Effect of metals on the lytic cycle of the Coccolithovirus, EhV86

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In this study we show that metals, and in particular copper (Cu), can disrupt the lytic cycle in the *Emiliania huxleyi* – EhV86 host–virus system. *E. huxleyi* lysis rates were reduced at high total Cu concentrations (> approximately 500 nM) in the presence and absence of EDTA (ethylenediaminetetraacetic acid) in acute short term exposure experiments. Zinc (Zn), cadmium (Cd), and cobalt (Co) were not observed to affect the lysis rate of EhV86 in these experiments. The cellular glutathione (GSH) content increased in virus infected cells, but not as a result of metal exposure. In contrast, the cellular content of phytochelatins (PCs) increased only in response to metal exposure. The increase in glutathione content is consistent with increases in the production of reactive oxygen species (ROS) on viral lysis, while increases in PC content are likely linked to metal homeostasis and indicate that metal toxicity to the host was not affected by viral infection. We propose that Cu prevents lytic production of EhV86 by interfering with virus DNA (deoxyribonucleic acid) synthesis through a transcriptional block, which ultimately suppresses the formation of ROS.

Keywords: copper, *Emiliania huxleyi*, phytoplankton, virus, cadmium, phytochelatins, thiols, glutathione
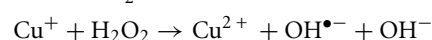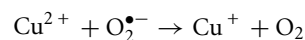
INTRODUCTION

Viruses have been shown to be the most ubiquitous biological entities detected in the ocean to date (Bergh et al., 1989; Sandaa, 2008), with over a quarter of organic carbon in the sea passing through the "viral shunt" (Wilhelm and Suttle, 1999). Viruses not only directly affect the abundance and diversity of the organisms they infect (e.g., Sorensen et al., 2009), but viral lyses results in the release of nutrients and organic carbon and thus influences the biogeochemical cycles of key elements such as carbon, nitrogen, and iron (Fe; Gobler et al., 1997; Mioni et al., 2005; Weinbauer et al., 2009). Therefore viruses are important players in global ecosystems (Suttle, 2007). The relationship between virus and host in the marine environment is thought to be complex, however the *Emiliania huxleyi* – EhV host–virus system has emerged as one of the best model systems to investigate algal host–virus interactions (Bidle and Vardi, 2011).

EhV86, a Coccolithovirus, is responsible for the termination of naturally occurring blooms of the coccolithophore *E. huxleyi* (Schroeder et al., 2002; Wilson et al., 2002). More recently, studies into the mechanisms of EhV86 infection have revealed an animal-like infection strategy (Mackinder et al., 2009), exploiting pathways previously thought to be restricted to higher multicellular organisms. These pathways include the synthesis of ceramide (Wilson et al., 2005; Han et al., 2006) inducing programmed cell death (PCD) in the unicellular coccolithophore (Bidle and Falkowski, 2004; Pagarete et al., 2009; Vardi et al., 2009). Notably, viral lysis of *E. huxleyi* by EhV86 also triggers the production of reactive oxygen species (ROS; Evans et al., 2006), which in turn induces caspase activity and consequently PCD (Bidle et al., 2007).

Cellular processes in micro-organisms are also strongly influenced by the chemistry of trace metals, which in turn influence the primary production and community structure in the ocean (Sunda, 1988; Morel and Price, 2003). Copper (Cu) and cadmium (Cd) are two trace metals that can be found at elevated levels in coastal environments (e.g., Braungardt et al., 2007). Copper is an essential cofactor of enzymes involved in a variety of physiological processes including respiration, photosynthesis, oxygen transport, and antioxidant defense. Therefore, Cu is required for growth by marine phytoplankton, but is toxic at elevated concentrations (Morel et al., 1978; Brand et al., 1986; Gledhill et al., 1997). Copper predominantly occurs as Cu(II) but biological systems utilize the ability of Cu to undergo a redox cycle. It is this property that gives Cu its toxic potential. Copper catalyzes the reaction between superoxide and hydrogen peroxide, producing the highly reactive hydroxyl radical via the following cycle:

$$Cu^{2+} + O_2^{\bullet-} \rightarrow Cu^+ + O_2$$
$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH^{\bullet-} + OH^-$$

The reaction with hydrogen peroxide will compete with the faster reaction between Cu^+ and $O_2^{\bullet-}$ (Voelker et al., 2000; Heller and Croot, 2010, 2011):

$$Cu^+ + O_2^{\bullet-} \rightarrow Cu^{2+} + H_2O_2$$
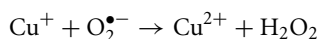
so that the production of the hydroxide radical will be influenced by the ambient redox environment (which is reducing within cells; Schafer and Buettner, 2001) and the relative concentrations of Cu and superoxide.

Hydroxyl radicals can cause oxidative damage to cellular components such as deoxyribonucleic acid (DNA), proteins, and lipids. For example, Cu causes damage to DNA by binding near guanine bases, where it is reduced to Cu(I) and then reoxidized to Cu(II) by reaction with hydrogen peroxide producing hydroxyl radicals. The radicals then mediate DNA strand breakage in close proximity to the bound Cu (Sagripanti and Kraemer, 1989; Aruoma et al., 1991). The redox properties of Cu also allow the metal to bind to several types of amino acid residues and therefore Cu could be inappropriately incorporated into proteins and enzymes that normally bind other metal ions. This results in a loss of function through inactivation or changes in conformational fold. In humans for example, Cu may contribute to the development of Alzheimer's disease, along with Fe and zinc (Zn), as it has been found to induce aggregation of the β-amyloid (Aβ) protein and has been found in high quantities (0.44 mM) in Alzheimer plaques (Lovell et al., 1998; Curtain et al., 2001). Moreover, these high Cu levels are linked to an increase in oxidative stress which plays a central role in neurodegenerative disorders (Permyakov, 2009).

