Title: The Role of Vitamin D in *Emiliania huxleyi*: A Microalgal Perspective on UV-B Exposure

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Abstract

An essential interaction between sunlight and eukaryotes involves the production of vitamin D through exposure to ultraviolet-B (UV-B) radiation. While extensively studied in vertebrates, the role of vitamin D in non-animal eukaryotes like microalgae remains unclear. *Emiliania huxleyi*, a microalga inhabiting shallow ocean depths exposed to UV-B radiation, is well-suited for this research. Our results show that *E. huxleyi* can produce vitamin D_2 and D_3, pointing to their potential role in the algal physiology. We further show that *E. huxleyi* algae respond to vitamin D at the transcriptional level, regulating the expression of protective mechanisms such as the light-harvesting complex stress-related protein (LHCSR) and heme oxygenase, and that vitamin D enhances the algal photosynthetic performance while reducing harmful reactive oxygen species buildup. Understanding the function of vitamin D in *E. huxleyi* has broader implications, shedding light on its role in non-animal eukaryotes and its potential importance in marine ecosystems. This research sets the stage for further investigations into the complex relationship between sunlight, vitamin D, and microalgal physiology, which contributes to our understanding of how eukaryotes adapt to diverse environmental conditions.

Introduction

Life on Earth has a complex relationship with sunlight, relying on its energy for certain processes while simultaneously requiring protection against its potential harmful effects. A molecular process that is tightly linked to sunlight is the formation of vitamin D following exposure to ultraviolet-B (UV-B) radiation emitted from the sun. Vitamin D (calciferol) comprises a group of steroids that result from the photochemical transformation of several sterol precursors by UV-B wavelengths[1]. The most common vitamin D species known to occur naturally are vitamin D_2 and D_3, originating from the conversion of ergosterol and 7-dehydrocholesterol, respectively[2,3].

In mammals and other studied vertebrates, vitamin D functions as a hormone, involved in the regulation of a multitude of intracellular and physiological processes vital for the organism survival and well-being[4]. Vitamin D is pivotal in facilitating the absorption and homeostasis of essential ions such as Ca^{2+} and PO_4^{3-} [5,6], and vitamin D deficiency has been linked to a range of physiological disorders[7,8]. Due to its key role in human health, vitamin D has been the focus of biological and pharmaceutical research endeavors. These efforts have been directed towards understanding the mechanisms through which it operates in humans and human models.

However, mounting evidence suggests that vitamin D has been a constituent of eukaryotes long before the emergence of vertebrates. This is evident not only in the identification of vitamin D in distant eukaryotic lineages like algae[9–11], plants[12], and fungi[13–15] but also in the preservation of vitamin D-related biomarkers, likely from an algal source, in marine sediments dating back over 600 million years[16].

Despite its widespread presence across diverse lineages, our understanding of the physiological role of vitamin D in non-animal eukaryotes remains limited. Non-animal eukaryotes, namely microalgae, have been suggested as potential sources of vitamin D for higher trophic levels in the marine environment[17–19]. But the processes underlying vitamin D production and regulation in microalgae remain largely unexplored[20].

While UV-B radiation is crucial for vertebrate health due to its role in vitamin D formation, it can also be detrimental, causing direct damage to biomolecules like DNA, leading to the generation of reactive oxygen species (ROS)[21–23], and can ultimately result in cell death[24]. Photosynthetic organisms, like algae, are particularly susceptible to UV-B damage, as their energy production

hinges on exposure to solar radiation[25,26]. Although water acts as a UV-B filter[27], significant intensities can still penetrate the upper layers of ocean surfaces[28], potentially impacting organisms such as algae[29].

Microalgae of the species *Emiliania huxleyi*, also named *Gephyrocapsa huxleyi*[30], are widely distributed in modern oceans and play key roles in various biogeochemical cycles[31,32]. These algae are known to flourish in high light environments at shallow depths of about 10 to 20 meters[33], where exposure to UV-B wavelengths is likely. Earlier findings provided intermittent indications that *E. huxleyi* algae might possess the inherent capability to synthesize vitamin D. These reports highlighted the algal capacity to generate vitamin D_2 upon exposure to UV-B irradiation[11], its cholesterol content[34,35], and the presence of a gene analogous to 7-dehydrocholesterol reductase (DHCR7) responsible for converting 7-dehydrocholesterol into cholesterol[36].

