

1 **Intragenus competition between coccolithoviruses: an insight on how a**
2 **select few can come to dominate many.**

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19 **ABSTRACT**

20 Viruses are a major cause of coccolithophore bloom demise in both temperate and sub-
21 temperate oceanic regions. Here we observe the competitive interactions between two
22 coccolithovirus strains, EhV-86 and EhV-207 during the infection of the cosmopolitan
23 marine micro-alga *Emiliana huxleyi*. EhV-207 displayed a shorter lytic cycle and increased
24 production potential than EhV-86, and was remarkably superior under competitive
25 conditions. The observation of such clear phenotypic differences between genetically distinct,
26 yet similar, coccolithovirus strains by flow cytometry and quantitative real time PCR allowed
27 links to the burgeoning genomic, transcriptomic and metabolic data currently available to be
28 made. We speculate on the tentative identification of the genetic source of the phenotypic
29 variation observed and the factors driving their selection (such as the functional relevance of
30 the encoded sphingolipid biosynthesis associated genes). This work illustrates that, even
31 within a family, not all viruses are created equally and the potential exists for relatively small
32 genetic changes to infer disproportionately large competitive advantages for one
33 coccolithovirus over another, ultimately leading to a few viruses dominating the many.

34

35 **Keywords:** coccolithoviruses, infection dynamics, competition, phenotypes, *E. huxleyi*

36 INTRODUCTION

37 Viruses are a major cause of coccolithophore bloom demise in both temperate and sub-
38 temperate oceanic regions (Bratbak et al., 1995; Martinez et al., 2007; Brussaard et al., 2008;
39 Sorensen et al., 2009). Their role in regulating coccolithophore populations has been firmly
40 established via studies of natural environmental systems (Wilson et al. 2002, Schroeder et al.
41 2003, Martinez et al. 2007), and induced semi-natural blooms in the Norwegian fjords
42 (Bratbak et al. 1993, Jacquet et al. 2002, , Pagarete et al. 2009, , Kimmance et al. 2014). In
43 addition to the direct reduction in total cell abundance, the species-specific nature of viruses
44 leads to the regulation of interspecies competition and succession within a mixed
45 phytoplankton community (Brussaard, 2004; Fuhrman, 1999).

46

47 Within a coccolithophore bloom, the success of specific coccolithovirus strains vary and is
48 influenced, among other factors, by the type of host strains present and the physico-chemical
49 environment in which they are found (Wilson et al., 2002a; Martinez et al., 2007, 2012; Rowe
50 et al., 2011; Coolen, 2011). Under bloom conditions, there is typically an initial and diverse
51 pool of low abundance virus (Sorensen et al. 2009) and host strains (Schroeder et al. 2003), a
52 subset of which become more dominant than others as the bloom progresses (Martinez et al.
53 2007; Sorensen et al. 2009), and an indication that some virus genotypes win over others
54 (Pagarete et al, 2014, Highfield et al, 2014). Virus abundance and diversity is controlled by
55 external (environmental) influences that drive evolutionary processes (Coolen et al. 2011;
56 Martinez et al. 2012). However, the molecular diversity observed within samples is a mere
57 snapshot of that particular time, and although useful, it does not reveal how and why
58 coccolithovirus strains vary with regards to infection strategies and mechanisms. Given that
59 there are clear genetic differences between coccolithovirus isolates (Allen et al. 2006,
60 Nissimov et al. 2011, 2012, 2014, Pagarete et al. 2013) it is likely that within a mixed

61 community of coccolithoviruses during bloom conditions, subtle variations in their
62 phenotypic properties, i.e. infection and lysis rates, may have a profound impact on shaping
63 the ultimate genetic richness and composition of the host and viral community.

64

65 However, the fundamental question remains as to how differences in coccolithovirus
66 phenotypes influence host growth dynamics, virus succession and population survival. A
67 previous study by Bidle and Kwityn (2012) demonstrated that different *Emiliania huxleyi*
68 strains vary in their susceptibility to infection by a single coccolithovirus strain, yet, to date,
69 the significance of coccolithovirus strain-specific differences have not yet been fully
70 investigated. Whether the enormous genetic potential contained within their large genomes
71 (~400-500 MBs) produces variations in the infection strategies employed by different
72 coccolithovirus strains, remains to be determined. Like in all other virus systems, intra-
73 familial competition among coccolithoviruses for successful infection and replication must
74 exist. The molecular encoded components at the interface of this ongoing evolutionary
75 struggle are currently unknown, yet our current whole genomic knowledge of a dozen
76 coccolithovirus strains suggests their identification is now possible.

77

78 Here we use an experimental approach to assess the phenotypic differences in two *E. huxleyi*
79 virus (EhV) strains, and their manifestation during infection of a single host strain. Infection
80 experiments were performed with two coccolithovirus strains which were originally isolated
81 from the same location in the English Channel: EhV-86 and EhV-207, and have been shown
82 previously to successfully infect the same host (*E. huxleyi* CCMP 2090) (Nissimov et al,
83 2011; 2012), yet differ in genomic composition (Nissimov et al. 2014). For simplicity, it was
84 decided that the focus would be to identify any potential differences in viral production and
85 host lysis during infection, and not to consider host strain variability here. The main goal was

86 to reveal the competitive interactions of two genetically distinct viruses infecting side by side,
87 and assess the implications for host growth and virus productivity. Strain specific primers
88 were designed for each coccolithovirus, and the change in EhV abundance was monitored for
89 one week post- virus addition by means of quantitative real time PCR (qPCR) and Analytical
90 Flow Cytometry (AFC).

91

92 **MATERIALS AND METHODS**

93 **Culture conditions and experimental setup**

94 Prior to the start of the experiment, cultures of *E. huxleyi* CCMP 2090 were grown in filtered
95 (30 kDa, TFF GE Healthcare), autoclaved natural seawater enriched with f/2 nutrients
96 (Guillard 1975), in 2 L InforsMinifors chemostat bioreactors (INFORS UK Ltd). Cultures
97 were maintained with a light and dark cycle of 16:8 hours, at a white light intensity of ~86
98 $\mu\text{M photons m}^{-2} \text{ s}^{-1}$, and a temperature of 18°C, with continuous gentle mixing and aeration.
99 *Emiliania huxleyi* abundance was measured daily using analytical flow cytometry (AFC, see
100 below for protocol), and when cellular density reached $1.5 \times 10^6 \text{ cells mL}^{-1}$ (i.e. beginning of
101 exponential growth, $\mu = > 0.7$), aliquots of 100 mL were distributed into 12 polystyrene,
102 sterile, 250 mL tissue culture flasks (Greiner, CellStar). The 12 algal subculture flasks and
103 nine additional flasks containing only f/2 media (controls; Table S1) were then returned to the
104 same light and temperature conditions as above, and left to acclimatise for 48 h.

105

106 The virus pathogens EhV strain EhV86 (Wilson et al. 2002) and EhV-207 (Nissimov et al.
107 2012) were obtained from the Plymouth Marine Laboratory virus collection and for each EhV
108 strain, a fresh batch of viral lysate was prepared prior to experiments to ensure a high amount
109 of infective virions. For this, 500 mL of an exponentially growing *E. huxleyi* CCMP2090
110 culture ($\sim 4 \times 10^6 \text{ cells mL}^{-1}$) was infected with 5-10 mL of an EhV lysate stock. When the

111 culture had lysed (complete loss of pigmentation, monitored using analytical flow cytometry,
112 AFC, see below) lysates were gently vacuum-filtered through 0.2 µm pore size sterile filters,
113 Millipore Express) to remove *E. huxleyi* cell debris, and stored in the dark at 4°C until
114 required. On the day of the experiment (t₀), the experimental flasks were set up to represent
115 seven different treatments (A-G) as described in Table S1. Ten minutes before the first
116 sampling point (t₀), flasks 1-3 (A) were inoculated with EhV-86 lysate at a virus:host ratio of
117 1:1; flasks 4-6 (B) were inoculated with EhV-207 at a virus:host ratio of 1:1; and flasks 7-9
118 (C) were inoculated with a combination of both EhV-86 and EhV-207, providing an overall
119 virus:host ratio of 1:1 (with a 1:1 ratio of each virus). Flasks 10-12 (D) were virus controls
120 that contained no host (only culture media), but were inoculated with the same volume of
121 EhV-86 lysate as flasks 1-3. Likewise, flasks 13-15 (E) contained only culture media and an
122 inoculum of the same volume of EhV-207 lysate as flasks 4-6. Flasks 16-18 (F) contained
123 only culture media and an inoculum of combined EhV-86 and EhV-207 lysates equivalent to
124 that in flasks 7-9. Finally, flasks 19-21 (G) were the negative controls containing host culture
125 with an addition of EhV-86 and EhV-207 lysates equivalent to treatments (C) and (F),
126 however prior to addition lysates were inactivated through a series of treatments: autoclaving
127 at 126°C, filter sterilization through 0.2 µm sterile cellulose acetate syringe filter (Gilson),
128 followed by overnight exposure to UV light in a PCR station (Labcaire), prior to their
129 addition to the control flasks.