Copper has long been known to possess antimicrobial and antiviral properties, and in the last decade studies have suggested that Cu surfaces could be reintroduced into hospitals to reduce the transmission of microbes such as methicillin resistant *Staphylococcus aureus* (Noyce et al., 2006a), *Escherichia coli O157* (Noyce et al., 2006b), and influenza A virus (Noyce et al., 2007). The mechanisms of Cu disruption of virus infection may vary depending on the virus and have yet to be fully understood (Karlstrom and Levine, 1991a,b; Sagripanti et al., 1997; Horie et al., 2008), however there is some evidence to suggest that inactivation of viruses can proceed via Cu mediated DNA damage as described above (Levinson et al., 1973; Sagripanti et al., 1997).

Cadmium (Cd) had been considered a non-essential metal, but more recently a unique biological role for Cd has been identified in marine diatoms. It has been shown that Cd can replace Zn as a metal of the Zn carbonic anhydrase (Price and Morel, 1990), and that Cd carbonic anhydrases can play a role in the acquisition of inorganic carbon for photosynthesis in the oceans (Lane et al., 2005). High levels of anthropogenic Cd in the coastal environment has also led to toxicological effects in exposed marine organisms. For example, Cd is reported to reduce reproduction rates in phytoplankton (Brand et al., 1986). The mechanism of Cd toxicity is known in animal systems where Cd complexes glutathione (GSH) and protein-bound sulfhydryl groups, resulting in enhanced production of ROS such as superoxide ion, hydroxyl radicals, and hydrogen peroxide (Stohs et al., 2001). Cadmium has been shown to inhibit the enzymatic activity of the Cu/Zn-superoxide dismutase (Cu/Zn-SOD) from rat liver (Hussain et al., 1987) and human

Cu/Zn-SOD (Huang et al., 2006). This occurs through replacement of the normally bound Zn(II) ion with a Cd(II) ion at the active site.

Elevated Cu and Cd concentrations in many marine eukaryotic phytoplankton are tolerated through the induction of phytochelatins, thiols of the general formula (γ-Glu-Cys)$_n$-Gly, where n commonly ranges between 2 and 4 (Ahner and Morel, 1995; Ahner et al., 1995; Kawakami et al., 2006b,c; Devez et al., 2009). Phytochelatins and other thiols may also be released into the surrounding media, reducing free metal concentrations, and potentially affecting metal bioavailability (Lee et al., 1996; Leal et al., 1999; Vasconcelos and Leal, 2001; Vasconcelos et al., 2002). Phytochelatins are synthesized from GSH, which is also known to respond to oxidative stress. However, while the intracellular abundance of PCs is thought to be linked to metal concentrations in the surrounding water (e.g., Ahner and Morel, 1995; Ahner et al., 1995; Morelli and Scarano, 2001; Dupont and Ahner, 2005; Le Faucheur et al., 2005; Kawakami et al., 2006b; Pawlik-Skowronska et al., 2007; Morelli and Fantozzi, 2008; Devez et al., 2009), cellular GSH abundance, and metal concentrations are not necessarily directly related (Kawakami et al., 2006c; Scheidegger et al., 2011). Glutathione has many metabolic roles (Mendoza-Cozatl et al., 2005), however, exogenous GSH is known to affect replication of *Herpes simplex* virus type 1 (HSV-1) by interfering with the very late stages of the virus life cycle, without otherwise affecting host cellular metabolism (Palamara et al., 1995).

Metals thus have the potential to impact host–virus interactions. However, to our knowledge, the effects of metals have not yet been assessed for any marine host–virus system. The aim of this study was therefore to undertake a preliminary investigation into interactions between trace metals and the *E. huxleyi* – EhV86 system. We subsequently examined (1) the impact of elevated Cu and Cd concentrations on the EhV86 lytic cycle and (2) the cellular mechanism involved in these interactions.

MATERIALS AND METHODS

Sterile trace metal clean techniques were used for culturing. Glassware and polycarbonate bottles (Nalgene) were acid washed (1 M HCl) for at least 24 h prior to use, 4 L polycarbonate culture vessels (Nalgene) were double bagged (Nalgene autoclavable plastic bags) prior to autoclaving at 120°C for 30 min.

CULTURE CONDITIONS

Emiliania huxleyi (strain CCMP 1516) was obtained from the Provasoli-Guillard Center for Culture of Marine Phytoplankton (CCMP). Experiments reported here focused on acute short term (4 days) effects. *E. huxleyi* was batch cultured in f/2 minus Si medium prepared using 0.2 μm filtered seawater collected from the North Atlantic Gyre in the Canary Basin (between 24.1 and 29.5°N and 23.4 and 27.6°W). The culture medium (pH = 7.8 ± 0.1) was allowed to equilibrate overnight and then filtered sterilized (0.2 μm, Sartorius) prior to seeding with *E. huxleyi*. Although it was possible that viruses already present in the seawater would have passed through the 0.2-μm filter, in practice we did not observed any evidence of lysis of *E. huxleyi* in our control cultures, indicating that this was not a problem in these experiments. Concentrations of the nutrients nitrate (NaNO₃) and phosphate

(NaH_2PO_4) added to the seawater were 3×10^{-4} and 1×10^{-5} M, respectively. Concentrations of trace metals added to the seawater were 10 nM Cu, 100 nM molybdenum, 4 nM Zn, 2.5 nM cobalt (Co), 23 nM manganese, 450 nM Fe, and 10 nM selenium. Media used for initial experiments with 2.5 μM added Cd, Co, Cu, and Zn were carried out in the presence of 5 μM ethylenediaminetetraacetic acid (EDTA). Experiments with different Cu concentrations were carried out in the presence and absence of 5 μM EDTA, and experiments investigating thiol production and RNA expression were carried out in the absence of EDTA. Cultures were maintained at $15 \pm 1°C$ under a light/dark cycle of 12:12 h and at an illumination of 150 μmol photons $m^{-2} s^{-1}$ in a growth cabinet (MLR-350, Sanyo).

