluctuations in the coccolithovirus diversity in relation to the host population (Martínez Martínez et al. 2012). This latter study was interesting since the underlying highly dynamic situation between coccolithoviruses and *E. huxleyi* hosts as revealed by DGGE analyses was completely masked from the relatively mundane numerical data that was collected in parallel that revealed only minor changes (Wilson et al. 2002a). Clearly suggesting that a wide range of data, including physicochemical and biological parameters, should be collected in parallel to help interpret the role of coccolithoviruses (Lehahn et al. 2014). Increasing use of metagenetic and metagenomic technologies will likely supersede gel-based fingerprinting methods such as DGGE. Indeed next generation sequencing will enable deeper inspection of the microdiversity of virus-host dynamics and may improve our understanding of the stable equilibrium often observed during bloom development (Pagarete et al. 2014).

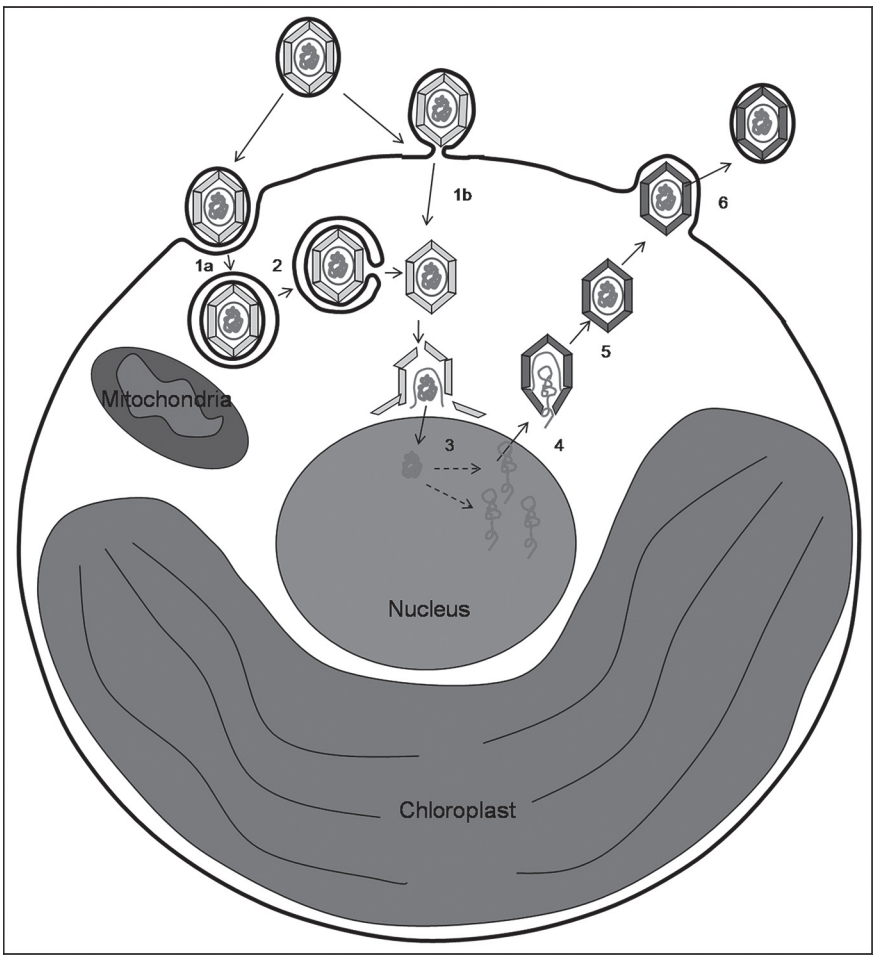

Fig. 3. Schematic of the proposed life cycle of coccolithovirus isolate EhV-86. Enveloped EhV-86 enters *E. huxleyi* with an intact capsid and nucleoprotein core either by an endocytotic mechanism (step 1a) followed by fusion of its envelope with the vacuole membrane (step 2) or by fusion of its envelope with the host plasma membrane (step 1b). The viral capsid encapsulated nucleoprotein core rapidly targets the nucleus where capsid breakdown releases the viral genome (step 3). The viral genome enters the host nucleus where early promoter sequences are expressed by host RNA polymerase. Mid-late genes are expressed by viral RNA polymerase within the cytoplasm where capsid assembly takes place, possibly by filling of a pro-capsid with viral DNA and core proteins (step 4). Early assembled viruses are transported to the plasma membrane (step 5) where they are released by a budding mechanism (step 6) (from Mackinder et al. (2009) with permission).