In this study we explore the overlooked role of vitamin D in *E. huxleyi*. Specifically, we investigate the relationship between vitamin D formation following exposure to UV-B and the regulation of cellular mechanisms that operate in response to harmful radiation.

Results

E. huxleyi algae produce vitamin D_2 and D_3

To investigate whether vitamin D species are formed by *E. huxleyi* algae upon exposure to UV, we cultivated algal cultures in a chamber with environmentally relevant UV-B radiation levels (see Materials and Methods). Metabolic analyses revealed the presence of both D_2 and D_3 in these algal cultures (Table 1). Our results show that D_2 was significantly enriched in UV-exposed cultures, with levels of approximately ~4 ng/mg dry weight, while it was barely detected in cultures that were not exposed to UV. The D_2 precursor ergosterol was found in both UV-treated and control cultures. Lower amounts of vitamin D_3 (~0.04 ng/mg dry weight) were detected in both UV-treated and control cultures.

Importantly, while D_2 detection was consistent across all analyzed UV-exposed samples, D_3 was identified only in part of the experiments during our research. Interestingly, when D_3 was detected, its precursor 7-dehydrocholesterol, was detected as well. Inconsistent detection of D_3

was previously reported in plants, and was attributed to the sensitivity of the analytical method used[37]. Our many efforts to resolve the variable measurements of D_3 were not successful (see detailed description of attempts in Materials and Methods). Collectively, our findings demonstrate that *E. huxleyi* algae produce D_2 and D_3, suggesting a possible cellular function for vitamin D.

E. huxleyi algae show a transcriptomic response to UV radiation

UV radiation is necessary for the formation of vitamin D[2]. Therefore, we sought to explore the transcriptomic response of *E. huxleyi* algae to UV exposure, aiming to elucidate cellular processes that may be related to vitamin D. To achieve this, we analyzed the *E. huxleyi* transcriptome using cultures that were grown under continuous UV exposure during the light period of the daily light/dark cycle, in comparison to algal cultures that were protected from the UV source. Cultures were sampled for RNA-sequencing at three time points representing different growth phases (days 7, 10, and 13, see Fig. S1).

The transcriptomic analysis revealed differential expression (DE) of 374 genes between UV-exposed and control cultures (Table S1). Of these genes, 172 were annotated with GO terms related to a known function or process. The annotated genes that were DE in the transcriptome under UV exposure were associated with various cellular processes including intracellular signaling pathways and stress response mechanisms. Notably, genes participating in the inositol

Compound	UV (ng / mg)	Control (ng / mg)
D_2	4.32 ± 1.39 *	0.09 ± 0.01
D_3	0.038 ± 0.001	0.039 ± 0.001
Ergosterol	83.01 ± 28.96	71.76 ± 9.53
7-dehydrocholesterol	0.24 ± 0.04 *	0.16 ± 0.02

Table 1. *E. huxleyi* algae produce vitamin D_2 and D_3. Metabolic analysis of vitamin D species and precursors under UV and control conditions, using algal cultures at day 10 of growth. Values are of ng / mg dry weight. Statistically significant differences ($P < 0.05$) between treatments are marked by *, calculated using two-sample t-test assuming equal variances. ± values indicate standard deviation based on 4 biological replicates.

3-phosphate/calcium (IP_3/Ca^{2+}) and the oxylipin signaling pathways were DE (Table 2), both playing key roles in stress response mechanisms across different organisms[38–42].

Furthermore, a substantial number of DE genes were involved in various stress responses, including DNA damage sensing and repair, oxidative stress mitigation, protective pigment biosynthesis, and maintenance of the photosynthetic machinery. Interestingly, several of the genes and pathways that were DE in *E. huxleyi* algae, are known to be associated with UV exposure and vitamin D biosynthesis in vertebrates. For instance, the IP_3/Ca^{2+} and prostaglandins pathways are involved in UV stress response in mammals[43,44], and vitamin D is involved in the regulation of these pathways[45–49]. In mammals, vitamin D also plays a role in oxidative stress mitigation, DNA repair, and the regulation of various enzymes related to stress response including heme oxygenase, glutathione peroxidase, and tyrosinase[50–54]. Considering the role of vitamin D in regulating stress response mechanisms in mammals, and the presence of similar mechanisms regulated by UV in *E. huxleyi*, it seems plausible that vitamin D plays a role in regulating the algal stress responses.