130

131 **Sampling procedure**

132 Once experimental conditions were set up in the flasks the cultures were left for ten minutes
133 to allow viruses to establish initial attachments/infections with the host cells. Then 3 mL of
134 sample were taken out from each flask at the following time intervals: 0 (t₀), 1 h (t₁), 2 h (t₂),
135 3 h (t₃), 4 h (t₄), 8 h (t₅), 12 h (t₆), 24 h (t₇), 48 h (t₈), 72 h (t₉), 96 h (t₁₀), 120 h (t₁₁), 144

136 h (t12), and 168 h (t13). The 3 mL samples were then further divided into three sub-samples:
137 1 mL was fixed with 0.5% (final conc.) glutaraldehyde for the enumeration of host abundance
138 using analytical flow cytometry (AFC), and 1 mL was centrifuged at 20,000g for 30 sec., of
139 which the top phase was carefully removed into a sterile, 2 mL Eppendorf polypropylene
140 tube, fixed with 0.5% (final conc.) glutaraldehyde, and the pellet re-suspended in 1 mL of
141 DNA free water and fixed in 0.5 % glutaraldehyde for the subsequent enumeration of free
142 and attached VLPs respectively by AFC. All samples were snap frozen in liquid nitrogen
143 (LN₂) and stored at -80°C for further analysis. The same procedure was performed with the
144 third 1 mL subsample, with the exception of the addition of the glutaraldehyde, as these
145 samples were used for DNA extractions and subsequent quantitative real time PCR analysis.

146

147 **Enumeration of host and virus abundance**

148 All samples were analysed *en masse* following the conclusion of the experiment. Fixed,
149 frozen samples were defrosted at room temperature and then analysed using AFC following
150 standard protocols (Marie et al. 2000; Brussaard et al. 2004). Samples were analysed on a
151 FACScan flow cytometer (Becton Dickinson, Oxford, UK) equipped with a 15 mW laser
152 exciting at 488 nm and with a standard filter set up. Counts of *E. huxleyi* were conducted at
153 high flow rate (~ 80 $\mu\text{L min}^{-1}$) and files were analysed using WinMDI 2.8 software (Joseph
154 Trotter, [<http://facs.scripps.edu>]). For virus analysis, sub-samples were diluted 500-fold with
155 TE buffer (10 mmol L⁻¹ Tris-HCL pH8, 1 mmol L⁻¹ EDTA), stained with SYBR Green 1
156 (Molecular Probes; Marie et al., 2000) at a final dilution of 5×10^{-5} the commercial stock,
157 incubated at 80°C for 10 min in the dark, then allowed to cool for 5 min before flow
158 cytometric analysis. Samples were analysed at a flow rate of ~ 20 $\mu\text{L min}^{-1}$ and EhVs were
159 identified on the basis of their RALS versus green fluorescence. Data files were analysed
160 using WinMDI 2.8 software (as above). The two EhV strains could not be separated by their

161 AFC profiles in the combined EhV-86 and EhV-207 treatments, as they gave identical
162 fluorescence vs. scatter signatures.

163 **Probe design and optimisation**

164 The selection for specific primer probes for the two viruses EhV-86 and EhV-207 was
165 performed on the IMG/ER analysis platform, in which the total protein coding genes of EhV-
166 86 and EhV-207 were BLAST searched against each other with a maximum E value of $1e^{-05}$
167 and minimum percent identity of 40%. Following this analysis, EhV-86 and EhV-207 strain
168 specific genes were identified (i.e. ehv290 [510 bp] and EQVG00465 [1059 bp],
169 respectively) and selected for the design of strain specific PCR primers. The final sequence
170 lengths that were to be amplified by PCR/quantitative real time PCR were 209 bp and 353 bp
171 for EhV-86 and EhV-207 respectively (primers sequence: qPCR(EhV-86)-F [5'-
172 GCACAACCTTCAACAATTCG-3']; qPCR(EhV-86)-R [5'-
173 TCAGCTCAACTTTTGGATCA-3']; qPCR(EhV-207)-F
174 [5'-CATAGGGTTGGCAATATTCA-3'] and qPCR(EhV-207)-R [5'-
175 TTCGAAACAACCTTGGTCAAC-3'], Sigma-Aldrich Company Ltd).

176

177 To first establish that the designed primers were strain specific, a standard PCR was
178 performed on fresh lysate stocks of EhV-86 and EhV-207 with the primers for ehv290 (i.e.
179 qPCR(EhV-86)-F and qPCR(EhV-86)-R) and EQVG00465 (i.e. qPCR(EhV-207)-F) and
180 qPCR(EhV-207)-R). Reactions were conducted in a VWR JENCONS Uno Thermal Cycler in
181 25 μ L final volume reactions (unless otherwise stated). PCR reactions were set as follows: 1
182 μ L of virus lysate template (or ~ 50 ng μ L⁻¹ of extracted DNA) was mixed with 5 μ L of 1 \times
183 PCR reaction buffer (Promega), 1.5 μ L of 25 mM MgCl₂, 0.1 μ L of *Taq* DNA polymerase
184 (Promega), 2 μ L of 10 μ M of each primer, 1.25 μ L of 2 mM dNTPs and DNA-free molecular
185 biology grade water (Sigma-Aldrich). PCR was conducted in triplicate and the conditions

186 were as follows: an initial denaturation step at 95°C for 3 min, followed by 34 cycles at 95°C,
187 60°C and 74°C for 30, 60 and 90 sec respectively, and a final cycle at 95°C, 60°C and 74°C
188 for 30 sec, 5 min and 5 min respectively.

189

190 **DNA extraction from top phase and pellet samples**

191 Phenol-chloroform DNA extraction was performed on the samples intended for qPCR
192 analysis based on the detailed protocol described by Schroeder et al, (2002). Briefly, 0.5 mL
193 aliquots from the 1 mL top phase and pelleted sub-samples collected at the different time
194 points during the experiment were placed into sterile tubes in a heating block at 90°C for 1 min
195 and then transferred onto ice for a further minute, repeating this three times. Then to the tubes
196 were added 20 µL 0.5 M EDTA pH 8.0 at a final concentration of 20 mM, 5 µL proteinase K at a
197 final concentration of 50 µg mL⁻¹, and 25 µL of 10% SDS at a final concentration of 0.5%, and
198 incubated in a water bath for 1 h at 65°C. After the incubation the tubes were transferred onto ice
199 and gently mixed with 60 µL of phenol. Then 500 µL of chloroform/isoamyl alcohol (24:1) was
200 added to the tubes, mixed gently, after which they were centrifuged at 10,000 rpm for 5 min.
201 After removing the top phase into a clean Eppendorf microfuge tube and adding 500 µL of 7.5 M
202 ammonium acetate, they were left at room temperature for 30 min. A centrifugation step at
203 10,000 rpm for 15 min followed after which the supernatants were placed into clean 2 mL
204 Eppendorf tubes. To these tubes 1 mL of 100% ethanol (ETOH) was added, leaving them to
205 precipitate for 3 h at 4°C, and then centrifuging them at 13,000 rpm for 30 min after which the
206 supernatants were removed and discarded. Finally, the pellets were washed by centrifugation for
207 10 min with 500 µL of 70% ETOH and air dried overnight. The genomic DNA pellets were re-
208 suspended in 50 µL of TE buffer and then quantified by spectrophotometer (i.e. NanoDrop) and a
209 1% agarose gel electrophoresis.

210

211 **Quantitative real-time PCR**

212 Quantitative real-time PCR (qPCR) assays on extracted DNA samples were carried out in
213 optical-grade 96-well plates in an ABI PRISM®7000 Sequence Detection System (Applied
214 Biosystems, UK) with the Qiagen Quantifast Sybr Green PCR kit containing a ready to use
215 master mix. The calibration curve (or standards) for the qPCR to which each experiment
216 DNA sample was compared to consisted of triplicates of the serial dilutions (10^{-1} to 10^{-10}) of
217 amplified and gel extracted PCR products of ehv290 (for EhV-86) and EQVG00465 (for
218 EhV-207) at initial DNA concentrations of $65.7 \text{ ng } \mu\text{L}^{-1}$ and $57.4 \text{ ng } \mu\text{L}^{-1}$ respectively (i.e.
219 predicted DNA copy number of 2.91×10^{11} and $1.51 \times 10^{11} \mu\text{L}^{-1}$ respectively). The
220 calibration curve diluted samples were loaded each time on the same plate as the DNA
221 samples from the experiment in order to reduce bias occurring due to small shifts in
222 fluorescence signal from one 96 well plate to another. The reactions of both standards and
223 samples consisted of $12.5 \mu\text{L}$ of Sybr Green master mix, $0.5 \mu\text{L}$ of primer qPCR(EhV-86)-F
224 or qPCR(EhV-207)-F (at a final conc. of $0.2 \mu\text{M}$), $0.5 \mu\text{L}$ of primer qPCR(EhV-86)-R or
225 qPCR(EhV-207)-R (at a final conc. of $0.2 \mu\text{M}$), $1 \mu\text{L}$ of template DNA (sample, standard, or
226 NTC [no template control]) and $10.5 \mu\text{L}$ of RNase free water (final volume of $25 \mu\text{L}$). The
227 thermal cycling conditions (on the ABI PRISM 7000 cycler) were as follows: an initial cycle
228 of 95°C for 10 min followed by 40 cycles at 95°C for 30 sec and 60°C for 30 sec. The
229 automated generation of the calibration curve by the ABI PRISM 7000 sequence detection
230 system allowed the logarithmic plotting of each standard concentration against the cycle
231 number at which the detected fluorescence signal increased above the threshold value- CT.
232 Then, the Sequence Detection System software calculated the target gene DNA copy number
233 (or concentration) from the CT value obtained for each of the samples with unknown
234 concentration. The calibration curve slope was used to determine the reaction efficiency (E)
235 using the following equation: $E = -1 + 10^{(-1/\text{slope})}$. For instance, if E equalled to 1 then this meant
236 a 100% product doubling in each amplification cycle.

