Occurrence of chlorophyll allomers during virus-induced mortality and population decline in the ubiquitous picoeukaryote *Ostreococcus tauri*

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Summary

During viral infection and growth limitation of the picoeukaryote *Ostreococcus tauri*, we examined the relationship between membrane permeability, oxidative stress and chlorophyll allomers (oxidation products). Chlorophyll allomers were measured in batch-cultures of *O. tauri* in parallel with maximum quantum efficiency of photosystem II photochemistry (*Fv*/*Fm*), carotenoids, and reactive oxygen species and membrane permeability using fluorescent probes (CM-H$_2$DCFDA and SYTOX-Green). Viral infection led to mass cell lysis of the *O. tauri* cells within 48 h. The concentration of the allomer hydroxychlorophyll *a* peaked with a 16-fold increase (relative to chlorophyll-*a*) just after the major lysis event. In contrast, cell death due to growth limitation resulted in a twofold increase in allomer production, relative to chl-*a*. Two allomers were detected solely in association with *O. tauri* debris after viral lysis, and unlike other allomers were not observed before viral lysis, or during cell death due to growth limitation. Conversely, the component chl-*a*$_{276}$ was found in the highest concentrations relative to chl-*a* in exponentially growing *O. tauri*. The components described have potential as indicators of mode of phytoplankton mortality, and of population growth.

Introduction

Phytoplankton assemblages are highly variable in their functionality, and at times large proportions (up to 95%) can be classified as dead and non-photosynthetically functional (Veldhuis *et al.*, 2001; Agustí and Sánchez, 2002). It is generally assumed that intact pigment-containing cells are viable and therefore capable of manifesting physiological processes such as cell growth, repair and division, or that they are dormant. It is clear, however, that whole, non-functional and non-viable cells can be abundant and therefore contribute to ocean colour, and hence to estimates of chlorophyll a biomass. After phytoplankton death, caused by environmental factors such as nutrient depletion, temperature stress (Agustí *et al.*, 2006; Alonso-Laita and Agustí, 2006) or light stress (Berman-Frank *et al.*, 2004; Liabrés and Agustí, 2006; Liabrés *et al.*, 2010), intact but dead cells may persist. The chlorophyll contained in dead cells is not photosynthetically active, which could cause an uncoupling of estimates of chlorophyll *a* biomass and primary production (Key *et al.*, 2010). Therefore, it is essential to distinguish functional from non-functional phytoplankton cells.

Chlorophyll transformation i.e. alteration to the chlorophyll molecule, can arise from any disruption to the phytoplankton cell (Jeffrey and Vesk, 1997) via many different processes, and transformation products have been linked with different modes of phytoplankton mortality (Head and Horne, 1993; Walker and Keely, 2004; Bale *et al.*, 2011). The allomer (chlorophyll oxidation product) hydroxychlorophyll *a* (HO-chl-*a*) is thought to be common in phytoplankton during cell demise (Franklin *et al.*, 2012; Steele *et al.*, 2015) and is routinely detected during pigment analyses (Hooker *et al.*, 2005) if the HPLC method used is adequate for its detection, in both high density (Walker and Keely, 2004; Bale *et al.*, 2015; Steele *et al.*, 2015) and low density (Steele, 2014; Steele *et al.*, 2015) algal populations. Hydroxychlorophyll *a* has also been associated with the onset of bloom decline in naturally occurring phytoplankton populations (Walker and Keely, 2004; Steele *et al.*, 2015) and has been found to increase in sinking particles (dominated by diatom-derived material, Bale *et al.*, 2015). Chlorophyll allomers have also been assigned as markers of fresh photoautotrophic organic matter, originating from coastal zones with high dissolved oxygen (Szymczak-Zyla *et al.*, 2011). However, few studies have investigated phytoplankton cell physiological state and chl-*a* alterations (Franklin *et al.*, 2012).
During cell death, when the mechanisms which protect the cell against oxidative stress break down, and/or programmed cell death (PCD) is induced, an increase in reactive oxygen species (ROS) inside the cell may increase the quantity of allomers relative to chlorophyll (Franklin et al., 2012). As HO-chl-a is the major product of chl-a in the presence of hydrogen peroxide, it is likely to be associated with conditions where peroxynitrous (e.g., hydrogen peroxide) are elevated (Walker et al., 2002), for example during oxidative stress. Intracellular ROS are by-products of regular cellular processes (e.g., photosynthesis) and are regulated by antioxidant mechanisms to avoid oxidative stress in the cell (Apel and Hirt, 2004). ROS are also known to be ubiquitous signalling molecules in the cell against oxidative stress break down, and/or processes. ROS appears to be stimulated by death of the chlorophyte Dunaliella tertiolecta (Segovia and Berges, 2009); and bloom-termination of the dinoflagellate Peridinium gatunense due to CO₂ limitation (Berman-Frank et al., 1994; Vardi et al., 1999). This induction of PCD by ROS also occurs during virus-induced cell death of E. huxleyi (Bidle, 2015), which mediates cellular phytoplankton death processes, for example during cell death due to environmental factors; including dark-induced death of the chlorophyte Dunaliella tertiolecta (Segovia and Berges, 2009); and bloom-termination of the dinoflagellate Peridinium gatunense due to CO₂ limitation (Berman-Frank et al., 1994; Vardi et al., 1999). Viruses are thought to be responsible for causing the release of about a quarter of all photosynthetically fixed carbon through cell lysis (Fuhrman, 1999), thereby regulating phytoplankton community structure (Murray and Jackson, 1992), biomass production and population density (Bratbak et al., 1993; Vardi et al., 2012). Virus-mediated death of phytoplankton is known to lead to degradation of photosynthetic pigments (Llewellyn et al., 2007), but the timing and specific effect on chlorophyll a during infection and lysis are not well understood. Host photosynthesis is not always required for viral growth but varies with species (Benson and Martin, 1981; Van Etten et al., 1983; Suttle and Chan, 1993; Seaton et al., 1995; Juneau et al., 2003). Hence, the effect of viral infection on host photosynthetic capacity is variable. Pigment transformations during viral lysis of Emiliania huxleyi have been described (Llewellyn et al., 2007; Bale et al., 2013), and there is some evidence for production of chlorophyll allomers (Bale et al., 2013). These studies however, lack concurrent physiological measurements that describe the average phytoplankton population cell state during virus infection and lysis.