VIRUS CULTURE MAINTENANCE

The Coccolithovirus EhV86 was propagated by using acclimated and synchronized batch cultures of *E. huxleyi* 1516 grown in f/2 medium without EDTA and Si (Schroeder et al., 2002). The occurrence of lysis was generally indicated by a change in the culture appearance, from a green to a chalky white color. The new virus stock solution was obtained from an *E. huxleyi* culture grown to a cell density of approximately 1×10^6 cells mL^{-1} at a multiplicity of infection of approximately 10. The new virus stock solution was labeled and stored in the dark at 4°C until required.

METAL AND VIRUS ADDITION

The virus and the single studied metal (Cd, Co, Cu, Zn) were added simultaneously. The addition of EhV86 virus, in excess for infection, was done to exponentially growing *E. huxleyi* host cultures approximately 4 days after subculturing. In initial experiments investigating effects of Cd, Co, Cu, and Zn, metals were added at a concentration of 2.5 μM in excess of concentrations already present in the media. A second experiment investigated a range of Cu concentrations between 125 nM and 1 μM. For the final experiment investigating the mechanism of the Cu virus interaction, Cu was added at a total concentration of 1.25 μM and Cd at 5.0 μM. Non-infected cultures with and without metal were used as a control in parallel for each virus/metal treatment.

Growth of the cultures was monitored daily by enumerating cells (Multisizer™ II coulter counter). Cell numbers were used to guide subsequent sampling frequency for PCs and viral ribonucleic acid (RNA). Cultures were sampled daily for virus counts and on alternate days for thiol content. RNA expression was sampled on days 5, 7, and 10 post infection for Cu and daily up to day 8 post infection for Cd. All analyses were carried out in duplicate. For virus counts, 1 mL was sampled and fixed using 50 μL of polyoxymethylene (paraformaldehyde, Sigma Aldrich, 1% final concentration) and subsequently stored at −80°C for later analysis by flow cytometry. For thiol analysis, 500 mL of culture solution was filtered (0.45 μm pore size nitrocellulose membrane filters, Whatman) under gentle vacuum pressure and stored at −80°C. For isolation of RNA, *E. huxleyi* cells were harvested via centrifugation and RNA was extracted from the pellets using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was DNase treated (Promega) to remove any DNA contamination and then quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific).

Cell counts from the coulter counter were used to calculate the average growth (μ) rates for *E. huxleyi* over the period of viral infection from the slope of a graph of ln(cells) against time. The viral lysis rate is then calculated from

$$\mu_{+virus} = \mu - \gamma_{lysis}$$

where γ_{lysis} is the *E. huxleyi* lysis rate, μ is the growth rate of *E. huxleyi* in control cultures or treatments containing the added metal, and μ_{+virus} is the growth rate of *E. huxleyi* in infected cultures or infected cultures containing the added metal.

FLOW CYTOMETRY

Determination of the abundance of viral particles and *E. huxleyi* cells was performed simultaneously (*FACSort, Becton Dickinson Biosciences*). EhV86 were discriminated based on their green fluorescence and side scatter. *E. huxleyi* cells were counted based on their red and orange fluorescence signatures upon staining with SYBR Green I DNA dye (Schroeder et al., 2002; Wilson et al., 2002).

Comparison of fresh (coulter counter) and fixed (Flow Cytometry) *E. huxleyi* cell counts showed that a good agreement was observed between these two counting approaches (*t* test, $p > 0.05$, $n = 48$).

DETERMINATION OF PARTICULATE THIOLS

The total concentrations of glutathione (GSH) and phytochelatins (PCs) in metal and virus exposed *E. huxleyi* cultures were determined according to the method reported by Kawakami et al. (2006a). Intracellular thiol measurements were performed in duplicate by reverse-phase high performance liquid chromatography (HPLC) with fluorescence detection.

Thiols were extracted on ice (5 min), following addition of 1.2 mL solution of 0.1 M HCl containing 5 mM diethylenetriamine pentaacetic acid (DTPA, Fluka Biochemica) to a 2-mL microcentrifuge tube (Fisher) containing the filter with *E. huxleyi* biomass. The extract was centrifuged (13000 g/20 min at 4°C) and syringe filtered (0.2 μm pore size cellulose membrane, Minisart RC4, Sartorius) prior to reduction (25 μL of a 20-mM 2-carboxyethylphosphine hydrochloride, 5 min, TCEP, Sigma). Further oxidation was minimized (5 mM DTPA) and the extract was buffered at pH 8.2 (200 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid, HEPES). After a further 5 min, 10 μL of a 100-mM of a sulfur-specific fluorescent tag monobromobimane was added (MBrB in acetonitrile, Fisher) followed by 465 μL of the HEPES/DTPA pH 8.2 solution. The derivatization procedure was carried out in a dark room under dim red light conditions. After 15 min, the reaction was stabilized and the derivatization of thiols by MBrB, stopped by addition of 100 μL of 1 M methanesulfonic acid (99%, Sigma). Vials were stored in the dark at 4°C until HPLC analysis.

Thiols were analyzed by reverse-phase HPLC with fluorescence detection (Kawakami et al., 2006a). The HPLC comprised a system controller (Shimadzu SCL-10A) and two pumps (Shimadzu LC-10ADvp), an autosampler (Shimadzu SIL-10ADVP) and a fluorescence detector (Shimadzu RF-10A XL) operating at 380 nm (excitation) and 470 nm (emission) wavelengths. Separation of the thiols was carried out using a 150 mm × 2.1 mm C-18 HPLC column (Ascentis, Supelco) with a 3-μm particle size and a gradient program of 0–5 min, 10% B; 5–18 min, 10–22% B; 18–40 min,

22–35% B; 40–50 min, 35–100%; 50–55 min, isocratic 100% B; 55–58 min, 100–10% B; 58–60 min 10% B, where A was 0.1% tri-fluoroacetic acid (TFA, Fluka) and B was acetonitrile. The flow rate was 0.2 mL min^{-1}.