237

238

239 **Oligonucleotide specificity**

240 PCR amplification of EhV-86, EhV-207 and EhV-86+EhV-207-combined lysates revealed
241 that the newly designed qPCR primers were strain specific (Fig. S1). When each set of
242 primers was used in a sample from which the target strain was not present (EhV-86 or EhV-
243 207), amplification was not detected; i.e. the amplification was specific to the set of primers
244 used and only samples that had the target strain produced PCR amplicons. The amplified
245 products of the EhV-86 and EhV-207 specific targets corresponded to the predicted size
246 fragments of 209 and 353 bp for EhV-86 and EhV-207 respectively.

247

248 **Calibration curve efficiencies of the quantitative real time PCR sample analysis**

249 The calibration curves (Fig. S2) that were conducted to establish the optimum conditions for the
250 qPCR analysis indicated that under the described PCR conditions, the serial dilutions of the
251 known concentrations of ehv290 (for EhV-86) and EQVG00465 (for EhV-207) PCR generated
252 DNA was log-linear for both, with a correlation coefficient (R^2) of 0.99. The calculated efficiency
253 ($E = -1 + 10^{(-1/\text{slope})}$) of the reactions were 102.21 % and 88.70 % respectively. The calibration
254 curves were interrogated to allow the accurate quantification of DNA template in both single
255 and dual infection samples.

256

257 **RESULTS**

258 **Genetically distinct viruses produce contrasting host lysis rates**

259 *Emiliana huxleyi* CCMP 2090 abundance increased steadily in all flasks during the first 24 h
260 to an average (\pm SD) maximum of $2.6 \times 10^6 \pm 4 \times 10^5$ cells mL⁻¹ (Fig. 1). However, by day 3,
261 both the EhV-207 and EhV-86+EhV-207-infected populations crashed dramatically, with a

262 96 % loss of cells in these flasks from $2.48 \times 10^6 \pm 2.73 \times 10^5$ (SD) to $9.83 \times 10^4 \pm 2.87 \times 10^4$
263 cells mL⁻¹ (SD). The crash was simultaneous between these two treatments (Fig. 1). In
264 contrast, during the same time period (24-72 h post-addition of virus), there was a 14%
265 increase in *E. huxleyi* abundance in the EhV-86 infected cultures to 3.15×10^6 cells mL⁻¹
266 $\pm 1.41 \times 10^5$ (SD). However by 96 h host density also began to decrease in the EhV-86
267 infected cultures, but at a much slower rate (~17 % reduction in host cell density) compared
268 to the EhV-207 and EhV-86+EhV-207-infected cultures (Fig.1). These differences were also
269 evident from the fluorescence profiles of *E. huxleyi* derived during the AFC analysis (Fig. 2)
270 in which the mean cellular red fluorescence (proxy for chlorophyll *a*) at 72 h post-infection,
271 was much lower in the EhV-207 and EhV-86+EhV-207-infected cultures than in those
272 infected only with EhV-86. By the end of the experiment (168 h), the majority of the *E.*
273 *huxleyi* cells in the EhV-86 infected cultures had lysed (Figs.1, 2). However a small,
274 relatively healthy (as indicated by high cellular red fluorescence observed during AFC
275 analysis) host population remained in the EhV-86 infected cultures, and the average (\pm SD)
276 cell abundance was 2.77×10^5 ($\pm 6.89 \times 10^4$ mL⁻¹), as opposed to 2.52×10^2 ($\pm 3.36 \times 10^1$ mL⁻¹)
277 and 1.18×10^3 ($\pm 1.24 \times 10^3$ mL⁻¹) in the EhV-207 and EhV-86+EhV-207-infected cultures
278 respectively. With regards to the control cultures that contained inactivated EhVs, *E. huxleyi*
279 abundance remained high at the end of the experiment with 4.32×10^6 cells mL⁻¹ $\pm 3.04 \times 10^5$
280 (SD), (Fig. 1, 2).

281

282 EhV abundance decreased in all non-control flasks two hours post-infection to an average
283 (\pm SD) of $2.91 \times 10^5 \pm 1.42 \times 10^4$ mL⁻¹ (Fig. 3). This suggests that by two hours post-virus
284 addition, almost half (45%) of the inoculated EhVs were either attached to the host cell
285 receptors or had penetrated into the cells for replication. The first round of mass EhV release
286 from the infected cells in all treatments was 3 h post-addition (Fig. 3). After this point there

287 appeared to be a separation between EhV treatments, with the samples containing EhV-207
288 producing a higher number of virions compared to those containing EhV-86. However, this
289 was not statistically significant until (8 h post-addition) when there was significantly more
290 free EhVs in the EhV-207 and EhV-86+EhV-207 infected cultures than in the EhV-86
291 infected treatments ($P < 0.05$). The trend of significantly lower and slower virion
292 production/release from the EhV-86 infected cultures continued until the end of the
293 experiment 7 days post-addition ($P < 0.01$). At this point, the amount of free EhV mL^{-1} in the
294 EhV-86 infected cultures ($5.19 \times 10^7 \pm 3.88 \times 10^6$ SD) was on average 92% less than in the
295 EhV-207-infected ($6.58 \times 10^8 \pm 1.09 \times 10^8$ SD) or EhV-86+EhV-207 ($7.04 \times 10^8 \pm 8.55 \times 10^7$
296 SD) combined treatments (Fig. 3). Throughout the experiment, there was no difference in the
297 number of EhV between cultures infected by EhV-207 and those infected with both EhV-
298 86+EhV-207 combined, with the exception of one hour post-infection, where the number of
299 free EhVs in the combined virus treatment was significantly lower ($P < 0.05$) (Fig. 3).

300

301 **Quantifying strain-specific differences in virus genome copy number**

302 Quantitative real time PCR analysis (qPCR) was used to determine EhV-86 and EhV-207
303 genome copy number (GCN) in both cell-free and cell-associated fractions. Total GCN
304 estimates were derived from a combination of the two fractions. Cell-associated GCN will
305 include genomes found within virions adsorbed to the cell surface, unreleased intracellular
306 virions, as well as unpackaged genomes undergoing replication. Cell-free GCN estimates, on
307 the assumption that one virus genome copy is found per free floating virion, can be directly
308 compared to the AFC measurements of non-cell associated virions. This comparison, of EhV
309 virion abundance determined by AFC with genome copy number quantified by qPCR,
310 revealed that there was a consistent 100-fold decrease in the predicted genome copy number
311 detected by the qPCR method, a phenomena most likely caused due to inefficiencies during

312 the phenol-chloroform DNA extraction step (Table S2). This reduction in apparent virus
313 abundance was consistent throughout the experiment and was thus taken into consideration
314 when interpreting the qPCR results of the dual virus infection treatments. In addition, the
315 resolution of the qPCR was accurate only 12 h post-EhV addition at GCN higher than 10^3
316 mL^{-1} , hence the qPCR results of $t_0 - t_5$ were below reliable detection levels and are not
317 shown here. Regardless of whether the two virus strains were added to the host cultures
318 combined or in isolation, EhV-207 and EhV-86 appeared to exhibit different infection
319 dynamics, and the qPCR results revealed more than what was initially observed with the AFC
320 analysis alone.