To assess the ubiquity of allomer formation and its potential co-occurrence with loss of algal cell function, it is important to expand the study of chlorophyll transformation during viral lysis beyond one genus. The cosmopolitan picoeukaryote Ostreococcus tauri was chosen for this study as it is an environmentally relevant primary producer for which a lytic virus has previously been isolated (thus, a model host: virus system was available, Derelle et al., 2008). Typically, picoeukaryotes are abundant in the world's oceans, have a relatively high rate of carbon fixation (Li, 1994; Worden et al., 2004) and are particularly important in the open ocean (Grob et al., 2011). Furthermore, the eukaryotic picoplankton genus Ostreococcus has a global distribution (coastal seas, the oligotrophic North Atlantic, the Mediterranean, the Indian and Pacific Oceans (Worden et al., 2004; Zhu et al., 2005; Countway and Caron, 2006), and a wide depth profile (surface waters to 120 m depth, Worden et al., 2004). Here we used a well characterised strain of Ostreococcus tauri (OTH95 RCC745), which has undergone complete genomic sequencing (Derelle et al., 2006; Robbens et al., 2007), to analyse the relationship between cell demise and chlorophyll allomers.

This study describes the formation of chlorophyll allomers in Ostreococcus tauri during two mortality pathways: viral infection and growth limitation, with particular focus on the period directly before, and at the onset of, population decline. We performed concurrent measurements of O. tauri growth, OtV5 virus abundance, membrane permeability, cellular un-scavenged reactive oxygen species, chlorophyll a (chl-a), chlorophyll b (chl-b) and their allomers, the chl-a precursor (chl-a₇₂₇₆), maximum quantum efficiency of photosystem II photochemistry (Fᵥ/Fₘ), and the carotenoid components of the non-photochemical quenching xanthophyll cycle, to describe in detail the timing of allomer formation in relation to population physiological state.

Results

Virus-infected cultures; physiological indicators

The virus OtV5, which infects O. tauri, was added to triplicate O. tauri cultures after 3 days of growth. The observed cycle of infection was consistent with previous work by Derelle et al. (2008). Infected cultures began to decline between 8 and 24 hours post-infection (hpi, days 3.3 and 4, Fig. 1A). At this time the O. tauri population density and Fᵥ/Fₘ decreased, while simultaneously SYTOX-Green staining for membrane permeability, increased (Fig. 1A–C). By 32 hpi (day 4.3), 93.4 ± 2.0% (mean ± SD) of the O. tauri population had lysed, and of the remaining cells, 52.5 ± 4.5% stained positively with SYTOX (Fig. 1C). The cytograms of O. tauri (Fig. 2) show cellular material which have retained a degree of red (chlorophyll-type)
fluorescence (in the lower left hand corner). For flasks inoculated with OtV5, these cell fragments, labelled as debris, show maxima on days 5 and 5.3, just after the main lysis event between days 4 and 4.3 (Fig. 1A). This debris may have also contributed to the increased Fv/Fm values. In the control flasks, the relative proportion of cell debris remained relatively constant over the same period.

Virus numbers had increased (80 000-fold) by 8 hpi (day 3.3), however, the major viral lysis event occurred between 24 hpi (day 4) and 32 hpi (day 4.3). During this time OtV5 abundance peaked at $9 \times 10^6$ mL$^{-1}$. After viral inoculation CM-H$_2$DCFDA staining for detection of reactive oxygen species (ROS) was significantly elevated. The percentage of cells stained CM-H$_2$DCFDA-positive increased 0.6-fold relative to the non-inoculated control flasks 24 hpi (Fig. 1D, Mann-Whitney $U = 4.5$, $P < 0.05$). This CM-H$_2$DCFDA staining increased significantly (Spearman rank; $R = 0.714$, $P < 0.05$) over time in inoculated cultures to a maximum of $0.75 \pm 0.14\%$, (a 7.3-fold increase) 56 hpi (day 5.3). Notably, the CM-H$_2$DCFDA stain was indicating non-scavenged ROS within intact O. tauri cells, i.e. those which had not lysed. A small residual surviving population of O. tauri began to regrow between 48 hpi and 56 hpi (days 5 and 5.3, Fig. 2), as identified previously in this O. tauri strain (Thomas et al., 2011), with the specific growth rate ($\mu$) of $0.07 \pm 0.01$ h$^{-1}$. This residual population was potentially resistant to infection by OtV5, and continued growing exponentially ($\mu = 0.69$) for at least 5 days. This residual population may account for the recovering photosynthetic capacity evident from Fv/Fm values (Fig. 1B).