Taxonomically, the genus *Coccolithovirus* has been assigned to the family *Phycodnaviridae* (Wilson et al. 2011). Based on DNA polymerase sequence, the phylogenetic affiliation of coccolithoviruses is still unresolved, and effectively sits out on a limb (Fig. 4) highlighting the novelty of this charismatic genus of viruses. Coccolithoviruses clearly sit within the major group of large DNA viruses termed Nucleocytoplasmic large DNA viruses (NCLDVs) (Iyer et al. 2001; Allen et al. 2006c; Iyer et al. 2006; Koonin & Yutin 2010). The taxonomy of this group is in constant flux with competing terms being proposed with the fast rate of discovery of giant viruses. These include the "Megavirales" which encompasses the NCLDV group and new discoveries based on a comprehensive set of core genes (Colson et al. 2013); and "Megaviridae" a clade of giant viruses deeply branching between domains Archaea and Eukarya domains, and controversially exhibiting the topology of a fourth domain in the Tree of Life (Claverie 2013). Though Yutin et al. (2014) refutes the fourth domain hypothesis, instead presenting evidence that these NCLDVs evolved from smaller DNA viruses and suggest that universal genes have been independently acquired by giant viruses from their eukaryotic hosts. Whatever the case, it is clear that coccolithoviruses represent a phylogenetic enigma sitting at the fringes of these groups. There is however, very clear evidence of horizontal gene transfer between coccolithovirus EhV-86 and its *E. huxleyi* host (Monier et al., 2009), however, these are genes involved in sphingolipid biosynthesis rather than universal virus genes, further suggesting the uniqueness of coccolithoviruses.

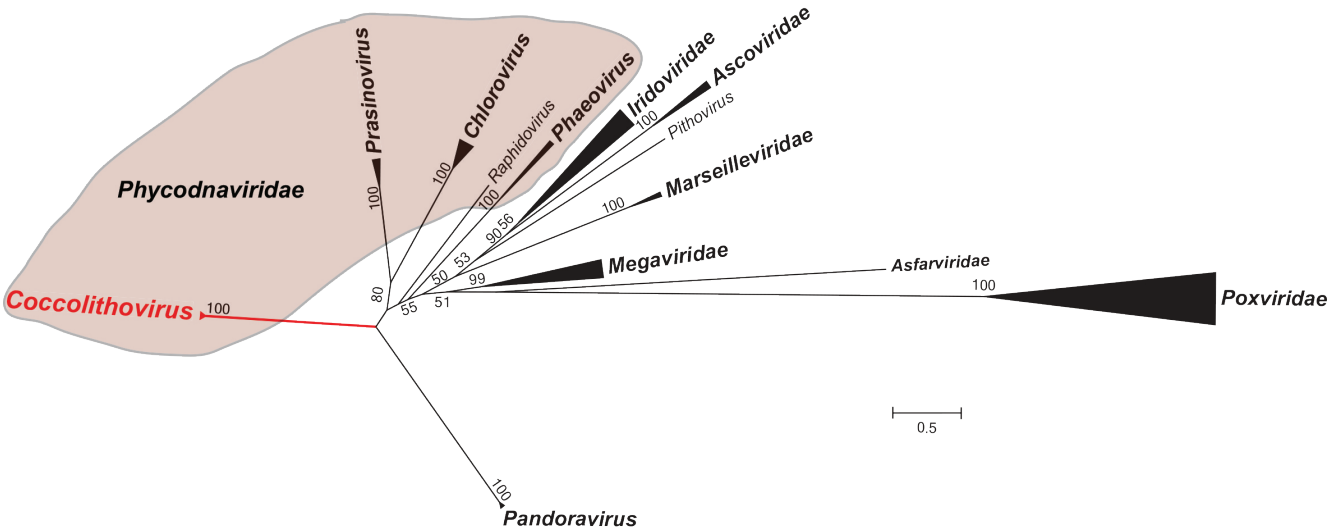

Fig. 4. Phylogenetic relationships among NCLDV DNA Polymerase B sequences based on ML inference. 968 positions were included in the analysis, bootstrap values >50 are displayed at the nodes. Coccolithovirus DNA Pol B sequences are all mostly identical, with EhV-163 showing some variation compared to all the other coccolithovirus sequences in the database. Scale bar corresponds to 0.5 amino acid substitutions per site. Courtesy of Ilana Gilg, Bigelow Laboratory for Ocean Sciences.