321

322 In the single virus-addition treatments, EhV-207 exhibited faster infection than EhV-86 and
323 replicated to produce new virions quicker; i.e. combined GCN was higher (Fig. 4). In the dual
324 infection treatments (EhV-207 + EhV-86), the presence of EhV-86 did not appear to reduce
325 the infection potential of EhV-207, and GCN produced was comparable to that in the EhV-
326 207-only infected treatments. In contrast, EhV-86 was affected by the presence of EhV-207
327 in the dual infection treatments and the amount of EhV-86 GCN at the end of the experiment
328 was 1000 fold less than in the single EhV-86 infected cultures ; i.e. $6 \times 10^3 \pm 2.86 \times 10^3$ (SD)
329 and $4 \times 10^6 \pm 1.28 \times 10^6$ (SD) mL^{-1} respectively (Fig. 4). Thus, EhV-207 appeared to be not
330 only a faster strain than EhV-86 with regards to its rate of infection under the conditions
331 studied, but also a ‘superior’ strain that out-competed its EhV-86 rival to infect a host
332 population when they were combined at equal abundance

333

334 To gain a deeper understanding of the competitive interactions between the two EhVs, we
335 also measured the GCN of each virus strain that was associated with the cellular fraction,
336 with the assumption that the GCN in the pelleted fraction represents viruses or synthesised

337 viral genomes within the cells or virus particles attached to the cell receptors (infecting or
338 still attached). Throughout the experiment, the amount of cell-associated EhV-207 GCN in
339 both dual and single virus treatments was higher than the amount of free EhV-207 GCN, with
340 the exception of 168 h post-infection. At this point the average (\pm SD) amount of free and
341 cell-associated EhV-207 GCN was equivalent: i.e. 6.9×10^6 ($\pm 2.6 \times 10^5$) and 5.59×10^6
342 ($\pm 1.01 \times 10^6$) respectively (Fig. 4). In comparison, the amount of cell-associated EhV-86
343 GCN in both dual and single virus treatments was also higher than the amount of free EhV-86
344 GCN throughout the experiment, with the exception of 12 h post infection, where the average
345 (\pm SD) amount of free and attached cell-associated EhV-86 GCN was equivalent: i.e. $1.27 \times$
346 10^3 ($\pm 1.85 \times 10^2$) and 1.69×10^3 ($\pm 8.6 \times 10^2$) respectively (Fig. 4).

347

348 The presence of EhV-86 in the dual infection treatments did not affect the amount of cell-
349 associated or free EhV-207, as the GCN of both fractions was similar to the GCN of the cell-
350 associated and free EhV-207 in the EhV-207 single virus treatments. At 48 h post-infection
351 the average (\pm SD) cell-associated EhV-207 GCNs in the dual and single virus treatments
352 were 5×10^7 ($\pm 2.83 \times 10^7$) and 4.47×10^7 ($\pm 1.52 \times 10^7$) respectively, while the average
353 (\pm SD) free EhV-207 GCNs in both the dual and single virus treatments were 3.89×10^6
354 ($\pm 2.62 \times 10^5$) and 5.25×10^6 ($\pm 7.07 \times 10^4$) respectively. In contrast, in the dual-infected
355 treatments the presence of EhV-207 decreased the GCN of both cell-associated and free EhV-
356 86. At 12 and 48 h post-virus addition there were 72 % and 65 % less cell-associated EhV-86
357 genomes, and 18 % and 42 % less free EhV-86 genomes in the dual infected treatments,
358 respectively, in comparison with the single virus treatments (Fig. 4).

359

360 By the end of the sampling period the cell-associated EhV-86 copy number in the dual
361 infection treatments was more than three orders of magnitude lower than that of cell-

362 associated EhV-207. Similarly, the average GCNs of free EhV-86 in both the single and dual
363 infected treatments were approximately 15 and 2500 times lower respectively, than the
364 average GCN of free EhV-207 after 168 h (Fig. 4).

365

366 **DISCUSSION**

367 To date, potential differences in infection rates between coccolithovirus strains have been
368 assessed briefly in only one previous study (Nissimov et al, 2013), and to our knowledge this
369 is the first time where these dynamics have been investigated under controlled laboratory
370 conditions that includes an in-depth quantitative coccolithovirus genome analysis. Strain-
371 specific genetic markers enabled us to differentiate production rates of two EhV strains
372 during dual-strain *E. huxleyi* infection experiments. Assessment of the competitive
373 interactions of these viruses during infection of the host identified major differences in their
374 infection strategies. When infecting in combination, EhV-207 was not affected by the
375 presence of EhV-86 whereas EhV-86 was quickly out-competed, and a significant reduction
376 in free and cell-associated EhV-86 was seen two days after the initial infection. Thus, when
377 infecting alongside EhV-207, the EhV-86 strain appeared to be “inferior” and its persistence
378 in this experimental setup was under threat. The significance of the results here are
379 fundamental in our understanding of how viruses interact with their hosts and with each
380 other. They provide an insight into the complex and competitive interactions between viruses
381 in the natural environment, interactions that we currently have a limited knowledge of.

382

383 **The losers and winners of the virus “fight club” – a numbers game**

384 Throughout the experiment EhV-207 appeared to be superior to EhV-86 (and perhaps more
385 potent) in that it was a faster replicating virus with regards to its rate of infection and lysis of
386 *E. huxleyi* CCMP 2090, regardless of the competition with EhV-86. As both virus strains

387 were fresh lysates produced immediately prior to the experiment, and both AFC and qPCR
388 analysis revealed no obvious differences between the physical properties of the two strains,
389 this suggests that they were of equivalent abundance and provenance. An equivalent number
390 of EhVs were added at the start of the experiment (in both single and dual-virus treatments)
391 and by 2 h post-inoculation EhV abundance in all EhV treatments decreased to an identical
392 amount, suggesting that at least initially, there was no advantage for EhV-207 with regards to
393 adsorption kinetics. However, by 8 h there was significantly more EhV-207 than EhV-86;
394 suggesting that EhV-207 was able to develop a fairly rapid competitive advantage following
395 adsorption. The dramatic increase in EhV-207 24 h post-infection in both the single and dual-
396 virus treatments and the rapid decrease in *E. huxleyi* thereafter, demonstrates that the initial
397 advantage following adsorption was not abated with time. If we consider this in the context of
398 the combined-virus treatments, by generating a significant advantage over EhV-86 as early as
399 8 h post-infection, EhV-207 was most likely responsible for the demise of the majority of the
400 host cells, subsequently leaving EhV-86 less hosts for its own propagation.

401

402 The mechanisms that allow EhV-207 to outcompete EhV-86 under these experimental
403 conditions are currently not known and a future study combining a complete transcriptomic
404 or microarray analysis during infection should be considered. Nevertheless, these results
405 suggest that EhV-207 may have a shorter latent period within the host cells than EhV-86, and
406 that under these experimental conditions, EhV-207 packages, assembles and releases new
407 virus progeny much quicker than its EhV-86 rival. A second possible explanation to the EhV-
408 207 dominance is that the amount of EhVs produced per infected host cell (burst size) was
409 much higher when hosts were infected by EhV-207 compared to EhV-86. Over multiple
410 rounds of reinfection, this would quickly result in EhV-207 dominating EhV-86. However,
411 only the second explanation explains the decreased productivity of EhV-86 in comparison

412 with EhV-207 in the single virus infection treatments. Indeed, if the former were true,
413 presumably with a longer reinfection cycle, EhV-86 would be expected to produce more, not
414 less, progeny since the host cells would have longer to grow and therefore have a greater
415 overall virus production potential. Lytic viruses typically exhibit a short latent period and a
416 low burst size (Parada et al. 2006), however an extension of the latent period is a strategy
417 employed by some temperate phage during periods of low multiplicity of infection and low
418 host abundance (Wilson and Mann 1997, Parada et al. 2006). Although not measured directly
419 here, we calculated the potential burst sizes of the two EhV strains (estimated as the ratio of
420 the maximal number of viruses produced to the maximum cell concentration reached by the
421 specific host before cell decrease (Jaquet et al. 2002), as 16.5 for EhV-86 and 248 for EhV-
422 207; thus, there was potentially over an order of magnitude difference in burst size between
423 the two virus strains. Although limited, these calculations suggest that differences in burst
424 size could be a critical factor in deciding the outcome of competition dynamics between virus
425 strains.

426

427 What strategies lytic viruses such as EhVs can utilise during periods of intense competition is
428 as yet unknown, but it is clear that EhV-207 confers a competitive advantage over EhV-86
429 (ultimately affecting burst size and/or lysis rates), and the cause of such an advantage will be
430 encoded upon their respective genomes. With regards to this, EhV-207 has an extra tRNA not
431 found in the genome of EhV-86 and also 49 genes (of which 47 have no assigned or predicted
432 function) that have no homologs with EhV-86. Among these are two genes predicted to
433 encode for glycosyl-transferases (data not shown). Although rare in viruses, glycosyl-
434 transferase encoding genes have been previously reported in bacteriophages, poxviruses,
435 herpesviruses and baculoviruses (Markine-Goriaynoff et al., 2004). In some bacteriophages,
436 glycosyl-transferases have the ability to modify the virus DNA in order to protect it from host

437 restriction endonucleases, and in *Chlorella* viruses such as PBCV-1 they have been
438 implicated in the synthesis of glycan components of the virus major capsid protein (Zhang et
439 al., 2007). Hence the presence of these genes could be beneficial to EhV-207 and aid in its
440 much increased rate of genomic assembly and capsid construction prior to release from the
441 infected cells. It is unlikely that the genetic features unique to EhV-86 that EhV-207 does not
442 contain (i.e. genes predicted to encode for a longevity-assurance (LAG1) family protein, a
443 PDZ domain protein, a putative DNA-binding protein, a putative helicase, and 55 extra
444 membrane proteins or proteins with an unknown function) act to inhibit the infection rate of
445 EhV-86 (data not shown), although this cannot be ruled out at this stage. Alternatively, it is
446 possible that the phenotypic difference observed is caused by functional variation in shared
447 genetic components.