**Virus-infected cultures; chlorophylls, allomers and chl-a precursor**

For chemical assignment of components, see below. All chlorophyll allomers reached their maximum concentration relative to their parent chlorophylls on day 5 (Fig. 3B–G), coinciding with the maximum proportion of cell debris observed in the cytograms (Fig. 2). The ratio of hydroxychlorophyll b (HO-chl-b) increased relative to chl-b above levels of the control flasks 24 hpi (day 4, $0.076 \pm 0.003$, mean $\pm$ SE, 0.9-fold increase), and reached a maximum of $0.42 \pm 0.04$ at 48 hpi (day 5, Fig. 3B, 9.9-fold increase. This timing of chl-b allomer production was matched by the dominant chl-a allomer produced during OtV5-infection, hydroxychlorophyll a (HO-chl-a) and its epimer. These HO-chl-a allomers, relative to chl-a, increased above levels of the control flasks 24 hpi, and reached their maxima of $0.110 \pm 0.008$ and $0.08 \pm 0.009$ respectively (equal to 26-fold and 78-fold increases) at 48 hpi (day 5, Fig. 3F and G), when $99.0 \pm 2.8\%$ of the O. tauri population had lysed. At this time, non-infected cultures maintained low levels of HO-chl-a of $0.004 \pm 0.000$.

After the major lysis event between 32 and 48 hpi, pigment samples contained methoxychlorophyll a-like (MeO-chl-a-like) and hydroxychlorophyll a-like (HO-chl-a-like) allomers, with average allomer to chl-a ratios of $0.015 \pm 0.003$ and $0.011 \pm 0.002$ respectively ($n = 6$, Fig. 3D and E). Unlike the other allomers, these components were not detected in the cultures before viral lysis, nor were they detected in the control cultures. The timing of the detection of methoxychlorophyll a-like and hydroxychlorophyll a-like coincided with high levels of cell debris...
Fig. 2. Cytograms of an *Ostreococcus tauri* OTH95 RCC745 population during death by OtV5 lysis and re-growth of residual population (left column), and death by environmental limitation (right column). Population density plots representing all events acquired with red fluorescence: FL3-H (red fluorescence) and FSC-H (forward scatter).

that showed high red fluorescence in the cytograms (days 4.3 and 5, Fig. 2).

Both chlorophyll b, and the chlorophyll a precursor chl-\textit{a}$_{p276}$ showed similar profiles relative to chl-\textit{a} in the infected cultures (Fig. 3A and H). Both components showed small relative increases on days 4.3 and 5, and maximum abundance relative to chl-\textit{a} on day 5.3 (Fig. 3A and H), coincident with the regrowth of the residual \textit{O. tauri} population (Fig. 2). Chl-\textit{a}$_{p276}$ is a precursor in chlorophyll \textit{a} biosynthesis, and chlorophyll \textit{b} is used as a light harvesting chlorophyll in the peripheral antenna. Their small increases relative to chlorophyll \textit{a} on day 4.3, during the main lysis event, may be due to their location in the peripheral antenna, whereas chlorophyll \textit{a} is located in the core antenna and reaction centre as well as the peripheral antenna (Scheer, 2006) and therefore could be subject to a different rate of destruction during cell lysis.

\textbf{Virus-infected cultures: carotenoids}

In \textit{O. tauri} (as well as higher plants and green-algae) the xanthophyll cycle consists of the conversion of violaxanthin to zeaxanthin via antheraxanthin. During viral infection and lysis, zeaxanthin increased relative to the total abundance of \textit{O. tauri} carotenoids (Fig. 3). Viral infection and lysis coincided with the lysis of \textit{O. tauri} and a drop in carotenoid abundance (Fig. 2). Viola and antheraxanthin showed increased relative abundance in the infected cultures (Fig. 3). The increased abundance of the xanthophylls is consistent with the decrease in chlorophyll \textit{a} due to viral lysis (Fig. 2).
of the xanthophyll cycle components, 24 hpi to 48 hpi (day 4–5, Fig. 3K). This coincided with a decrease in violaxanthin relative to the xanthophyll cycle pigments over the same period (Fig. 3I), indicating active conversion of violaxanthin to zeaxanthin during and after viral lysis. 

\-\-carotene, neoxanthin and dihydrolutein all decreased relative to chl-\a between 24 hpi and 48 hpi (day 4 and day 5, Fig. 3L–N), and \-\-carotene and dihydrolutein then increased relative to chl-\a at day 5.3, coincident with population regrowth.

**Growth limited cultures; physiological indicators**

Prior to the study, the *O. tauri* cultures were closely monitored and maintained in semi-continuous batch culture conditions with specific growth rate (\(\mu\)) between 0.3 and 0.7 d\(^{-1}\). The non OtV5-infected control populations of *O. tauri* were kept incubated under standard growth conditions and progressed through a typical batch culture cycle. These populations began to decline after 16 day of growth, most likely due to nutrient limitation, although nutrients were not directly measured (Fig. 4A). Assuming cells were using nutrients in the Redfield ratio; by calculation, phosphate was potentially the initial nutrient to become limiting, as F/2 media has an N:P ratio of 24:1. The concentration of chl-\a increased throughout the stationary phase (days 3–15, Fig. 4E), then declined in line with the *O. tauri* population between days 15 and 29. \(F_v/F_m\), a measurement of PSII photosynthetic efficiency, declined gradually after the population growth rate slowed (day 6, Fig. 4B), and further after 15 day, as the *O. tauri* population declined. SYTOX staining, indicating the proportion of cells with permeable membranes, increased from day 10 and peaked on day 12 (with 8.2 ± 0.3% of the population stained with SYTOX, a 24-fold increase), before declining (Fig. 4C). CM-H\(_2\)DCFDA staining (Fig. 4D), indicating the relative amount of reactive oxygen species within the cells, peaked at the onset of population density decline (day 15), to a maximum of 8.5 ± 1.6% (a 65-fold increase). After the onset of population decline (day 16) CM-H\(_2\)DCFDA and SYTOX staining increased again (from days 17 and 20 respectively) as cell physiological state further deteriorated.