Phytochelatin concentrations were standardized with GSH (reduced form, purity 99%, Sigma) assuming that the fluorescence response was directly proportional to the number of thiol groups (Kawakami et al., 2006a). We used PCs directly produced by *Phaeodactylum tricornutum* under metal stress for identification of retention times for PC$_2$, PC$_3$, and PC$_4$. GSH eluted at 11.4 min and PC2, 3, and 4 at 18.8, 21.7, and 24.2 min, respectively. Cellular GSH and PC concentrations were normalized to the number of cells and are thus expressed in amol SH cell^{-1}. The limit of detection, calculated from three times SD of a 5-pmol GSH standard, was 0.1 pmol with a 100-μL injection volume. Analytical variability within standards and samples was less than 10%. The recovery of GSH added to samples prior to derivatization was determined to be $86 \pm 29\%$ ($n = 11$).

ONE-STEP REVERSE TRANSCRIPTION-PCR

RT-PCR detection of virus-related gene expression was undertaken using primers designed to amplify four viral genes, DNA polymerase (DNA pol), Helicase, proliferating cell nuclear antigen (PCNA) protein, and major capsid protein (MCP; **Table 1**). One-step RT-PCR was used to amplify 10 ng RNA in 25 μL reactions containing $1 \times$ One-step sensimix QPCR mix (with SYBR green), 7.5 pmol forward primer, 7.5 pmol reverse primer, and 5 units RNase inhibitor. Reactions were carried out in a Rotor-gene 6000 QPCR machine (Corbett Research) using the following conditions: reverse transcription at 49°C for 10 min, polymerase activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 54°C for 15 s (60°C for MCP), and 72°C for 15 s. Fluorescence was acquired at the end of each extension step on the green channel. RT-PCR reactions were subjected to melt curve analysis to ensure a single product had been generated by gradual melting from 72 to 95°C and fluorescence acquisition at each 1°C increment. RT-PCR products were verified by gel electrophoresis on a 2% (w/v) agarose gel in $1 \times$ TAE buffer and viewed on a UV transilluminator (Syngene).

STATISTICAL ANALYSIS

As analysis was only performed in duplicate, estimates of errors in growth, and lysis rates were calculated from the square route of the sum of squares of the SEs of the slopes of ln(cells) against

time. The lack of experimental replication meant that the statistical significance of our results could not be tested and comparisons between treatments are thus qualitative.

RESULTS

E. HUXLEYI – EhV86 INFECTION DYNAMICS IN THE PRESENCE OF VARIOUS METAL IONS

Preliminary experiments, carried out with a range of metals (Cu, Zn, Co, and Cd) on the *E. huxleyi* – EhV86 host–virus system indicated that viral lysis of *E. huxleyi* cells was disrupted in the presence of Cu, but was similar to controls for the metals Cd, Co, and Zn (**Figure 1A**). In this study we have interpreted our data qualitatively as lack of sufficient replicates in our experimental design precludes more quantitative estimates of the statistical significance of our results. However, Cu was consistently observed to disrupt viral lysis of *E. huxleyi* in all the experiments undertaken as part of this study. Furthermore varying the concentration of Cu in the absence and presence of EDTA indicated that viral lysis rates decreased with increasing Cu concentration and were lowest in the absence of EDTA (**Figure 1D**). Copper is known to be toxic to marine algae at high concentrations (e.g., Sunda and Guillard, 1976; Brand et al., 1986; Gledhill et al., 1997; Levy et al., 2007, 2008; Debelius et al., 2009), and indeed the growth rate of *E. huxleyi* was reduced at the highest Cu concentration when compared to control cultures (**Figures 1A,C**). However the cumulative effect of metal plus virus on host growth was only observed in Cu treatments and thus appeared to be a specific effect of Cu. Further short term exposure experiments aimed at understanding the interaction between Cu and the *E. huxleyi* virus–host system focused on Cd and Cu as these two metals are both known to be toxic and they exhibited contrasting behaviors in our preliminary experiments. Furthermore EDTA was omitted in order to maximize the effect of trace metals on both host and virus.

PRODUCTION OF GLUTATHIONE AND PHYTOCHELATINS BY E. HUXLEYI

The intracellular content of GSH and PCs in infected and non-infected *E. huxleyi* cells were determined in order to investigate oxidative stress and trace metal homeostasis during the course of the experiments. Post virus and metal addition growth curves for *E. huxleyi* in the thiol expression experiments are presented in **Figure 2**. Initial cell numbers were different for each experiment when virus and metals were added ($4.0 \pm 0.6 \times 10^5$ cells mL^{-1} for the Cu experiment and $1.4 \pm 0.2 \times 10^5$ cells mL^{-1} for the Cd experiment), however, calculated growth rates were similar for

Table 1 | Primers used in this study.