448

449 Indeed, previous studies have reported that coccolithoviruses biochemically hijack the host
450 sphingolipid biosynthesis pathway (Pagarete et al, 2009; Bidle and Vardi, 2011; Michaelson
451 et al, 2010) and produce virally-encoded glycosphingolipids that in turn trigger ROS (reactive
452 oxygen species), caspase activity and PCD (programmed cell death) in the infected host cells
453 (Bidle et al, 2007; Vardi et al, 2009, 2012). The rate limiting step in this *de novo* sphingolipid
454 biosynthesis pathway is the first step in the reaction where serine palmitoyltransferase cleaves
455 palmitoyl Co-A or myristoyl Co-A with serine (Han et al, 2006; Monier et al, 2009). Recent
456 protein structural analysis has suggested that there is a difference in the protein fold of the
457 SPT enzyme encoded by different coccolithoviruses, that may affect the rate and efficiency of
458 this first step in the virally-encoded sphingolipid biosynthesis pathway (Nissimov et al,
459 2013). These potential differences between coccolithovirus strains in viral SPT activity in the
460 first few hours post-infection may prove to be crucial in determining which strain dominates
461 during competitive interactions such as those shown in this study.

462

463 Another facet that is worthy of consideration is the possibility that a single host cell was
464 simultaneously infected by both virus strains. In this case, an internal intra-cellular battle over
465 the host cellular metabolic machinery would have occurred between the two viruses. Such a
466 scenario although theoretically possible, is maybe less likely due to the mutual exclusion
467 theory proposed by Luria and Delbruck (1943) in which was suggested that a virus particle
468 that infects first a host cell alters it to the extent of which a second infection by another virus
469 is unlikely. This was indeed shown to be the case in the co-infection of *Chlorella* by two
470 closely related viruses PBCV-1 and NY-2A (Greiner et al., 2009). In this previous study,
471 infection by the PBCV-1 virus depolarised the host cell membrane to exclude further
472 infections by NY-2A. Genomic comparison among EhV strains (Allen et al, 2006, Nissimov
473 et al, 2011a, 2011b, 2012a, 2012b, 2014, and Pagarete et al, 2013) suggests that many genes
474 have indeed been transferred between closely related virus strains, possibly a result of co-
475 infection and retrovirus involvement (Nissimov J., PhD thesis; 2013). Hence it is not
476 currently known whether mutual exclusion mechanisms such as the ones observed in
477 *Chlorella* exist in coccolithoviruses, but it is worth considering and investigating further.
478 Regardless of the mechanisms involved, EhV-86 appeared to be a poor competitor to EhV-
479 207 and lost the battle over infection and replication when placed into direct competition with
480 its intragenus opponent.

481

482 Finally, investigating the differences in free EhV GCN compared to the cell-associated EhV
483 GCN revealed an aspect never previously observed in the study of coccolithovirus
484 replication. For both EhV strains, cell-associated GCN was higher than the free GCN,
485 suggestive that not all the newly synthesized genomes were able to be packed into virions and
486 released from the cells. This is indicative of a nucleotide independent factor that limits the

487 burst size of these two EhV strains and ultimately the amount of new virus progeny produced.
488 The EhV virion consists of dsDNA genomic material encased within a protein shell
489 (predominantly composed of the major capsid protein), enveloped by a lipid membrane. GCN
490 clearly indicates the production of genomic material is not limiting, therefore either protein
491 production or membrane lipid availability and integrity are the likely limiting factors. The
492 latter is of particular interest considering the acquisition of the near complete pathway for the
493 synthesis of sphingolipids from the host by the coccolithoviruses, and the prominent role of
494 such lipids in the formation of membrane rafts to instigate virus release.

495

496 **The ecological significance of the virus “fight club”**

497 To date, virus-induced decline of algal populations in the natural environment are often
498 viewed as a one dimensional battle between hosts and their viruses. However the additional
499 element of competition between viruses is fundamental when trying to predict viral-induced
500 impacts on primary productivity and the role of viruses as ecological drivers of diversity.
501 Although a laboratory study, the results here may also be representative of natural systems,
502 where indeed there is mounting evidence that such processes occur. For instance, natural
503 coccolithophore assemblages in the North Sea, Norwegian fjords, English coast, and the vast
504 Atlantic Ocean, were characterised by extremely numerous and diverse co-occurring
505 coccolithovirus communities (Wilson et al., 2002; Martinez et al., 2007, 2012, Rowe et al,
506 2011, Nissimov et al, 2013). In a recent mesocosm experiment in the Norwegian fjord
507 (Sorensen et al., 2009), it was observed that the number of distinct EhV genotypes decreased
508 with the propagation of an *E. huxleyi* infection; i.e. early on during the development of the
509 bloom the EhV community was more diverse, and there were more distinct EhV genotypes
510 (detected by DGGE) than towards the end of the bloom (Martinez et al, 2007, 2012; Sorensen
511 et al, 2009). Hence the dominance of certain coccolithovirus strains over others will be a

512 direct consequence of competitive interactions and the specific phenotypic characteristics
513 manifested during host-virus infection dynamics. Depending on the virus strain type/s present
514 in a particular environment and its/their succession over other virus strains, such variation in
515 phenotypic characteristics will affect the overall propagation of an infection, its outcome with
516 regards to the demise of the host population, and subsequently the rates of carbon and
517 nutrient recycling (Wilhelm and Suttle, 1999). Thus if we exclude other factors such as
518 grazing and nutrient limitation from the equation, the ecological significance under the
519 described scenario (i.e. EhV-207-like viruses dominating) would be a localised short-time
520 rapid increase in the rate of carbon export and recycling of nutrients, but a reduced overall
521 carbon and nutrient export, as the host population growth would be rapidly diminished.
522 Alternatively, if EhV-86-like genotypes were to be hypothetically the “winners” of these
523 competitive interactions, then this would have resulted in slower infection kinetics which
524 would have allowed the host population to reach higher densities, resulting in a greater total
525 amount of carbon and nutrient re-circulation, over a longer time scale. Ultimately, in a
526 changing marine environment it is important to understand both the phenotypic and genotypic
527 diversity changes that occur within a microbial community and how these changes affect
528 globally important ecological and biogeochemical processes.

529

530 The evolutionary significance of intragenus virus competition is the fuelling of the co-
531 evolutionary arms race between the host and its virus. During the described competition
532 scenario, if the two strains were the only ones present in a given environmental niche, then an
533 increase in the fitness of EhV-207 would have resulted in the decrease in fitness of EhV-86 to
534 the point of its extinction or near extinction. Viruses possessing these phenotypes, will infect
535 the most active coccolithophore species and/or strains in consistence with the “killing the
536 winner” hypothesis (Thingstad and Lignell, 1997, Winter et al. 2010), essentially

537 transforming the host “winners” to “losers” with time. Then, the new host “winners” will
538 most likely be a sub-population that is resistant to EhV-207-like strains but possibly more
539 sensitive to EhV-86-like strains (or other similarly low activity genotypes that have optimal
540 infection strategies for these new host “winners”). This fits within the “virus-host stable co-
541 existence” theory in which was hypothesised the phenotypic plasticity of the algal hosts and
542 their ability to recover post-virus infection, is what makes the co-existence of these hosts and
543 their viruses possible, both on short and also on evolutionary time scales (Thyrhaug et al.
544 2003).

545

546 The emergence of novel viruses with niche-specific characteristics for infection, are to a large
547 extent, a result of these competitive interactions. For instance, in plants, the occurrence of
548 more than one RNA virus and their environmental association with their host is common
549 (Roossinck, 2005). If these viruses are similar to one another then they will be in direct
550 competition, whilst if they are not similar then they will not be. Indeed it was shown that
551 plant RNA virus evolution occurs due to “survival of the fittest” scenario, during which
552 closely related viruses increased the positive selection of some of these viruses over others
553 (Roossinck and Palukaitis, 1995). Thus, the competitive interactions between the closely
554 related coccolithoviruses EhV-86 and EhV-207 may not only drive the fitness and evolution
555 of their hosts, but also their own.