To further explore the observed transcriptomic response induced by UV using an independent approach, several genes that were regulated in the algal transcriptome were analyzed by qRT-PCR. RNA was extracted from cultures that were exposed to UV for a duration of 1 hour at day 10 of growth (Fig. 1, S2). Under these conditions, the investigated genes showed significant upregulation. The differences observed between the two transcriptomic assays, could be the result of a different response elicited by a prolonged compared to a brief UV exposure.

Vitamin D upregulates expression of UV-regulated genes

Next, we explored whether vitamin D is indeed involved in regulating stress response mechanisms in *E. huxleyi* under UV. If vitamin D is synthesized upon UV exposure and plays a role in regulating the algal stress response to UV, external addition of vitamin D could potentially induce algae to react as if they were exposed to UV. Therefore, we

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Gene ID	Putative protein or domain	Differential expression			Protein function
Genes related to intracellular signaling		d. 7	d. 10	d. 13	
G10384	Lipoxygenase domain	-0.46	-1.27	2.04	Oxylipin biosynthesis
G14992	Prostaglandin F(2-alpha) synthase	0.38	0.22	1.75	Oxylipin biosynthesis
G12340	Cytosolic phospholipase A2 domain	-0.51	-0.82	1.70	Oxylipin biosynthesis; Intracellular signaling
G14502	Ca-binding domain	-0.57	-1.12	1.67	Shares similarity to *A. thaliana* calmodulin-like protein; Intracellular signaling.
G21784	Phosphoinositide phospholipase C	-0.52	-0.62	1.50	Ip_3 signaling initiation; cytosolic calcium regulation
G15496	Phosphoinositide 5-phosphatase	0.54	0.74	1.46	IP_3 signaling; cytosolic calcium regulation
G25467	Steroid hydroxylase	1.15	1.49	1.16	Shares similarity to rat *cyp1a2* with a suggested 25-cholesterol hydroxylase activity
G1648	Mannosyl phosphorylinositol ceramide synthase	-0.37	-0.21	0.98	Potentially involved in Calcium signaling
G19702	Phosphatidylinositol 3-kinase	-0.17	0.00	0.95	IP_3 signaling
G27192	Calcineurin B-like interacting protein kinase	-0.34	1.25	-0.3	Involved in Ca-mediated signaling; Abiotic stress response
Genes related to stress response					
G27084	Deoxyribodipyrimidine photolyase	0.93	1.9	4.29	UV-damage DNA repair
G647	3-dehydroquinate synthase domain	-0.31	5.33	4.07	Shikimate pathway; potentially involved in the production of UV-protective compounds
G22973	Sirtuin 2	1.16	0.89	1.49	DNA transcription and repair
G16746	Tyrosinase Cu-binding domain	-0.54	-1.09	1.46	Involved in synthesis of protective pigments and antioxidants
G18590	Light-harvesting protein	0.51	1.22	1.13	Shares similarity to to *C. reinhardtii* LHCSR; Alleviates photo-oxidative stress
G12503	Poly ADP-ribose polymerase Zn-finger domain	0.62	1.13	1.08	DNA damage sensor
G18115	Deoxyribodipyrimidine photolyase	0.24	0.25	0.99	UV-damage DNA repair
G6690	Glutathione peroxidase	0.05	0.32	0.98	Oxidative stress mitigation
G25108	Chalcone synthase 2	-0.36	-0.43	0.82	Potentially involved in UV protection
G2876	DNA-(apurinic or apyrimidinic site) lyase	0.56	0.52	0.81	DNA repair
G26038	ATP-dependent DNA helicase	-0.34	-0.76	0.79	DNA stability and repair
G8907	(6-4)DNA photolyase	0.07	0.43	0.78	UV-damage DNA repair
G2511	Heme oxygenase	0.37	0.96	0.67	Oxidative stress mitigation
G16133	Formamidopyrimidine-DNA glycosylase	0.49	0.89	0.53	DNA repair
G832	Protochlorophyllide reductase	1.23	-0.41	0.2	Chlorophyll biosynthesis
G22197	5'-tyrosyl DNA phosphodiesterase	0.83	0.22	0.10	DNA repair