| Primer name	Target (CDS)	Sequence (5′–3′)	Amplicon size (bp)	Reference
EhVpol_F	DNA polymerase (ehv030)	TATAATGCACGCCAACTTGC	98	This study
EhVpol_R		GCAATTGCACCAAGTGGATA		
EhVpcna_F	PCNA (ehv440)	GGGCATTTCATTTGCCATAC	157	This study
EhVpcna_R		ATTCTCCGTCGACAATACGC		
EhVhel_F	Helicase (ehv104)	GCCAACTGGTACAGGGAAAA	184	This study
EhVhel_R		CATCCATGCATGTGTGACAA		
MCP_F2	MCP (ehv085)	GACCTTTAGGCCAGGGAG	134	Schroeder et al. (2002)
MCP_R2		GTTCGCGCTCGAGTCGAT		
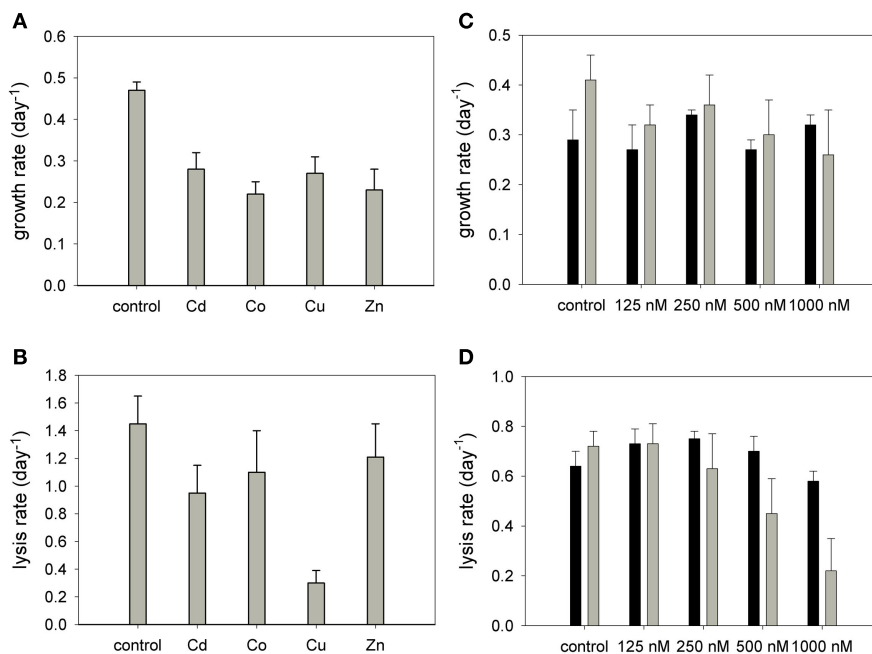
FIGURE 1 | Growth rates (A,C) and viral lysis rates (B,D) for the EhV86 Coccolithovirus in the presence of (A,B) Cd, Co, Cu, and Zn and (C,D) at Cu concentrations between 125 and 1000 nM in the presence (black bars) and absence (gray bars) of 5 μM EDTA.
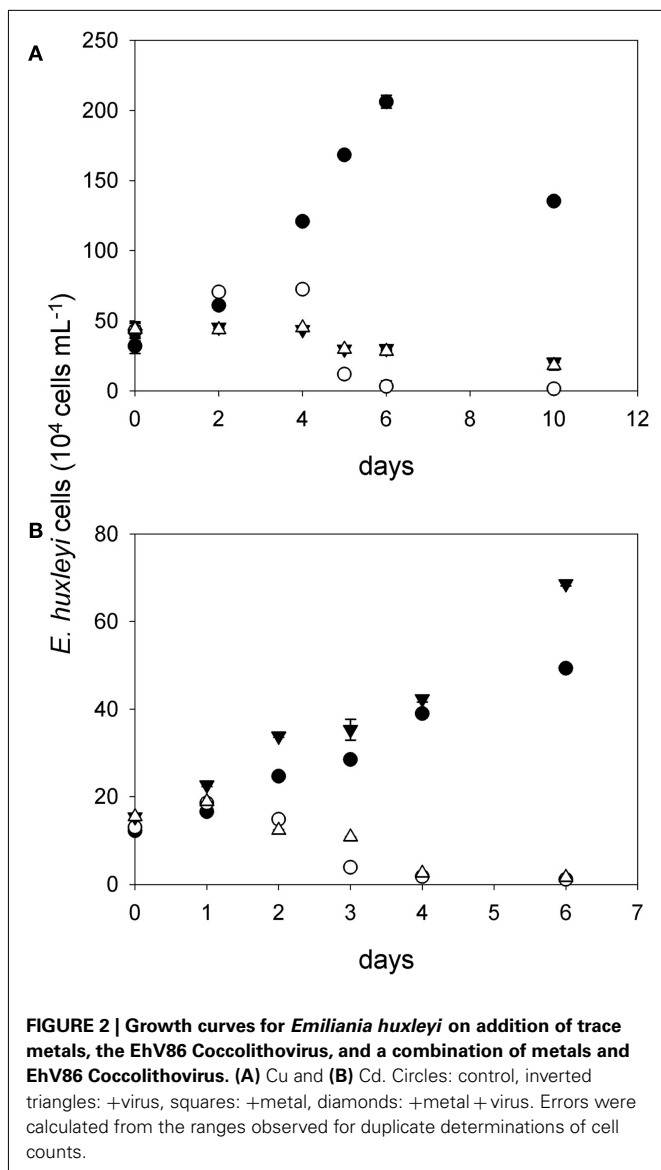
Errors were calculated from the square root of the sum of squares of the errors in the slopes obtained from linear regression of ln(cells) against time for the period over which lysis occurred in the +virus treatment.

the controls in each experiment for the post infection period and reflected results observed in preliminary experiments (**Figure 1**). Cellular GSH concentrations (**Figure 3**) were 66 ± 10 amol cell^{-1} on day 0 for the Cu experiment and 120 ± 20 amol cell^{-1} for the Cd experiment and are similar to those reported previously for *E. huxleyi* and marine phytoplankton (Ahner et al., 1995; Kawakami et al., 2006c). Cellular GSH content increased in the infected treatments (**Figure 3**). For the Cu experiment, GSH remained similar in both Cu and +Cu + Virus treatments, while in the Cd experiment, cellular GSH levels in the Cd treatment were similar to the control, while levels increased in the +Cd + Virus treatment (**Figure 3**). A previous study on infected *E. huxleyi* cells indicated an increased intracellular occurrence of ROS and oxidative stress (Evans et al., 2006). The increase in GSH in virus infected cells in this study is consistent with a response to increased oxidative stress on viral infection. Glutathione content in the +Cd + Virus treatment increased to an average of 216 ± 11 amol cell^{-1} between days 4 and 6 after infection and was closer to that of the virus treatment (316 ± 27 amol cell^{-1}) than the Cd treatment (46 ± 29 amol cell^{-1}) and control (50 ± 11 amol cell^{-1}). In contrast the cellular GSH content of the +Cu + Virus treatment was 55 ± 16 amol cell^{-1}, similar to that of the Cu treatment (55 ± 19 amol cell^{-1}), but less than GSH in the virus treatment (124 ± 85 amol cell^{-1}), although there was a marked increase in GSH variability in this treatment and the difference between the virus treatment and the control was not as marked as for the Cd experiment (**Figure 3**). These results indicate that the ROS event associated with viral infection is reduced by Cu.