556

557 The competitive interactions displayed here by coccolithoviruses raise exciting questions
558 with regards to the ability of these different strains to evolve strategies for the utilisation of
559 new resources; i.e. the infection of a new host. Recently it was shown that resource
560 competition between bacteriophages (i.e. competition over host availability for infection and
561 replication) promoted the evolution of novel bacteriophage phenotypes with the ability to

562 utilise new hosts, suggesting that this sort of competition was essential for driving the
563 evolution of host range expansion (Bono et al., 2013). Although coccolithoviruses are
564 fundamentally different from bacteriophages with regards to their rate of mutation (much
565 slower), a similar resource competition over a larger time scale may explain the large range
566 of susceptible infectious hosts to some coccolithovirus strains (personal observations).

567

568 Furthermore, resource expansion depends most likely also on whether a virus is an r or a K
569 strategist, and the type of trade-offs adapted by viruses and their hosts; a concept that has
570 been applied recently to viruses from the classical life history theory (De Paepe and Taddei,
571 2006, Winter et al, 2010,) . The question of whether a particular virus strain is an r strategist,
572 whereby able to quickly utilize its resource in order to produce abundant virus progenies
573 (usually characterised by a lower percentage of potent virus particles and therefore poor
574 competitors); or a K strategist, whereby the emphasis is on fewer but highly potent,
575 competitive virus progenies, remains to be seen. Based on the classical description of K and r
576 selection theory one would have expected that EhV-86 would have been a better competitor
577 than EhV-207 as it is characterised by slower multiplication rates. However it appears that
578 the latter is a stronger competitor that utilises the available resources quicker and produces a
579 larger number of progeny. Hence both viruses studied here, under optimal conditions, employ
580 aspects of both an R and a K specialist. However, additional factors such as the potential
581 trade-off between high multiplication rates and increased virion decay rates (De Paepe and
582 Taddei, 2006), should also be considered in future studies, particularly under resource-
583 limiting conditions.

584

585 **CONCLUSIONS**

586 Whether a particular coccolithovirus strain (and all its associated genomic, proteomic, and
587 metabolomic characteristics) will proliferate, is determined by its ability to co-evolve with
588 one or more host genotypes and outcompete other viruses less fit in a particular environment.
589 One can only imagine the complexity of the interactions described here in naturally occurring
590 blooms where at any given time there are many different coccolithophore genotypes (and
591 subsequently phenotypes) and an even larger number of distinct coccolithoviruses, often
592 experiencing annual variable environmental conditions. The dominance of a select few virus
593 genotypes at the end of a bloom or an infection event is a paradox of which the drivers still
594 remain anonymous. However specific characterisation of coccolithovirus genotypes and
595 phenotypic interrogation of their infection dynamics, in tandem with the analysis of their
596 phylogenetic history and functional biodiversity, can shed new light on coccolithovirus
597 evolution and ultimately their role in microbial oceanography.