Table 2. Differential expression (DE) of genes associated with signaling and stress response mechanisms in *E. huxleyi* that were upregulated under UV. DE values calculated according to the transcriptomic analysis are given for days 7, 10 and 13 of growth (designated d. 7, d. 10 and d. 13). Genes are ordered according to DE values at day 13. Full DE and NCBI accession data is presented in Table S1 and Data S1.

supplemented algal cultures with vitamin D and monitored the expression of stress response-related genes via qRT-PCR.

Algal cultures were treated with D_2, D_3, or with a combination of both, as this combination had a synergistic impact on algal growth compared to each species alone (Fig. S2). Control cultures were not treated with vitamin D and were exposed to either normal growth conditions or to UV radiation for 1 hour. RNA was collected from all cultures 1 hour post treatment.

Our analyses revealed four genes that exhibit upregulated expression both upon vitamin D treatment and UV radiation (Fig. 1). The upregulation of these genes by UV was observed also in the transcriptomic analysis (Tables 2, S1). Notably, in vitamin D-treated cultures, the upregulation was only observed following the addition of both D_2 and D_3. The four upregulated genes encode for light-harvesting complex stress-related protein (LHCSR, G18590), heme oxygenase (G2511), steroid hydroxylase (G25467), and a Ca-binding protein (G26534). Both LHCSR and heme oxygenase are proteins related to the algal stress response.

The LHCSR gene is known to exhibit increased expression in moss and green algae under UV-B and high-light stress, promoting excess energy dissipation in the light-harvesting complex and thereby reducing photo-oxidative stress[55–57]. Heme oxygenases are enzymes involved in the formation of antioxidants in plants and animals, with known upregulated expression in response to UV-B and other ROS-forming stressors[58–60]. UV-B radiation is a fundamental component of high-light environments, and vitamin D is produced under UV-B radiation (Table 1) and triggers the upregulation of oxidative- and photooxidative-stress mitigation pathways. Therefore, it is likely that vitamin D is part of a cellular signaling cascade that reports on and reacts to harmful light intensities or radiation.

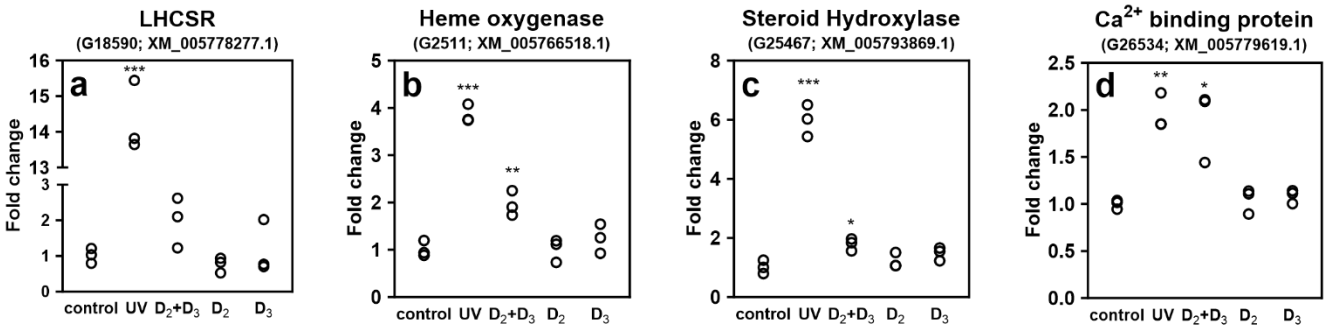

Figure 1. Combined treatment of vitamin D_2 and D_3 upregulates UV-responsive genes. qRT-PCR analysis of genes following 1 hour of UV exposure or vitamin D treatments. Top title denotes gene products. In brackets: gene identifier in *E. huxleyi* CCMP3266[94] and matching gene transcript in the *E. huxleyi* CCMP1516 reference genome[95]. Statistical significance compared to control was calculated using two-tailed t-test assuming equal variances. One, two or three asterisks indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