**Fig. 4.** Physiological changes of *Ostreococcus tauri* OTH95 RCC745 during batch culture growth, stationary and death phases (indicated by dashed lines). Including (A) Population density and specific growth rate (\(\mu, \text{d}^{-1}\)) of *O. tauri*; (B) maximum quantum efficiency of PSII photochemistry (\(F_v/F_m\)); (C) Percentage of *O. tauri* cells stained with SYTOX-Green (for membrane permeability), (D) Percentage of *O. tauri* cells stained with CM-H\(_2\)DCFDA (for reactive oxygen species), and mean fluorescence intensity, (E) chlorophyll a content per cell. Mean and SE bars shown (\(n = 3\)).
Growth limited cultures; chlorophylls, allomers and chl-a precursor

All allomers showed increases relative to their parent chlorophyll during the death phase, between days 20 and 29 (Fig. 5B–E). The HO-chl-b to chl-b ratio increased during the death phase to a maximum of 0.075 ± 0.013 on day 29 (Fig. 5B and C, a 1.1-fold increase relative to day 5). HO-chl-a and HO-chl-a’ reached maxima of 0.0074 ± 0.0005 and 0.0072 ± 0.0007 (increases of twofold and sevenfold respectively from day 5, Fig. 5D and E). These increases were small compared to maximum levels observed after viral infection.

Chlorophyll b also increased during the death phase (Fig. 5A), and also reached a lower maximum than that observed after viral infection.

The component chl-a_{P276} was found in the highest concentration, relative to chl-a, in exponentially growing *O. tauri* (days 0–3, when \( \mu \geq 0.69 \), Fig. 5F), consistent with its assignment as a precursor to chl-a. Like chlorophyll b, it also increased relative to chl-a between days 20 and 29.

Growth-limited cultures; carotenoids

Throughout the stationary and death phases the conversion of violaxanthin to zeaxanthin is evident (Fig. 5G–I). Notably, the maximum ratios of zeaxanthin to xanthophyll cycle components were similar for both fate processes (Fig. 3K and Fig. 5I). \( \beta \)-carotene increased (relative to chl-a) during the stationary phase, but decreased during the death phase (Fig. 5K). Conversely, neoxanthin and dihydrolutein increased relative to chl-a during the death phase (Fig. 5K and L).

Photosynthetic pigments; chemical assignment

Pigment extracts from OTV5-infected *Ostreococcus tauri* cultures and non-infected cultures were analysed for...
Assignment of chlorophylls a and b were made by comparison of their retention times to standards, comparison of UV/vis absorption spectra (Table 1) and major ions observed during LC/MS analysis (Table 2), to published data (Airs et al., 2001; Bale et al., 2011; Franklin et al., 2012). The extracts gave rise to several peaks which eluted in the region expected for chlorophyll allomers (Walker et al., 2002), prior to chlorophyll a (peak IX, Fig. 6), with UV/vis absorption spectra (Table 1) consistent with chlorophyll a allomers (Franklin et al., 2012). An array of peaks exhibiting chlorin-like UV/vis spectra were also detected eluting prior to chlorophyll b (Peak V, Fig. 6).

Peak I was present only in pigment extracts from the OtV5-infected cultures; it was not detected at any time in the non-infected cultures. During LC/MS/MS analysis with post-column addition of acid (Airs et al., 2001), peak I gave rise to precursor and daughter ions in the same pattern as methoxychlorophyll a (Table 2; Franklin et al., 2012), but with all components exhibiting an increased mass of 4 Da. The component is therefore assigned as methoxychlorophyll a-like allomer.

Peak II gave rise to a UV/vis absorption spectrum, as expected for a chlorophyll a allomer but with an additional absorption band at 481 nm (Table 1). From LC/MS analysis with post-column acidification, peak II gave rise to one major ion at m/z 887 [M+H-Mg]+. The MS2 spectrum contained a major ion at m/z 593 (Table 2), corresponding to a loss of 294 Da. Notably, the phytyl chain of chlorophylls a and b usually cleaves at the C-O bond with H+ transfer to the charge retaining fragment during MS/MS resulting in the loss of the entire substituent with H+ transfer (294 Da) instead of 278 Da. The mass of the entire phytyl constituent is, however, 295 Da. It is possible therefore, that peak II was a chlorin esterified by phytol, but in such a stereochemical configuration to promote fragmentation via the loss of 294 Da instead of 278 Da. Notably, the MS2 spectrum shows a less abundant ion at m/z 609, arising from the loss of 278 Da from the parent ion, providing support.