598

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606

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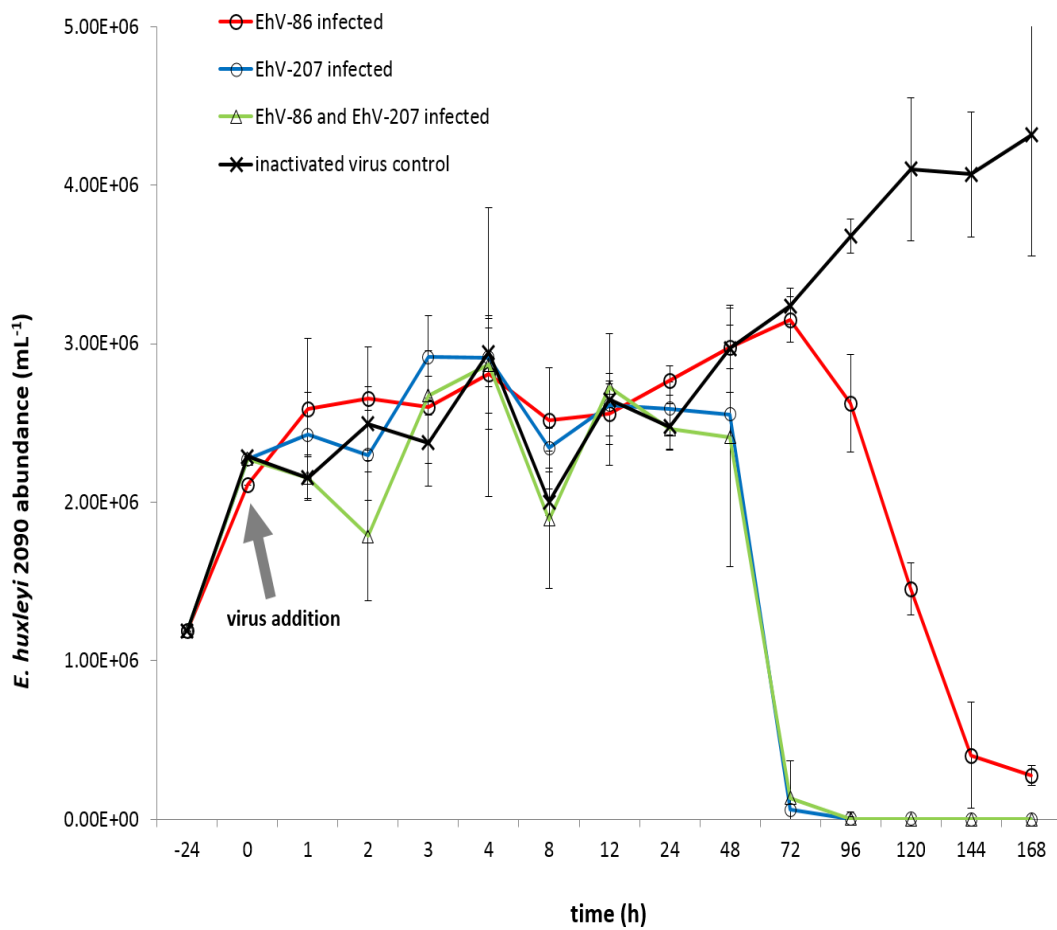
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743 **Fig. 1.** *Emiliania huxleyi* CCMP 2090 average cells mL⁻¹ (triplicates \pm SD) following
 744 infection by EhV-86 (red line) EhV-207 (blue line), and combined EhV-86 and EhV-207
 745 (green line). Control cultures containing inactivated viruses are shown by the black line. The
 746 first measurements of the host cultures taken one day before the addition of the virus stocks
 747 (i.e. t -24 h).
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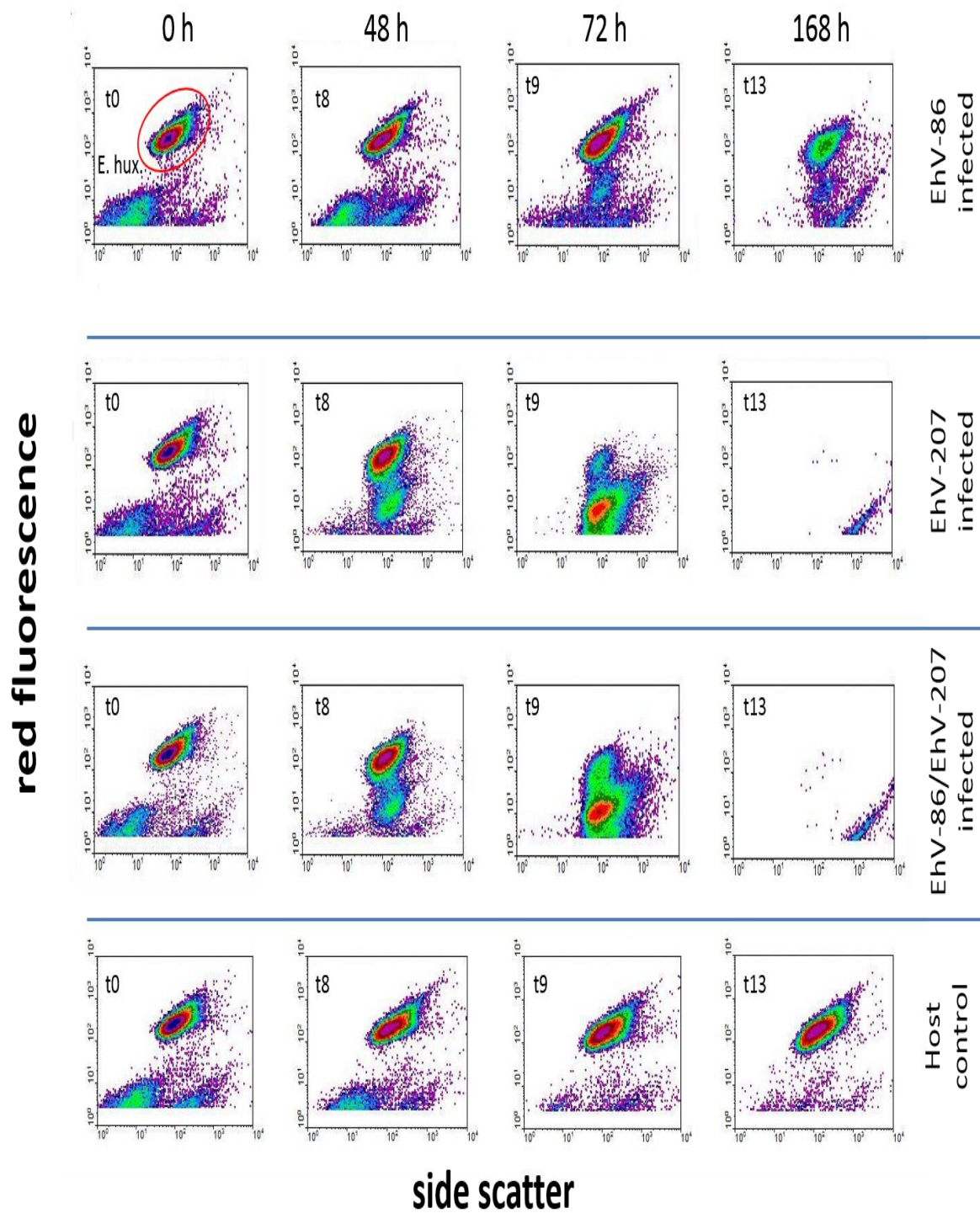
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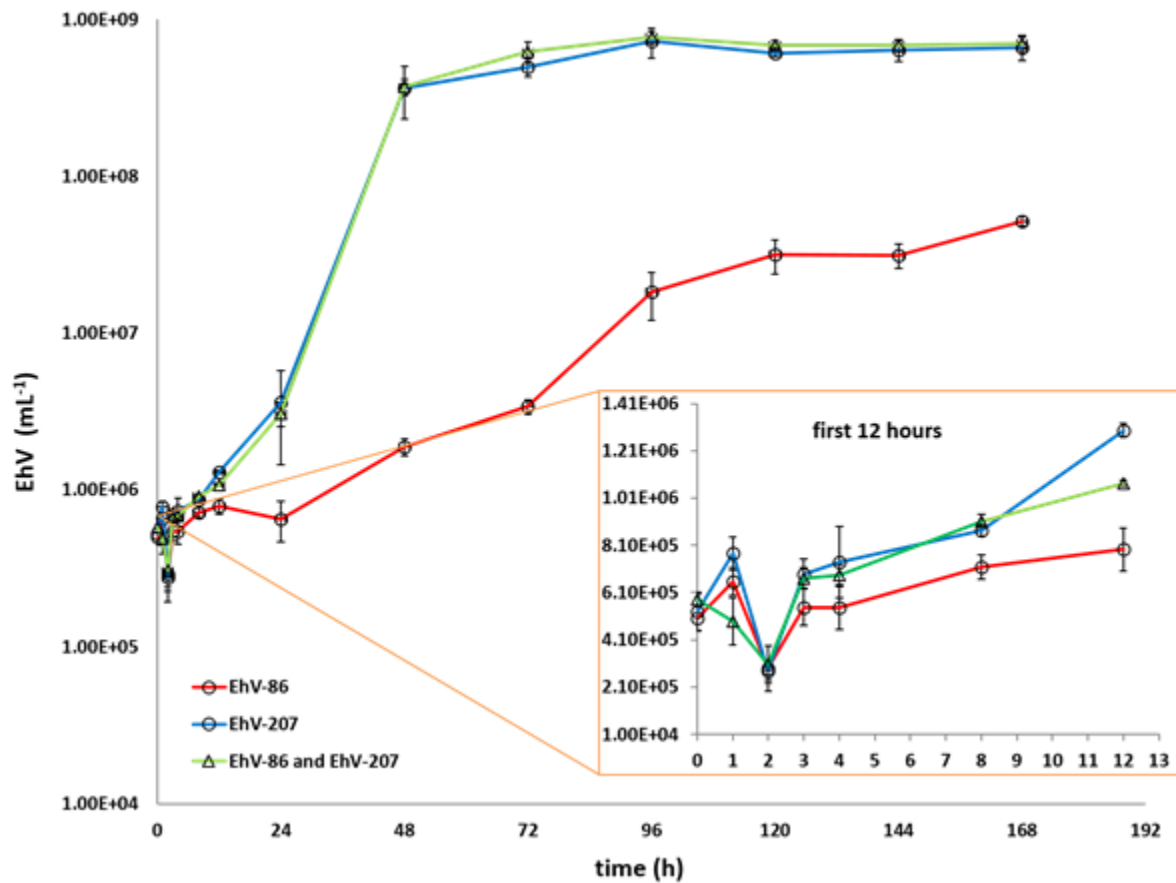
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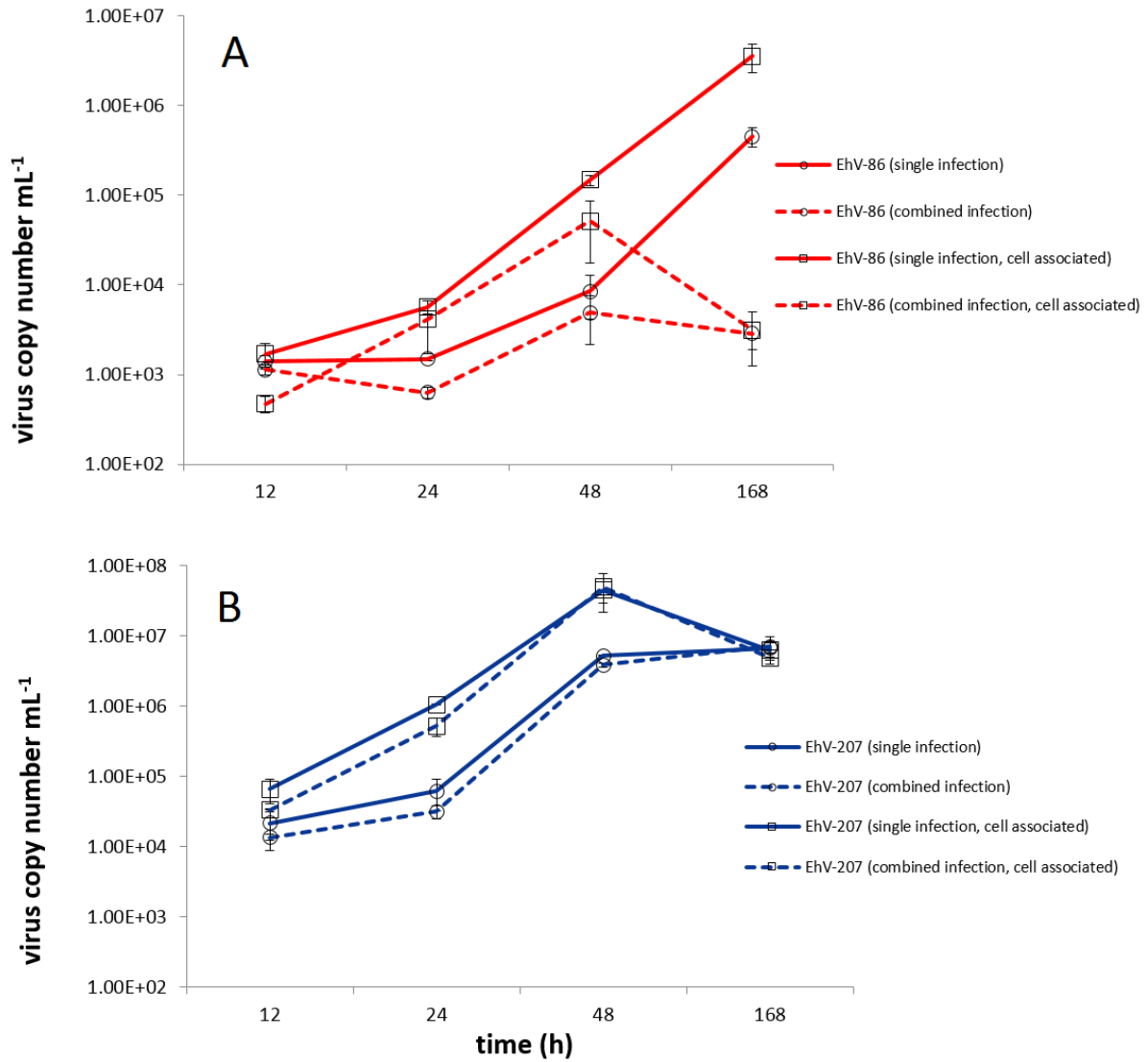
757 **Fig. 2.** Flow cytometry plots of red (chlorophyll) fluorescence versus side scatter at 0 h and
 758 168 h during the time course experiment for infected (EhV-86, EhV-207 or combined EhV-
 759 86&EhV-207 co-infected) and control *Emiliana huxleyi* cultures.

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Fig. 3. Average density mL⁻¹ of EhV-86 (red line), EhV-207 (blue line), and combined EhV-86&EhV-207 (green line) during the time-course experiment. Data points represent triplicate measurements (\pm SD). The abundance of free EhVs was enumerated using AFC (green fluorescence vs side scatter).