Phytochelatin concentrations increased after addition of both Cu and Cd (**Figure 4**). Virus treatments showed only a slight increase in PC concentrations, although the small *E. huxleyi* biovolume in these treatments resulted in very low absolute PC concentrations and the calculated errors are likely underestimated. Cellular phytochelatin concentrations were higher in the Cd and +Cd + Virus treatments than in the Cu or +Cu + Virus treatments. Thus in contrast to the cellular GSH content, PC concentrations were observed to vary with metal treatment and not with infection of *E. huxleyi* by EhV86.
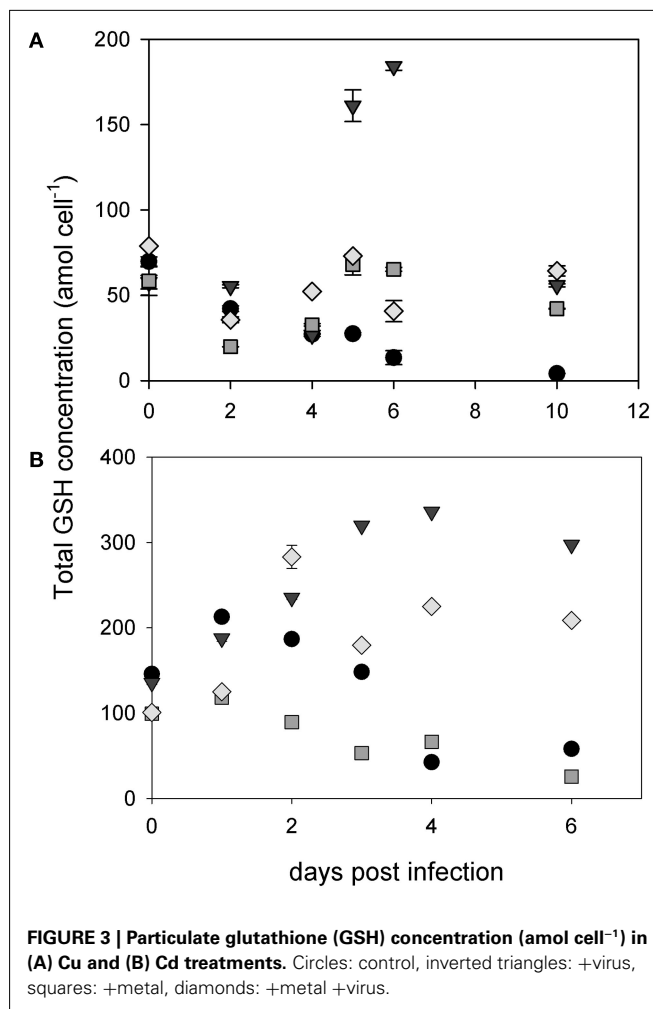
EXPRESSION OF VIRUS-RELATED RNA

In order to confirm the presence of EhV86 within Cu and Cd exposed *E. huxleyi* cells and to determine whether EhV86 was transcriptionally active when exposed to elevated levels of Cu and Cd, RT-PCR on four EhV86 encoded genes – DNA dependent DNA pol – ehv030, PCNA protein – ehv440, Helicase – ehv104 and MCP – ehv085 – was carried out in duplicate before and after treatment (**Tables 2** and **3**). Particulate material from Cu and Cd experiments in the absence of virus were also analyzed and viral RNA was not detected in any treatment not infected with the virus (**Tables 2** and **3**). Genomic studies have previously revealed that all four genes are required for successful infection (Wilson et al., 2005; Allen et al., 2006). DNA pol and helicase are required for DNA synthesis and therefore virus replication. The MCP is required for virion assembly and PCNA is known to be involved in several metabolic pathways, including Okazaki fragment processing, DNA repair, translesion DNA synthesis, DNA methylation, chromatin remodeling, and cell cycle regulation. PCNA in mammalian cells

FIGURE 2 | Growth curves for *Emiliania huxleyi* on addition of trace metals, the EhV86 Coccolithovirus, and a combination of metals and EhV86 Coccolithovirus. (A) Cu and (B) Cd. Circles: control, inverted triangles: +virus, squares: +metal, diamonds: +metal + virus. Errors were calculated from the ranges observed for duplicate determinations of cell counts.

FIGURE 3 | Particulate glutathione (GSH) concentration (amol cell^{-1}) in (A) Cu and (B) Cd treatments. Circles: control, inverted triangles: +virus, squares: +metal, diamonds: +metal +virus.

appears to play a key role in controlling several reactions through the coordination and organization of different partner proteins (Maga and Hubscher, 2003). DNA pol, helicase, and PCNA genes have been found to be expressed at around 2 h post infection, while MCP was only first detected 4 h post infection (Allen et al., 2006). We found that Cd had no observable effect on the transcription of all four genes (**Table 3**), consistent with observations of virus synthesis and culture lysis (**Figures 3** and **4**). However, in the presence of Cu, only PCNA, and MCP genes were expressed. These results show that Cu did not therefore prevent virus entry into *E. huxleyi* cells. However, viral replication within *E. huxleyi* appears to have been reduced by Cu via an unknown mechanism of transcriptional control.