Genomics to function

A significant milestone in the analysis of coccolithovirus EhV-86 was the sequencing of it's 407,339 bp genome (Wilson et al. 2005). At the time it was the second largest virus genome ever sequenced; that was soon to change since it was at the dawn of the giant virus revolution (La Scola et al. 2003; Raoult et al. 2004; Claverie et al. 2006), but soon became labelled the "tiny giant" (Wilson et al. 2009). Among the 472 protein coding sequences (CDSs or predicted genes) of the EhV-86 genome was a range of unexpected genes; most notably those involved in biosynthesis of ceramide, a sphingolipid known to induce apoptosis. The first and rate-limiting step in the pathway is catalysed by serine palmitoyltransferase (gene ehv050), further functional characterization of the gene revealed it exhibited a novel substrate preference for myristoyl-CoA rather than palmitoyl-CoA (Han et al. 2006). Existence of a functional viral sphingolipid pathway provided clues to physiological observations being conducted around the same time focussing on the role of host programmed cell death activation as a mechanism to prevent viral infection (Bidle & Falkowski 2004; Bidle et al. 2007). Now with the presence of a virally encoded sphingolipid pathway, it opened the possibility that the virus could circumvent the host's antiviral strategy; essentially an arms race with ecological significance, akin to Red Queen theory (Van Valen 1973; Vardi et al. 2009; Bidle & Vardi 2011; Bidle 2015). In addition, viral glycosphingolipids had the potential to act as diagnostic lipid biomarkers to specifically detect virus infection in the ocean (Vardi et al. 2009). Though it was cautioned that the EhV-86 genome only encodes a subset of the biosynthetic apparatus required to generate a novel sphingolipid, and it has been suggested its synthesis is likely the result of coordinated interactions between *E. huxleyi*- and coccolithovirus-encoded enzymes (Michaelson et al. 2010). Experimental evidence from *E. huxleyi*-dominated seawater mesocosms off the coast of Norway revealed a critical role for glycosphingolipids and programmed cell death in regulating *E. huxleyi*-coccolithovirus interactions at both transcriptional (Pagarete et al. 2009; Pagarete et al. 2011) and physiological (Vardi et al. 2012) resolution. This was supported by culture studies in parallel with a combination of comprehensive transcriptomic and metabolomic assessment that demonstrated how rapid remodeling in *E. huxleyi* primary metabolism redirects essential substrates to coccolithovirus-derived sphingolipid biosynthesis, eventually leading to virus assembly (Rosenwasser et al. 2014). Their data also demonstrated the central role of sterol metabolism in coccolithovirus assembly and host defense.

The majority of EhV-86 CDSs exhibited no similarity to proteins in the public databases; a mere 21% of the CDSs contain protein-protein BLAST results that matched an E value lower than 0.01. Uniquely for algal viruses, EhV-86 also contained 6 RNA polymerase subunits and a novel promoter, suggesting that EhV-86 encoded its own transcription machinery. Norwegian coccolithovirus isolates

EhV-99B1, EhV-163, EhV-V1, and EhV-V2 uniquely encode an intein (a selfish DNA element found within coding regions of host proteins) in the DNA-dependent RNA polymerase subunit 2 gene, compared to the English Channel coccolithovirus counterparts (Allen et al. 2011); which along with the absence of a phosphate permease (Fig. 5), is a significant differentiator between English Channel and Norwegian coccolithovirus isolates. EhV-86 homologues of known genes also encoded mRNA capping enzyme, DNA polymerase, DNA ligase, DNA topoisomerase, sphingolipid biosynthesis enzymes, eight proteases, major capsid protein, phosphate permease, 2 thioredoxins, ribonuclease and ribonucleoside-diphosphate reductase. Many of these CDS's were part of the core set of conserved genes found in the NCLDVs which affirmed it's phylogenetic affiliation (Allen et al. 2006c). In addition, 3 distinctive repeat families were found within the genome and have been predicted to function as non-coding promoter elements, proline rich coding regions and an origin of replication (Allen et al. 2006d). A further 8 coccolithovirus isolates were partially sequenced (EhV-84, EhV-88, EhV-201, EhV-202, EhV-203, EhV-207 and EhV-208, all isolated from the English Channel off Plymouth; and EhV-163 and EhV-99B1 isolated from south west Norway off Bergen) (Allen et al. 2006a; Nissimov et al. 2011a; Nissimov et al. 2011b; Nissimov et al. 2012a; Nissimov et al. 2012b; Pagarete et al. 2012). Although their genomes are largely structurally similar to EhV-86 (reviewed in Liu et al. (2015)), hybridisation of their DNA to an EhV-86 microarray illustrated some of the subtle differences between the genomes (Fig. 5) (Allen et al. 2007).