### Table 1. Main UV/vis absorption bands and assignment of components in Fig. 6.

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Main UV/vis absorption bands (nm)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>427, (462), 653</td>
<td>Methoxychlorophyll a-like allomer</td>
</tr>
<tr>
<td>II</td>
<td>430, 481, 658</td>
<td>Hydroxychlorophyll a-like allomer</td>
</tr>
<tr>
<td>III</td>
<td>430, 660</td>
<td>Hydroxychlorophyll b</td>
</tr>
<tr>
<td>IV</td>
<td>430, 660</td>
<td>Hydroxychlorophyll b'</td>
</tr>
<tr>
<td>V</td>
<td>460, 648</td>
<td>Chlorophyll b</td>
</tr>
<tr>
<td>VI</td>
<td>430, 663</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>VII</td>
<td>420, 663</td>
<td>Hydroxychlorophyll a</td>
</tr>
<tr>
<td>VIII</td>
<td>430, 663</td>
<td>Hydroxychlorophyll a</td>
</tr>
<tr>
<td>IX</td>
<td>430, 663</td>
<td>Chlorophyll a</td>
</tr>
</tbody>
</table>

### Table 2. Abundant ions of peaks I-VII (see Fig. 6) observed during LC/MS/MS analysis of O. tauri pigment extracts.

<table>
<thead>
<tr>
<th>Relationship to molecular ion</th>
<th>Structural assignment</th>
<th>Peak no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MH</td>
<td>[M+H]+</td>
<td>I 927</td>
</tr>
<tr>
<td>MH-22</td>
<td>[M+H-Mg]+</td>
<td>II 905</td>
</tr>
<tr>
<td>MH-32</td>
<td>[M+H-MeOH]+</td>
<td>III 873</td>
</tr>
<tr>
<td>MH-22-278</td>
<td>[M+H-Mg-C20H38]+</td>
<td>V 627</td>
</tr>
<tr>
<td>MH-22-294</td>
<td>[M+H-Mg-C20H38O]+</td>
<td>VI 623</td>
</tr>
<tr>
<td>MH-22-276</td>
<td>[M+H-Mg-C20H38]+</td>
<td>VII 623</td>
</tr>
<tr>
<td>MH-22-276-60</td>
<td>[M+H-Mg-C20H38-CO2Me]+</td>
<td>VIII 609</td>
</tr>
<tr>
<td>MH-22-18</td>
<td>[M+H-Mg-H2O]+</td>
<td>I 883</td>
</tr>
<tr>
<td>MH-22-294-18</td>
<td>[M+H-Mg-C20H38H2O]+</td>
<td>IV 531</td>
</tr>
<tr>
<td>MH-278-60</td>
<td>[M+H-C20H38-CO2Me]+</td>
<td>V 589</td>
</tr>
<tr>
<td>MH-278-32</td>
<td>[M+H-C20H38-MeOH]+</td>
<td>VI 617</td>
</tr>
</tbody>
</table>
for the assignment of phytol as the esterifying alcohol, and indicating that both fragmentation mechanisms were taking place. It is important to note that the relative intensity of the ions in the MS² spectrum was low, and therefore the spectrum must be interpreted cautiously. Other fragmentations indicate the presence of a CO₂Me group (60 Da loss to produce an ion at m/z 533), and HO-substituent (18 Da loss to produce an ion at m/z 575), consistent with a hydroxychlorophyll a (HO-chl-a) structure. The component is therefore tentatively assigned as a hydroxychlorophyll a-type structure with unusual stereochemistry (HO-chl-a-like).

Peaks III and IV eluted in the region expected for chlorophyll b allomers, and were assigned as hydroxychlorophyll b and its epimer by comparison to published MS/MS data (Hyvärinen and Hynnin, 1999). Similarly, components VII and VIII were assigned as hydroxychlorophyll a and it’s epimer based on MSⁿ data (Table 2; Walker et al. 2002).

Peak VI had a UV/vis absorption spectrum similar to chl-a (Airs et al., 2001) (Table 1). From LC/MS/MS with post-column addition of acid, peak VI gave rise to a major ion at m/z 869 corresponding to [M + H-Mg]⁺ (Table 2). On resonance induced fragmentation, the ion at m/z 869, gave rise to m/z 593 in the MS² spectrum, equating to a loss of 276 Da indicating an extra double bond in the phytol chain. The component at peak VI is therefore identified as a biosynthetic precursor to chlorophyll a (Rüdiger, 2006), chlorophyll a₈₂₇₆ (chl-a₈₂₇₆). This component has been detected previously in Thalassiosira pseudonana, Emiliania huxleyi (Franklin et al., 2012) and Pavlova gyrans (Bale et al., 2011).

**Discussion**

Chlorophyll a allomers were produced in Ostreococcus tauri cultures 24 h post-infection (hpi) with OtV5, with an increase in the ratio of total chl-a allomers to chl-a of approximately 28-fold by 48 hpi (day 5). Allomers were also produced during O. tauri growth-limitation but in smaller amounts, with approximately a twofold increase in the ratio of total allomers to chl-a, 14 days after the onset of population decline. In flasks inoculated with OtV5, and in growth-limited flasks, the maximum ratio of allomers to chl-a coincided with the maximum proportion of cell debris detected in cytograms (day 5 and day 29 respectively, Fig. 2). Although the proportion of cell debris retained in the cell pellet during pigment sample collection via centrifugation is unknown, it can be assumed to be consistent between days 4.3 and 5.3, as the size spectrum of the debris, indicated by the forward scatter of the cytograms, did not change significantly during this period (Fig. 2). To be detected by flow cytometry (in this case), the cell debris must have exhibited red fluorescence, which arises from chlorophyll-type structures. Notably the debris showed maximum fluorescence on days 4.3 and 5 in samples from the infected flasks, and day 29 in the growth-limited flask (Fig. 2), coincident with the maximum increase in chlorophyll allomers relative to parent chlorophylls. The cell debris is likely to comprise a ‘soup’ of cellular components, including fragments of membrane and chloroplasts, and likely to be a prime source of reactive oxygen species, due to the presence of illuminated chlorophyll, and the disintegration of cellular machinery to prevent ROS formation and provide effective scavenging. Indeed, staining of the cell debris by CM-H₂DCFDA was observed, but not quantified (data not shown). Therefore, we propose that the increase in chlorophyll allomers observed after viral lysis and growth-limitation were formed in the cell debris, rather than in unlysed cells. Two allomers have been found solely in association with O. tauri lysis by OtV5; a methoxychlorophyll a-like allomer and a hydroxychlorophyll a-like allomer. These components were not detected prior to viral infection, or during growth limitation, and therefore may be specific to viral infection and lysis.