DISCUSSION

Our experiments indicated that metals have a direct effect on virus production and consequently infection success in the *E. huxleyi* – EhV86 system. Furthermore it appears that the effect is stronger for Cu than for Cd, Zn, and Co so that Cu caused a reduction in lytic infection in short term exposure experiments at high concentrations of total Cu (> approximately 500 nM). Although these concentrations are higher than commonly observed in the marine environment, such high concentrations have been recorded in contaminated estuarine systems (Braungardt et al., 2007). Furthermore, these experiments represented an acute exposure to high concentrations of metal. Further investigations are thus required in order to assess effects of long-term metal exposure.

The mechanism of the interaction between Cu and the *E. huxleyi* – EhV86 system is currently unknown. Our observations are summarized in **Figure 5**. Our data show that EhV86 did not have an impact on the production of PCs in *E. huxleyi* and thus was unlikely to have affected the toxicity of Cu to the host. The primary effect of Cu on the lytic cycle of EhV86 thus appears to result from greater sensitivity of the virus to Cu toxicity relative to the host. Viruses require metals for the functioning of their DNA and RNA replication enzymes, so it has been previously suggested that viral inactivation by Cu involves cleavage or damage of viral DNA or RNA (Sagripanti et al., 1997). Recent studies have linked
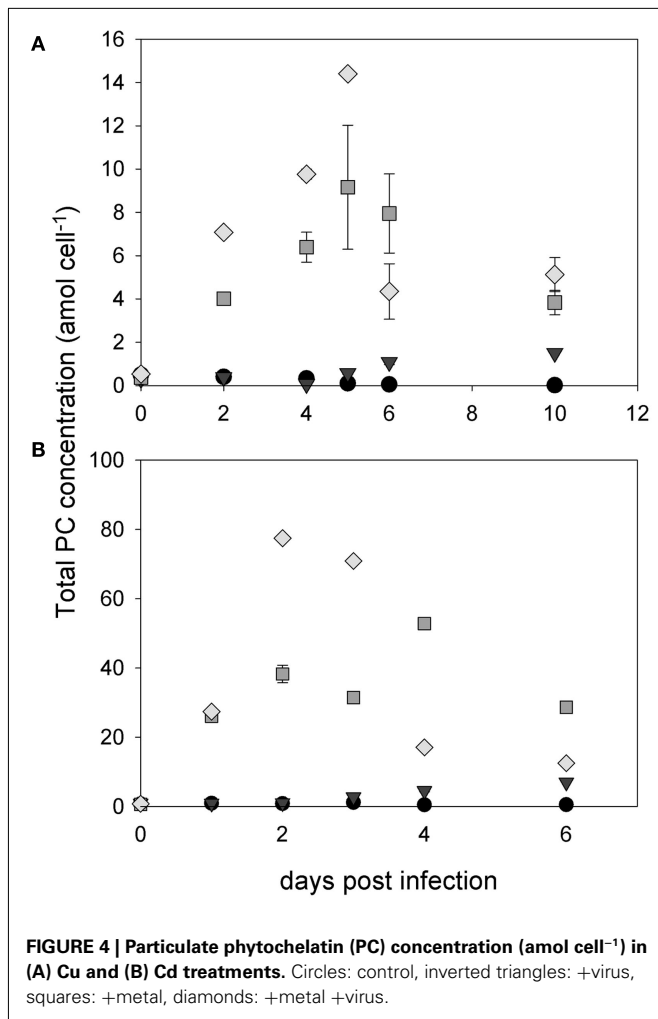
FIGURE 4 | Particulate phytochelatin (PC) concentration (amol cell⁻¹) in (A) Cu and (B) Cd treatments. Circles: control, inverted triangles: +virus, squares: +metal, diamonds: +metal +virus.

Table 3 | RT-PCR results for Cd exposure experiments.

	EhV86 genes			
	DNA pol	**Helicase**	**PCNA**	**MCP**
EhV86 day 4 (v0*)	–	–	–	–
EhV86 day 5 (v1*)	+	+	+	+
EhV86 day 6 (v2*)	+	+	+	+
EhV86 day 7 (v3*)	+	+	+	+
EhV86 day 8 (v4*)	+	+	+	+
Cd day 4	–	–	–	–
Cd day 5	–	–	–	–
Cd day 6	–	–	–	–
Cd day 7	–	–	–	–
Cd day 8	–	–	–	–
Cd + EhV86 day 4 (v0*)	–	–	–	–
Cd + EhV86 day 5 (v1*)	+	+	+	+
Cd + EhV86 day 6 (v2*)	+	+	+	+
Cd + EhV86 day 7 (v3*)	+	+	+	+
Cd + EhV86 day 8 (v4*)	+	+	+	+
NTC^	–	–	–	–

Days post EhV86 addition; ^no template control.