153 Vitamin D treatment improves the algal photosynthetic performance following excess light

154 To investigate the involvement of vitamin D in the cellular response towards harmful light
155 intensities, we tested the impact of vitamin D treatment on algal physiology, namely
156 photosynthetic performance, under excess light. To this end, algal cultures were exposed to low,
157 non-saturating light levels that are regularly used during incubation[61] (130 µmol photons m^{-2} s^{-1},
158 or PAR), and to saturating light[62] (1000 PAR) for 2 hours. Algal cultures under each light regime
159 were either treated with both D_2 and D_3, or not treated as control. Algal photosynthetic
160 performance was evaluated by measuring nonphotochemical chlorophyll fluorescence
161 quenching (NPQ) as a proxy for the ability of algae to dissipate excess absorbed light energy
162 into heat[63]. In addition, the maximum PSII quantum yield (F_v/F_m) was measured as an indicator
163 of photosynthetic efficiency[64]. Our results showed that algae under saturating light display
164 decreased NPQ and F_v/F_m, indicative of photoinhibition[61] (Fig. 2a, b). Furthermore, vitamin D-
165 treated cultures exhibited significantly higher NPQ following exposure to excess light (Fig. 2a,
166 S4), in comparison to untreated cultures. The cultures that were treated with vitamin D also
167 demonstrated enhanced F_v/F_m (Fig. 2b). These findings emphasize the potentially central role
168 of vitamin D in algal physiology under harmful light levels. Vitamin D appears to activate cellular
169

processes that enhance excess light energy dissipation and improve photosynthetic quantum yields.

Vitamin D alleviates ROS accumulation under excess light

Under excess light, photosynthetic organisms are likely to experience oxidative stress[65], and NPQ is a key process in mitigating oxidative stress induced by excess light. Our analyses revealed elevated NPQ in vitamin D-treated cultures following excess light. Therefore, we assessed whether addition of vitamin D to algal cultures under excess light, indeed alleviates oxidative stress resulting in decreased cellular levels of ROS. To achieve this, we subjected algal cultures to excess light (1000 PAR) and assessed the intracellular ROS levels using the cell-permeable fluorescent probe 2,7-Dichlorodihydrofluorescein diacetate (H_2DCF-DA). The tested algal cultures were either treated with both D_2 and D_3 or were untreated. Additionally, control cultures were exposed to regular light intensities (130 PAR) and subjected to the same treatments. Our findings revealed a substantial decrease in intracellular ROS levels in algae that were treated with vitamin D and exposed to excess light, compared with untreated algae under the same light regime (Fig. 2c). These findings further support a cellular role of vitamin D in