Measurement of ROS within the cells showed a response in OtV5-infected cultures—a 7.3-fold increase in the percentage of CM-H₂DCFDA-positive cells, but this was due to the small residual population. In the growth-limited population cellular ROS increased as growth limitation progressed, however, a peak in CM-H₂DCFDA-positive cells also occurred just prior to population decline (day 15). A peak in SYTOX-positive cells (with compromised membranes) occurred prior to this (day 12). Cell cycle arrest in a part of, or in the whole population during stationary phase may have allowed this build-up of ROS. A peak in ROS before population decline has also been detected in the diatom Thalassiosira oceania (D. J. Steele, unpublished). The proportion of cells detected with compromised membranes during OtV5-infection was much greater (maximum 51.8%), although as the lysis process happens quickly, there was only a brief window when membranes were compromised but cells were still relatively intact. This level of SYTOX staining is comparable to that of natural picoplankton (Veldhuis et al., 2001; Baudoux et al., 2008), and small eukaryote (Veldhuis et al., 1997) populations in mixed natural assemblages, where between 3% and 75% of the cells stained SYTOX-positive.

It is possible that the retention of CM-H₂DCFDA was very poor in O. tauri, as only a small percentage of cells were stained (maximum 8.5%). Low levels of CM-H₂DCFDA staining have also been observed during viral infection of Emiliania huxleyi CCMP 1516 with mean probe intensity increasing 0.4-fold (approx) 48 hpi, up to twofold (approx.) 76 hpi (Evans et al., 2006); and in E. huxleyi CCMP 2090, where staining increased 0.5-fold (approx.) 72 hpi (Sheyn et al., 2016). Elevated intracellular ROS (up to 40% CM-H₂DCFDA-positive) was observed during the
late lytic phase of viral infection of a natural coccolithophore population (Vardi et al., 2012). The physiological differences in cell type between *E. huxleyi* ( Prymnesiophyte) and *O. tauri* (Prasinophyte) may account for differences in CM-H<sub>2</sub>DCFDA uptake. Even after careful optimisation and validation, probe response was limited in *O. tauri*. However, it appears that the cells capacity to buffer ROS, in particular hydroxyl radicals and peroxynitrite anions, which the probe is most sensitive to (Haugland, 2010), was maintained during OtV5-infection and growth-limitation via ROS scavenging and non-photochemical quenching.

The xanthophyll cycle dissipates excess excitation energy which would otherwise lead to the formation of destructive singlet oxygen (1O<sub>2</sub>). The product of this xanthophyll cycle, zeaxanthin, deactivates excited singlet chlorophyll (Niyogi et al., 1998) and is also an antioxidant in the lipid phase of the thylakoid membrane (Havaux et al., 2007). The increase of zeaxanthin 24 hpi indicates that the xanthophyll cycle, a non-photochemical quenching (NPQ) process, increased in rate, during decreased photochemical quenching, reflected by decreased F<sub>v</sub>/F<sub>m</sub> 24 hpi (Fig. 1B). β-carotene is a precursor to the xanthophylls, and is also a ROS scavenger (Fiedor et al., 2001; 2005), specifically of 1O<sub>2</sub> (Telfer, 2002). β-carotene decreased relative to chl-a 24 hpi. As β-carotene is situated in the reaction centres (Young, 1993; Fiedor et al., 2001; 2005; Telfer, 2002), it also may be more prone to photooxidation (Llewellyn et al., 2007). Therefore, its decrease may be due to 1O<sub>2</sub> scavenging and photodegradation of the photosystem by ROS. This, along with the increased rate of non-photochemical quenching, prevented the build-up of cellular ROS, hence chlorophyll allomers remained low until after the major lysis events, due to OtV5-infection, and growth-limitation.

This capacity of *O. tauri* to buffer ROS accounts for the minimal chlorophyll allomer production of cultures before cell lysis. Increased formation of cellular HO-chl-a during cell lysis by viral action has been observed previously in *E. huxleyi* CCMP 1516 (Bale et al., 2013); an increase of fourfold in absolute mass per cell, where the ratio of HO-chl-a to chl-a increased from approx. 0.004 at the beginning of population decline (4 days post-infection), increasing to approx. 0.02 after the loss of 94% of the population (16 days post-infection). Notably this also represents cellular HO-chl-a plus HO-chl-a in cell debris, which would have been collected by filtration. Here, hydroxyclychrophyll a production in growth-limited *O. tauri* cultures, was consistent with previous observations during senescence of *Isochrysis galbana* (Prymnesiophyte, Bale et al., 2011) and *Thalassiosira pseudonana* (Diatom, Franklin et al., 2012). Chlorophyll a allomers have been observed not to increase during population decline of *E. huxleyi* (Franklin et al., 2012), however it was hypothesised by Franklin et al. (2012) that the *E. huxleyi* population, under N-limitation, may have undergone a physiological change, possibly in preparation for meiosis, rather than mortality (Franklin et al., 2012). Also *E. huxleyi* can maintain PSII repair through periods of nitrogen depletion (Loebl et al., 2010), limiting PSI photo-inactivation and ROS production (Holt et al., 2004; Key et al., 2010), which may decrease the formation of the oxidation product HO-chl-a. Rates of PSII repair are lower in *O. tauri* and lower still in *T. pseudonana* (Six et al., 2009; Key et al., 2010), which is reflected in the higher ratios of HO-chl-a to chl-a in *T. pseudonana* (Franklin et al., 2012) compared to *O. tauri*, during growth limitation.