and furthermore, RNA transcripts for both PCNA and MCP were detected. The lack of DNA pol and helicase transcripts indicates that a specific mechanism of Cu inhibition was at play. DNA pol and helicase are both associated with DNA synthesis and replication, hence it appears that Cu disrupted virus replication within the host cell, although further work is needed to identify whether this is a result of exposure of the virus to elevated free Cu within the host cell or in the media prior to entry into the host. However since neither MCP or PCNA are affected, we hypothesize that virion assembly, DNA repair, and other cellular functions associated with PCNA were not regulated at the transcriptional level by Cu. Thus, despite being involved in DNA replication and repair, viral PCNA was not regulated in the same way as dedicated DNA replicative enzymes, DNA pol, and helicase. It is interesting to note that both DNA pol and helicase encode for magnesium containing proteins, while PCNA and MCP do not, although whether this factor might influence the inhibition of transcription of the associated RNA is unclear. Intracellular free Cu concentrations in eukaryotes and prokaryotes are generally thought to be tightly controlled, with Cu being buffered within the cell by thiols and chaperoned into Cu containing enzymes (Robinson and Winge, 2010; Rae et al., 1999). The intravirion behavior of metals however is less well known, but our studies indicate that EhV86 may be less able to regulate Cu. Previous studies on the effects of Cu on the transcriptional control of genes have indicated regulation of Cu is achieved by modulating the transcription of genes encoding proteins directly involved in Cu binding. Transcription of the Ctr1/3 and Fre1 genes are regulated by the DNA-binding protein Mac1 (Yamaguchi-Iwai et al., 1997). During Cu starvation, Mac1 binds to DNA initiating transcription. However, in Cu replete conditions, Jensen and Winge (1998) demonstrated that Cu ions bind to the protein, initiating a conformational change which inhibits transcription. Transcriptional regulation of these Cu uptake genes
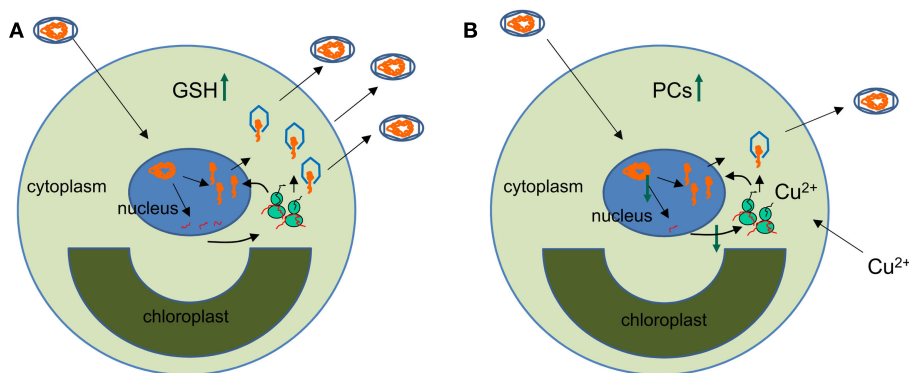
Table 2 | RT-PCR results for Cu exposure experiments.

	EhV86 genes			
	DNA pol	**Helicase**	**PCNA**	**MCP**
Control day 5	–	–	–	–
Control day 7	–	–	–	–
Cu day 5	–	–	–	–
Cu day 7	–	–	–	–
EhV86 day 7 (v2*)	+	+	+	+
EhV86 day 10 (v5*)	+	+	+	+
Cu + EhV86 day 5 (v0*)	–	–	–	–
Cu + EhV86 day 7 (v2*)	–	–	+	+
NTC^	–	–	–	–

Days post EhV86 addition; ^no template control.

Cu exposure to a complete decline in virus particles confirming antiviral properties of Cu-based agents and surfaces against major opportunistic pathogens (Noyce et al., 2006a,b, 2007; Huang et al., 2008; Weaver et al., 2008). However, in our study, virus particles were not observed to decline completely in the presence of Cu,

FIGURE 5 | Schematic of the interactive effect of Cu on the infection cycle of EhV86. (A) Simplified schematic of the normal infection cycle of EhV86 (after Mackinder et al., 2009). The virion particle (blue hexagon) enters the cell and targets the nucleus where it releases the viral genome. Viral genes are transcribed (red) first by host RNA polymerase in the nucleus and then by viral RNA polymerase in the cytoplasm. Virus DNA replication (orange) also takes place in the nucleus, while the capsid is assembled in the cytoplasm and released via a budding mechanism.

Cellular glutathione (GSH) increases (green arrow) as a result of reactive oxygen species generation, which occurs during capsid assembly. **(B)** Copper down-regulates (green arrow) the infection cycle prior to capsid assembly via transcriptional control of specific viral RNA. Cellular phytochelatin concentrations (PCs) increase in response to elevated copper concentrations, but GSH concentrations are similar to those in uninfected cells as capsid assembly is highly reduced. The ultimate result is a reduction in virus production.

has therefore been linked directly to cellular Cu concentrations (Graden and Winge, 1997).

The second observed effect of Cu in our study was on particulate GSH concentrations, which were similar to controls in infected *E. huxleyi* cells in the presence of Cu, but increased in *E. huxleyi* cells undergoing lysis, even when the concentrations of phytochelatins were higher (**Figure 3**). These results indicate that GSH, known to mitigate against ROS, is produced by the host when the virus replicates probably as a result of the viral induction of the hosts ceramide and apoptosis pathways (Schwarz, 1996; Stohs et al., 2001; Wilson et al., 2005; Bidle et al., 2007; Vardi et al., 2009). Moreover, GSH can also directly interfere with virus replication, mainly at the late stages of virion assembly (Palamara et al., 1995). Therefore, in our Cu exposed virus treatments, GSH production decreased when compared to virus treatments because the virus was unable to complete its replication and infection cycle and GSH was thus not induced to the same extent in the host.

To the authors knowledge, this is the first report of an interaction occurring between metals, virus, and host in a marine system. We have identified a specific interaction between Cu, *E. huxleyi* and its virus EhV86, however, further work is necessary in order to understand the mechanism involved in this interaction. Concentrations of Cu required to initiate the inhibitory effect on virus replication and virion synthesis were found to be comparable to those observed in highly Cu contaminated estuaries (Braungardt et al., 2007). However the study focused on short term acute exposure to Cu. It is currently unknown if any effects would be observed on long-term exposure to lower concentrations. However, the increased use of Cu-based antifouling paints in marine systems (Schiff et al., 2004; Singh and Turner, 2009a,b) and the importance of phytoplankton communities in underpinning global climate control (Richardson and Schoeman, 2004; Bouvy et al., 2011) highlights the need to understand such mechanisms. The immediate medical benefit that can be had by fully characterizing the Cu inhibitory effort on the *E. huxleyi* – EhV86 model system for developing antiviral drugs and protocols against animal virus infections should also be considered.

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