Use of the same microarray provided a detailed temporal transcription profile of EhV-86 CDSs over the first 4 hours of infection; essentially the latent period prior to viral release (Allen et al. 2006b). Virus transcription was divided into 2 broad stages: a primary stage in the first hour post infection in which a distinctive subgroup of localized CDSs associated with a putative promoter element (Allen et al. 2006d) are transcribed; and a secondary stage over the next 3 hours during which CDSs are transcribed regardless of their genomic location. The function of the primary stage is difficult to ascertain, since the vast majority of the CDSs expressed have little or no database homologues. CDSs from this region have been shown to have some of the highest levels of expression during the infection process (Wilson et al. 2005), presumably due to their early and then constant high levels of expression, suggesting that they are of vital importance to the infection strategy. It is noteworthy that this region is also the most genetically diverse in the coccolithovirus pan genome (Fig. 5) (Allen & Wilson 2006; Allen et al. 2007; Pagarete et al. 2014). Separate culture studies reveal that viral transcription completely overwhelms host transcription between 12–24 hours post infection (Kegel et al. 2007; Kegel et al. 2010).

Virus life, physiological plasticity and the coccolithovirocell (CLVC) concept

One of the more persuasive arguments for viruses as life is to think of the infected cell as a novel organism that produces virions (Forterre 2010, 2011). Hence, it is the infected cell or the so-called virocell that is the alternative form of life, rather than thinking of virions (virus progeny) as life. It is akin to a multicellular organism producing genetic material in the form of sperm. Forterre (2010, 2011) describes the 2 forms of life as ribosome encoding organisms (cells) and capsid encoding organisms (virocells). The infected cell is a multifaceted life form with a unique viral-driven metabolism compared to its uninfected wild-type counterpart. It has unique strategies for replication, survival, defence, evolution, dissemination, and communication; and is epitomised by the coccolithovirus-*E. huxleyi* virus pairing which I propose we term the coccolithovirocell (CLVC). The physiological thesis for the CLVC concept is explored in excellent recent reviews (Bidle & Vardi 2011; Bidle 2015). Such ideas build on cellular process studies of CLVCs and the environmental factors that influence them and include: photophysiology and role of UV (Jacquet & Bratbak 2003; Bidle et al., 2007; Kimmance et al. 2014); the interplay between biogenic sulphur compounds such as dimethylsulphide (DMS) and reactive oxygen species (ROS) (Bratbak et al. 1995; Wilson et al. 1998; Wilson et al. 2002a; Evans et al. 2006a); the role of biogenic sulphur compounds in active antiviral activity (Evans et al. 2006b) and grazing activity (Evans et al. 2006b; Evans et al. 2007); fatty acids metabolism (Evans et al. 2009); photosynthetic pigment transformation (Llewellyn et al. 2007; Bale et al. 2013); and calcification regulation (Frada et al. 2012; Kegel et al. 2013). In addition, there are a wide range of environmental factors that influence infectivity including: inorganic nutrient ratios and availability (Egge & Heimdal 1994); influence of trace metals (Egge & Heimdal 1994; Gledhill et al. 2012); CO_2 availability (and acidification) (Carreira et al. 2013); selective grazing of CLVCs by microzooplankton (Evans & Wilson 2008); grazing and dispersal of coccolithoviruses by zooplankton (Frada et al. 2014); and temperature control of resistance mechanisms (Kendrick et al. 2014). Resistance mechanisms also include the so-called Cheshire Cat hypothesis (Frada et al. 2008) where controls on host *E. huxleyi* life cycle dynamics (Frada et al. 2012) can influence the infection susceptibility. Haploid stages change the morphological appearance of the cell to small, scaled and flagellated form that appear to inhibit coccolithovirus infection. Physiological resistance associated with PCD cellular processes (Bidle et al. 2007; Bidle & Kwityn 2012) are also important and discussed in depth by Bidle (2015).