To date, in the published studies of chlorophyll allomers in phytoplankton cultures, HO-chl-a has been detected ubiquitously across taxa (Bale et al., 2011; 2013; Franklin et al., 2012), but production during cell death varies with species and mode of death. Therefore, the total HO-chl-a in a mixed, natural population will vary according to community composition and the modes of mortality taking place. In natural phytoplankton populations the main causes of mortality are grazing (Walsh, 1983), viral lysis (Suttle et al., 1990; Brussaard, 2004) and senescence due to environmental factors (Walsh, 1983). The contribution of each of these factors varies greatly with season and region. HO-chl-a has been detected in various natural waters (Walker and Keely, 2004; Steele, 2014; Bale et al., 2015; Steele et al., 2015). Increased concentrations of HO-chl-a have been reported in sinking particles, i.e., during a diatom bloom terminated by nutrient-limitation (Bale et al., 2015), and after declines of various phytoplankton blooms in the Western English Channel, (Steele et al., 2015). Unfortunately, none of the reports of HO-chl-a in natural waters (to date) have included viral enumeration. In *O. tauri*, the total amount of allomers (relative to chl-a) varied depending on the mode of death, from 13.8 ± 2.1% during viral-infection to 3.1 ± 0.8% during growth limitation. Hence when a bulk measurement of total chlorophyll a is taken from a population dominated by *O. tauri*, the contribution to allomers of this total will be greatly increased if viral-infection is occurring.

Here, the observed cycle of infection and lysis of *O. tauri* by OtV5 within 2 days of the maximum population density, with rates of population decline from 0.06 d<sup>-1</sup> (24 hpi) to 3.69 d<sup>-1</sup> (48 hpi), was comparable to the decline of two consecutive *O. tauri*-like (picoalga resembling *O. tauri*) algal blooms in West Neck Bay (Maine). The *O. tauri*-like algal cells contained virus-like particles, but were also grazed by heterotrophic nanoflagellates (O’Kelly et al., 2003). The blooms had maximum population densities of 2 × 10<sup>5</sup> and 5 × 10<sup>5</sup> cells mL<sup>-1</sup>, and collapsed within 4 days and 6 days respectively. Following the bloom declines, the population of *O. tauri*-like cells began to regrow. The longer decline period was probably due to a
lower maximum population density, lower density of viruses and competition between viruses and grazers. This time-frame of bloom termination by a combination of viral lysis and grazing has been recorded for other phytoplankton taxa, with similar lysis rates e.g. *Emiliania huxleyi* (Prymnesiophyte, Bratbak *et al.*, 1993; 1995; Castberg *et al.*, 2001; Vardi *et al.*, 2012; *Micromonas* spp (Prasinophyte, Evans *et al.*, 2003); and Phaeocystis globosa (Prymnesiophyte, Baudoux *et al.*, 2006). If the different rates of cell mortality caused by grazing and viral lysis changed, favouring viral lysis, our study suggests that allomer production would increase. For example, if the *O. tauri*-like blooms observed by O’Kelly *et al.* (2003) had declined with the maximum rate observed here (3.69 d⁻¹), the populations would have declined by 97% within 24 h of the population maxima, and resulted in the production of chl-a allomers (HO-chl-a + HO-chl-a’ + HO-chl-a-like- + MeO-chl-a-like) at a ratio of approx. 0.2 with chl-a. If a bulk measurement of chl-a were taken at this time, the total chl-a measurement coming from the *O. tauri* population, would consist of 20% allomers.

Given previous (Bale *et al.*, 2011; Franklin *et al.*, 2012) and present observations; elevated hydroxycchlorophyll a levels should be considered an indicator of phytoplankton death. The occurrence of the methoxhydroxycchlorophyll-a-like and hydroxycchlorophyll a-like allomers during this study (present after viral lysis of *O. tauri*), provides evidence that these allomers occur during termination by viral lysis. The ratio of allomers and chl-a precursors can be used to determine if the dominant population at a particular location is growing or declining—providing physiological context.