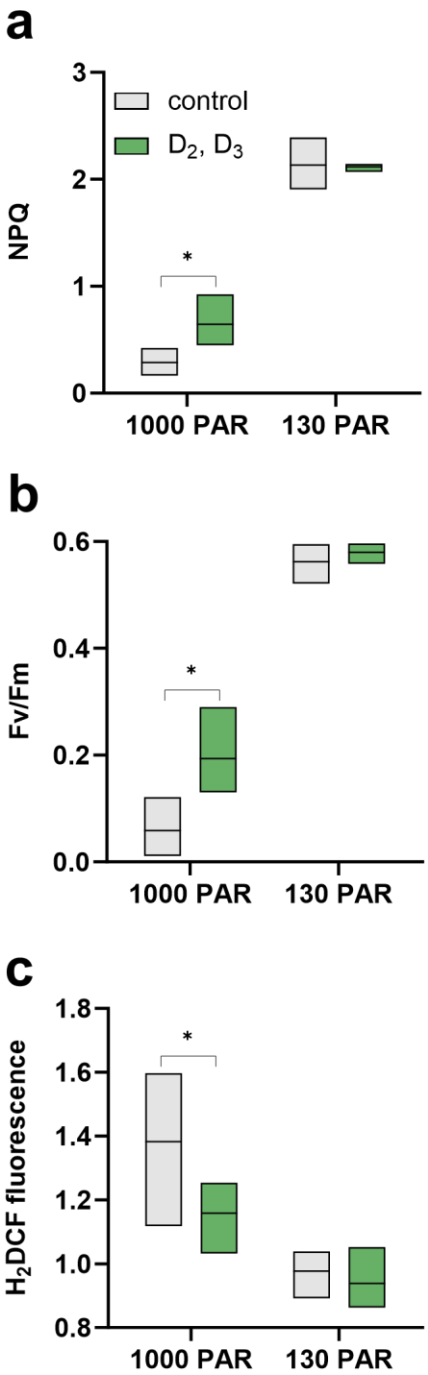

Figure 2. Vitamin D treatment improves photosynthetic performance and alleviates oxidative stress following exposure to excess light. (a) Non photochemical quenching (NPQ) and (b) F_v/F_m values of vitamin D-treated and control algae, exposed to regular (130 PAR) or excess (1000 PAR) light intensities. (c) Fluorescence values of vitamin D-treated and control algae stained with the intracellular reactive oxygen species (ROS) probe H_2DCF-DA, exposed to regular (130 PAR) or excess light (1000 PAR) intensities. Statistically significant values ($P < 0.05$) compared to control are marked by *, calculated from three biological replicates using two-tailed paired t-test for (a,b) and one-tailed paired t-test for (c).

algal cells under excess light conditions, demonstrating that vitamin D is involved in mitigating ROS-induced oxidative stress.

Discussion

The current study reveals the photoprotective role of vitamin D in a globally abundant marine algal species. Our findings introduce a novel cellular mechanism that utilizes the photochemical transformation of vitamin D as an indicator of exposure to harmful radiation, consequently enhancing the algal physiological response to excess-light stress. This enhanced response is manifested by an increase in photosynthetic efficiency following exposure to conditions that inhibit photosynthesis, along with an overall reduction in the development of ROS (Fig. 2). Furthermore, the elevation in NPQ observed after vitamin D treatment, coupled with the upregulation of LHCSR, suggests that a significant aspect of the response triggered by vitamin D is aimed at mitigating photo-oxidative stress. Additionally, the fact that the physiological response in *E. huxleyi* algae to vitamin D is only observed following saturating light conditions may hint at a light-dependent regulatory mechanism.

Vitamin D can serve as a sensitive light indicator in phytoplankton. For example, phytoplankton residing in surface mixed layers of oceans often encounter significant fluctuations in PAR intensity throughout the day. These environments, which can extend depths of over 200 meters[66], may result in cells being transported over considerable vertical distances[67], leading to rapid change in light intensity of hundreds of PAR within a matter of hours[33,68]. In such a dynamic scenario, as cells ascend, they experience an increase in UV-B and subsequently may generate vitamin D. The specificity of the photochemical conversion of vitamin D under UV-B wavelengths, coupled with its relatively high photochemical quantum yield[69], suggests that it could function as an independent and sensitive proxy for assessing exposure to UV-B radiation, high light levels, or fluctuations in light intensity.

Interestingly, the impact of vitamin D on gene expression became evident only under a combined treatment with D_2 and D_3 (Fig. 1). Similarly, the combined treatment led to a lower cell density compared to separate D_2 or D_3 treatments (Fig S2). Decreased cell densities might be a result of cell cycle arrest, which is a phenomenon previously described in *E. huxleyi*[70] and is known to

occur in response to UV radiation across various organisms[71–73]. Whether vitamin D influences the algal cell cycle, and why a combination of D_2 and D_3 drives a detectable response, merits further investigation.

When studying algal physiology, it is essential to acknowledge the significance of UV-B as an influential environmental factor. In experimental setups aimed at studying algal physiology and ecology, UV-B radiation has traditionally been excluded due to its detrimental effects. However, this contradicts the natural conditions in which algae thrive, where they regularly encounter low levels of UV-B. While the omission of UV-B simplifies experimental conditions for precise variable isolation, our study unveils the profound influence of vitamin D, a product of UV-B exposure, on the physiological response of *E. huxleyi* to environmental stress. These findings shed light on the potentially advantageous role of UV-B for algae facing excess-light stress, encouraging further exploration of the interplay between algae and this often-overlooked environmental factor.