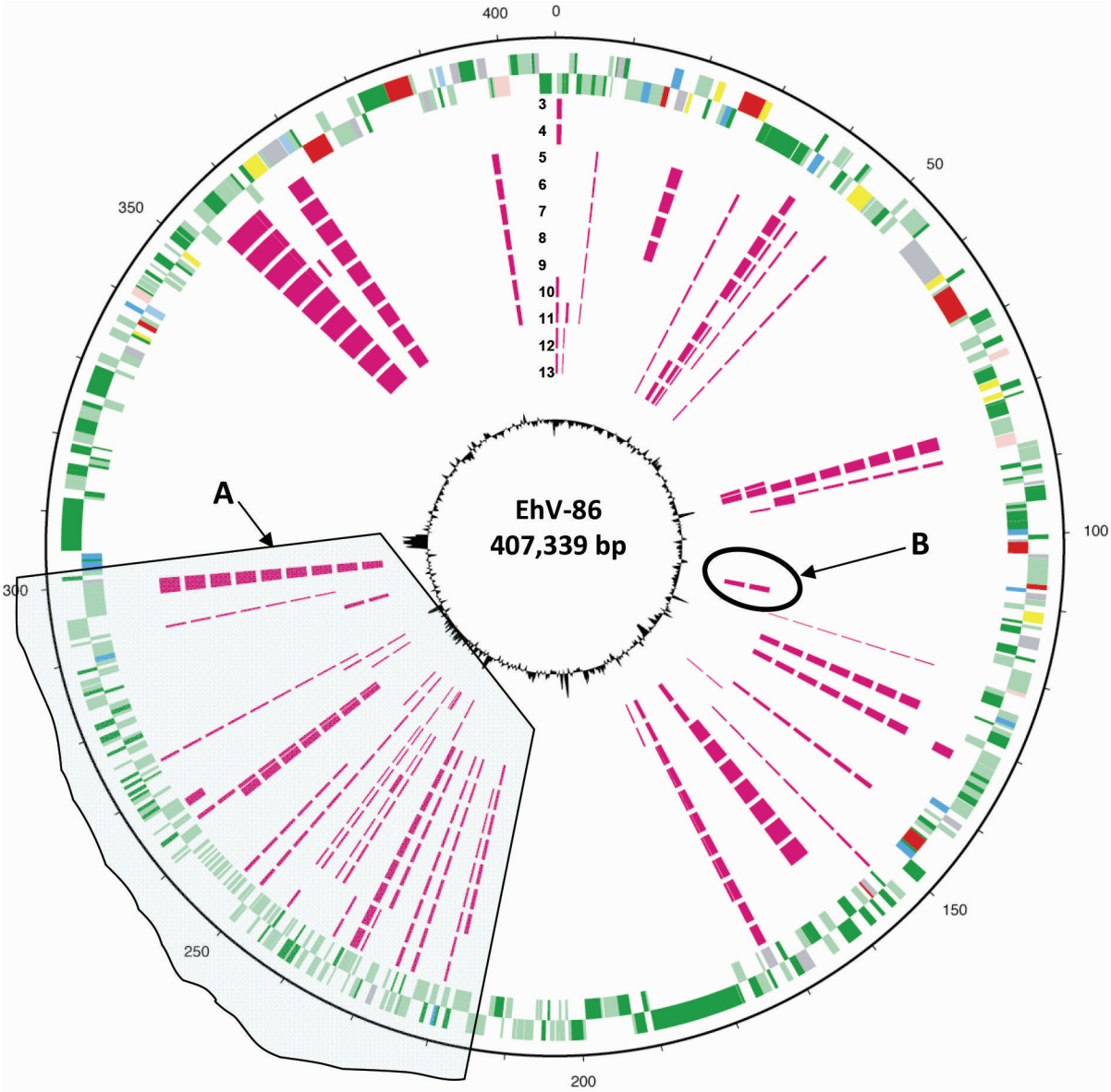

Fig. 5. Circular representation of the EhV-86 genome. The outside scale is numbered clockwise in kbp. Circles 1 and 2 (from outside in) are CDSs (forward and reverse strands, respectively), starting with CDS ehv001 at position 276 bp. CDSs are colour coded by putative function: light green, no know function; dark green, no known function but contains transmembrane helices; grey, miscellaneous; sky blue, degradation of large molecules; red, information transfer; yellow, metabolism; pink, virus specific; and light blue, kinases. Circles 3–13 are the positions, relative to the EhV-86 genome, of ***negative*** hybridising CDSs from microarrays hybridised with labelled genomic DNA from EhV-84, EhV-88, EhV-201, EhV-202, EhV-205, EhV-206, EhV-207, EhV-208, EhV-209, EhV-163 and EhV-V2, respectively. Circle 14, G+C content. All English Channel isolates except EhV-163 & EhV-V2 which are from south-west Norway off Bergen. **A)** Region of high intensity of variation between virus isolates; genes in this region are also transcribed early during the infection cycle and contain a novel promotor element thought to facilitate their high levels of expression. **B)** Phosphate permease gene absent in Norwegian isolates EhV-163 and EhV-V2. Adapted from Allen et al. (2007).

Future directions

Already a great deal has been discovered about the coccolithovirocell, yet we have only scratched its surface. Arguably the biggest challenge will be to decipher the functional capacity of the coccolithovirus pan genome in what remains a hotbed of novelty with between half and three-quarters of its genes as database orphans. We already know that at least 75% of coccolithovirus genes are transcribed during infection (Allen et al. 2006b); understanding their function should be a priority for future research. Advances in genetic tools, in particular those that will allow us to conduct gene knockouts using e.g. RNAi, will significantly improve our chances of understanding gene function. There is also scope for commercial biodiscovery of novel CLVC metabolites (Reid et al. 2011). With an ancient evolutionary lineage, it is likely CLVCs have experienced a wide range of environmental selection pressures over time that will have signatures of their impact in the coccolithovirus pan genome. This may account for some of the 25% untranscribed genes. Currently, with rapid environmental change (Danovaro et al. 2011), a warmer more acidic ocean may functionally express these genomic signatures to resurrect ancient physiological conditions in the CLVC that will provide protection through defence, replication, signalling, or resistance co-evolutionary strategies, hence ensuring