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776 **Fig. 4.** EhV-86 (A) and EhV-207 (B) virus copy number averages (triplicates \pm SD) from
 777 *Emiliania huxleyi* cultures infected with either EhV-86 (single infection), EhV-207 (single
 778 infection) or combined EhV-86&EhV-207 (combined infection), 12 h, 24 h, 48 h and 168 h
 779 post infection; performed with qPCR strain specific primers for the discrimination of one
 780 coccolithovirus strain from the other.

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795 **Table S1.** Virus treatments A-G and their initial volume* at the beginning of the experiment
796 (t0). Each treatment was performed in triplicate (i.e. a total of 21 flasks; 12 containing *E.*
797 *huxleyi* CCMP 2090 at a cellular density of $1.5 \times 10^6 \text{ mL}^{-1}$). + indicates the presence of EhV-
798 86, EhV-207, or host in a given flask, – indicates inactivated virus.
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Flask n ^o	Volume of f/2 media (mL)*	EhV-86	EhV-207	<i>E.huxleyi</i> host	Experimental treatment
1	100	+		+	A
2	100	+		+	
3	100	+		+	
4	100		+	+	B
5	100		+	+	
6	100		+	+	
7	100	+	+	+	C
8	100	+	+	+	
9	100	+	+	+	
10	100	+			D
11	100	+			
12	100	+			
13	100		+		E
14	100		+		
15	100		+		
16	100	+	+		F
17	100	+	+		
18	100	+	+		
19	100	-	-	+	G
20	100	-	-	+	
21	100	-	-	+	

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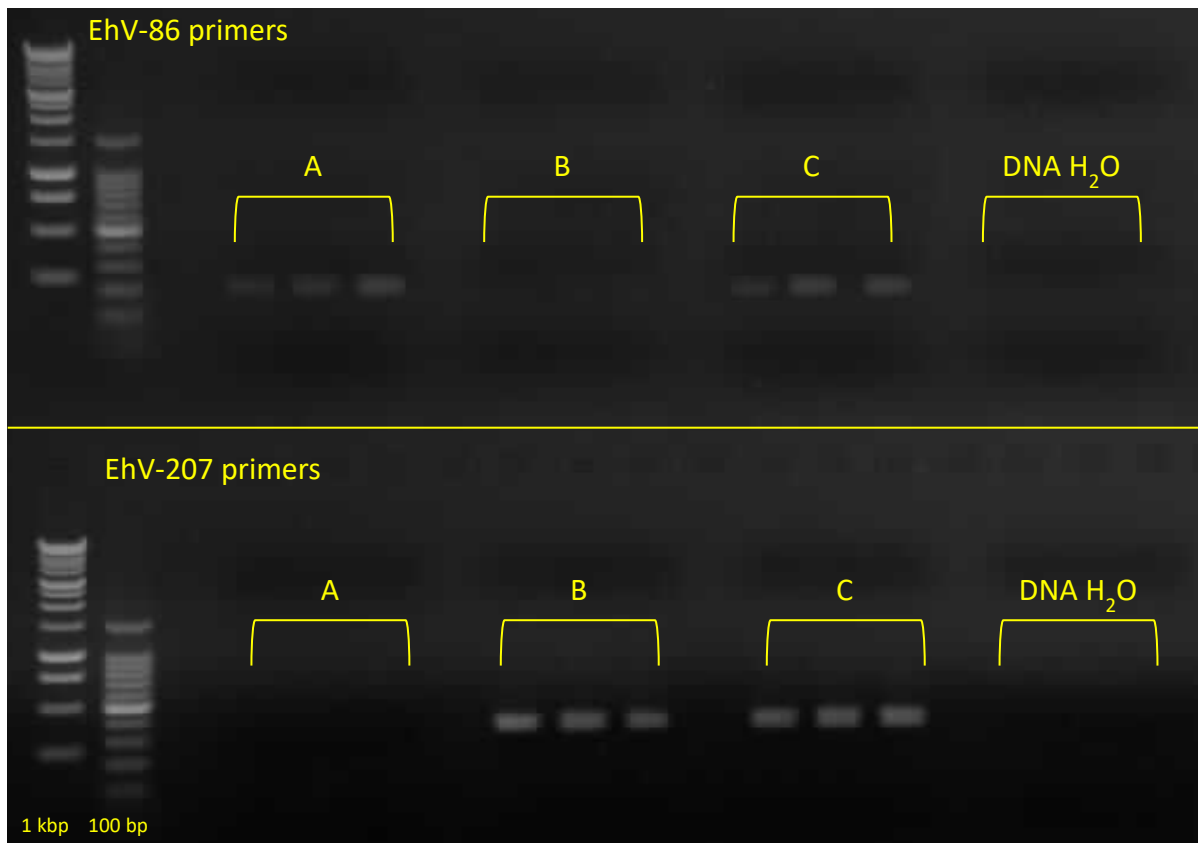
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812 **Table S2.** Free, unattached, average (triplicate) EhV abundance and virus copy number
 813 (VCN) per mL, detected by analytical flow cytometry (AFC) and quantitative polymerase
 814 chain reaction (qPCR) respectively, 12 h and 168 h post-addition of viruses; either EhV-86 or
 815 EhV-207.
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time (h)	Analysis type	AFC	qPCR	AFC	qPCR
	EhV-86 (solo free)	EhV-86 (solo free)	EhV-207 (solo free)	EhV-207 (solo free)	
12	7.92×10^5	1.40×10^3	1.30×10^6	2.14×10^4	
168	5.19×10^7	4.51×10^5	6.58×10^8	6.71×10^6	

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834 **Fig. S1.** Gel electrophoresis of PCR products conducted with primers specific to EhV-86 and
835 EhV-207. The products are amplified regions from lysates taken from three different culture
836 conditions: EhV-86 infected host (A), EhV-207 infected host (B), and host infected
837 simultaneously by both EhV-86 and EhV-207 (C). In the last two lanes to the right (top and
838 bottom) are the control DNA free water samples.
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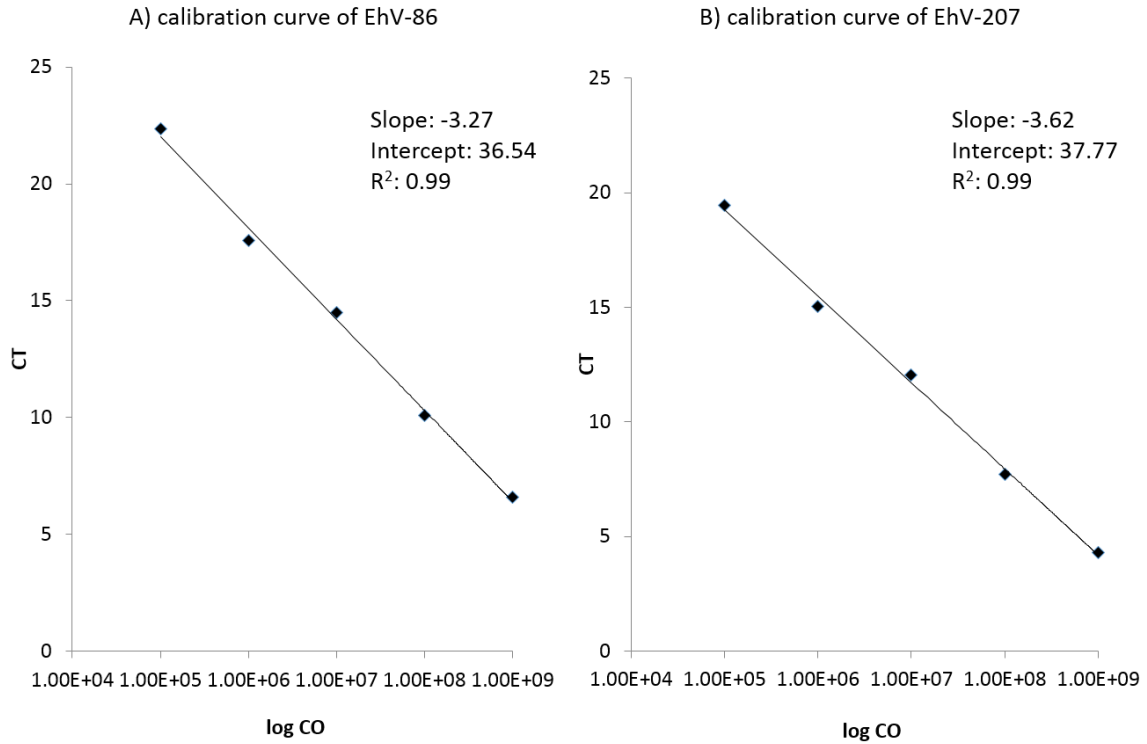
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851 **Fig. S2.** Calibration curve for the qPCR amplification of known amounts of purified DNA of
 852 EhV-86 (ehv290) and EhV-207 (EQVG00465). CT= cycle number, (log CO= known
 853 concentrations of purified EhV-86 (A) and EhV-207 (B) DNA products.

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