**Experimental procedures**

**Cultures and viral infection**

Unialgal triplicate cultures of *Ostreococcus tauri* (O'TH95 RCC745) were grown in 5 L conical flasks containing 1 L artificial seawater base media ESAW (Harrison and Berges, 2005) enriched with F/2 nutrients (Guillard and Ryther, 1962). Bacterial contamination was minimised by regular sub-culturing (every 3 days) prior to the study. Illumination from cool white fluorescent tubes was provided at 100 - 130 μmol photons m⁻² s⁻¹ on a 16: 8 h, light: dark cycle, at 20°C constant temperature. The populations were monitored daily, with samples taken at 9:00 h until virus addition, and twice daily thereafter (at 9:00 h and 17:00 h). Exponentially growing cultures (μ ≥ 0.69) were infected in triplicate with OtV5 lyase (Derelle *et al.*, 2008) at a host to virus ratio of 1. Sampling of the OtV5-infected flasks ceased after the population had crashed. The non-infected control flasks were sampled daily until their population density began to decline (day 29), and formed the growth-limited study. The maximum quantum efficiency of PSII photochemistry (Fv/Fm, 15 min dark acclimation) was determined as soon as possible after sampling using a FITE Fluorometer (Satlantic). Filtered, non-enriched ASW media (0.2 μm) was used as a blank and data was processed using FIRe-WORX software (Copyright 2007 Audrey B. Barnett).

**Algal staining and enumeration**

SYTOX-Green (Invitrogen S7020) was used to measure changes in membrane permeability (Veldhuis *et al.*, 1997); was applied at 0.5 μM L⁻¹ final concentration with incubation in the dark at 20°C for 15 min. CM-H₂DCFDA (5-and 6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, Invitrogen C6827), used to measure the relative amount of reactive oxygen species within a cell, was applied at 5 μM L⁻¹ final concentration (dark, 20°C for 30 min). Incubation conditions were optimised prior to the study using positive controls (Peperzak and Brussaard, 2011); for SYTOX, heat-killed cells (80°C, 5 min) and for CM-H₂DCFDA, hydrogen peroxide treated cells (10 μM L⁻¹ final concentration). Uptake of the stain was compared with unstained cells by flow cytometry, using an excitation laser of 488 nm, on a green (530/30 nm) versus red fluorescence (670 nm) plot. *O. tauri* population density was also quantified by flow cytometry (Accuri C6, BD Biosciences): Milli-Q was used as sheath fluid and analysis was triggered on forward scatter and red fluorescence. A core size of 22 μm was used and the event rate was kept below 1000 events s⁻¹ to avoid coincidence; when necessary samples were diluted with filtered media (0.2 μm). Flow rate was set to 66 μL min⁻¹ and measured daily by uptake of Milli-Q over 5 min (by mass).

**Viral enumeration**

Samples (1 mL) were fixed with glutaraldehyde (0.5% final concentration for 15 min at 4°C), flash frozen in liquid nitrogen and stored at −80°C until analysis. Defrosted samples were diluted in TE buffer (10 mM Tris HCL, 1 mM EDTA, pH = 8) and stained with SYBR Green-1 at a final concentration of 5 × 10⁻⁵ commercial stock (Invitrogen S7585) (Brussaard *et al.*, 2000) and incubated for 10 min at 80°C, and then 5 mins at room temperature. Samples were run on a FACSscan flow cytometer (Becton Dickinson) triggered on green fluorescence and set to ‘low’ flow (~20 μL min⁻¹) for 1 min, with event rates between 100 and 500 cells s⁻¹.

**Photosynthetic pigments**

The sample volume for photosynthetic pigments varied over the course of the study. For the OtV5 treatment flasks sample volumes were as follows: 50 mL on days 0, 1 and 2; 30 mL on day 3; 15 mL on days 3.3 and 4; 10 mL on day 4.3 and 7 mL thereafter. The same volumes were collected from the control flasks from day 0 to day 4, 15 mL was sampled from control flasks on day 4.3; and 50 mL from day 5 onwards until the termination of the study. The culture samples were centrifuged (at 5300 × g for 20 min at 8°C), and the pellet was flash frozen in liquid nitrogen and stored at −80°C until analysis. Exhaustive extraction of pigments used acetone (90% in Milli-Q) under dim light by sonication (at 40 W, Vibra Cell Probe; Sonics) for 40 s. The extract was clarified by centrifugation at 17,000 × g (Thermo Scientific). A 200 μL aliquot of extract was mixed with 80 μL water in the autosampler, and 25 μL of
this mix was injected onto the column. Reversed-phase high performance liquid chromatography (HPLC) was carried out using an Accela system (Thermo Scientific) with photodiode array detector, controlled using ChromQuest software. Chromatography was performed using a Waters Symmetry 3.5 μm C8 column (2.1 × 150 mm) with pre-column of the same phase. Elution used a mobile phase gradient composed of methanol, acetonitrile, aqueous pyridine (0.25 mol L⁻¹) and acetone (all HPLC grade) at a flow rate of 0.2 mL min⁻¹ (Method B in Zapata et al., 2000). Assignment of chlorophyll allomers was carried out by LC/MSⁿ using an Agilent 1200 HPLC with photodiode array detector, coupled via an atmospheric pressure chemical ionization (APCI) source to an Agilent 6330 ion trap mass spectrometer. HPLC conditions were as described above with instrument control and analysis performed using Chemstation software. The following MS settings were used (positive ion mode): drying temperature 350°C, APCI vaporizer temperature 450°C, nebulizer 60 psi, drying gas 5 L min⁻¹, capillary voltage −4500 V, scan range m/z 400–1100. Formic acid was added to the HPLC eluant at 300 μL h⁻¹ to aid ionisation (Airs and Keely, 2000). MSⁿ settings were as follows: SPS on, number of precursor ions 2, isolation width 3 m/z.

**Acknowledgements**

We would like to thank Nigel Grimsley for providing the *O. tauri* OTH95 RCC745/O1V5, host/virus system. This work was supported by the Natural Environment Research Council [grant number NE/IS28034/1].

**References**