longer term survival of CLVCs through Red Queen scenarios. The coccolithoviruses remain a phylogenetic enigma and tight group at the fringes of the *Phycodnaviridae* (Fig. 4). Although it is useful having model CLVC systems to work with, it is surprising given the large volume of research in this area there are actually so view isolates in circulation, and they are all genetically very similar (Fig. 5) from geographically narrow collection sites. It would be interesting to increase the geographical range to isolate a wider diversity of coccolithoviruses; progress in flow cytometry sorting techniques may provide the tool for isolation (not forgetting traditional isolation techniques). In addition, virus sorting by flow cytometry can be used as a high throughput tool to investigate wide temporal and spatial changes in coccolithovirus and CLVC diversity (Martínez Martínez et al. 2011; Yoon et al. 2011; Martinez et al. 2014). Indeed, given their distinct flow cytometry signatures (Jacquet et al. 2002; Martínez Martínez et al. 2011), coccolithoviruses and CLVCs are the ideal models for single virus sorting applications and for determining the genetic and physiological interplay between as yet undiscovered virus-host systems. We don't currently understand how the numerous environmental selection pressures impact on CLVCs in particular the role of inorganic nutrient availability; grazing pressure and its knock on effects in terms of virus dissemination; role of changing weather patterns on aerosol formation and again its role in virus dissemination (Aller et al. 2005). Many of the factors that ensure the annual reappearance of coccolithoviruses are not understood, although open questions, all likely interlinked involve: resistance mechanisms; cell-signalling (are there novel signalling or sensing infochemicals?); physical transport processes (down- and upwelling, and horizontal currents); and even adoption of secondary infection or persistence strategies through links with grazers akin to terrestrial insect systems (Cory 2015).

Conclusion

Coccolithoviruses are lubricants in the engine that drives *E. huxleyi* bloom dynamics. The integral cog in the engine is the virus-infected *E. huxleyi* cells, the coccolithovirocells (CLVCs). Paradoxically, CLVCs are necessary for the survival of these globally omnipresent Gaian features. Their unique genetic structure encodes a constantly evolving physiological and metabolic superhighway that feeds into macro-scale weather processes; global biogeochemical cycling; food chain dynamics; and even geological processes. It is a truly remarkable virus that has far-reaching global consequences.

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