Our study offers comparative insights on the role of vitamin D in vertebrates and in *E. huxleyi* algae. The extensive knowledge on vitamin D biology primarily originates from research on humans and other vertebrates. Transposing this knowledge to *E. huxleyi* presents challenges due to significant phylogenetic and physiological differences between multicellular organisms and unicellular entities. Nevertheless, parallels can be drawn. Vitamin D was shown to enhance cellular defense in human and mice keratinocytes against UV-induced oxidative stress and DNA damage[53,54]. Vitamin D has also been shown to mitigate oxidative stress in rat liver and intestine[50,74], partly through the upregulation of heme oxygenase, a response mirrored in vitamin D-treated algal cells. Furthermore, the upregulation by vitamin D of certain genes in *E. huxleyi,* potentially involved in intracellular signaling cascades (Fig. 2c, d), suggests the involvement of vitamin D in initiating or contributing to signaling mechanisms. In mammals, vitamin D plays a role in the activation of key signaling proteins, such as phospholipase C (PLC), phospholipase A_2 (PLA_2) and phosphatidylinositol-3 kinase (PI3K)[75], which are essential for the rapid generation of secondary messengers such as Ca^{2+} and IP_3. While *E. huxleyi* cells demonstrated the upregulation of PLC, PLA_2, and PI3K following prolonged UV exposure (Table 1), further investigation is needed to determine if vitamin D also participates in regulating these proteins in algae. Interestingly, the involvement of Ca^{2+} signaling in photo-oxidative stress mitigation has

been reported in other marine algal species[76]. Another possible similarity between vertebrates and *E. huxleyi* is the activation of vitamin D. In vertebrates, vitamin D requires enzymatic modifications to become hormonally active. These modifications include the hydroxylation of the 1st and 25th carbons, to produce 1,25-$(OH)_2$-vitamin D[4]. Similar hydroxylated D_2 and D_3 species were identified in the current research (Fig. S5) based on multiple reaction monitoring (MRM) profiles published previously[77]. Additional work is necessary to validate the detection of these hydroxylated species and the corresponding biosynthetic pathways in algae.

Vitamin D synthesis likely has ancient origins, given its presence across various lineages of eukaryotes[9–15]. Sterols are a defining feature of eukaryotes, and the enzymatic pathways leading to the production of ergosterol and 7-dehydrocholesterol, which are precursor molecules to D_2 and D_3 forms of vitamin D, may have existed in the last eukaryotic common ancestor (LECA)[78,79]. Consequently, it can be hypothesized that the earliest eukaryotes were already synthesizing vitamin D when exposed to solar radiation. While previous studies had postulated the ancient evolutionary origins of vitamin D[20,80], the understanding of its role beyond the animal kingdom had remained limited. Eukaryotes rely on oxygen (O_2), and their evolution is thought to have necessitated proximity to oxygenic photoautotrophs inhabiting the sunlit ocean surface[81]. This is because during their proposed emergence in the late Archean to early Proterozoic eras[82,83], the deep ocean was likely devoid of O_2 [84–86]. Considering the antioxidant properties exhibited by vitamin D in both animals and *E. huxleyi* algae, coupled with its evolutionary links to oxidative agents such as UV-B and O_2, it prompts the question of whether vitamin D evolved in early eukaryotes as a means of sensing oxidative environments. A similar evolutionary role has been proposed to sterols, and chiefly to cholesterol that has been widely studied in the context of environmental O_2[87,88]. Like sterols, vitamin D possibly played a role in the early stages of life on Earth.

Materials and methods

Algal strain and growth conditions

The axenic algal strain of *E. huxleyi* CCMP3266 was purchased from the National Center for Marine Algae and Microbiota (Bigelow Laboratory for Ocean Sciences, Maine, USA). Algae were grown in artificial sea water according to Goyet and Poisson[89] and supplemented with L1

medium according to Guillard and Hargraves[90], with the exception that Na_2SiO_3 was omitted following the cultivation recommendations for this strain. Algae were grown in standing cultures in borosilicate Erlenmeyer flasks with an initial inoculum of 330 cells/ml, placed in a growth chamber at 18°C under a light/dark cycle of 16/8 hr. PAR intensity during the light period was 130 µmoles photons m^{-2} s^{-1}. Cultures volume was 50 ml except for cultures used to measure growth under vitamin D treatment (Fig. S3) which were grown in 20 ml.

Continuous UV irradiation was achieved by placing a UV-emitting light source (Exo Terra Reptile UVB150, Hagen, Montreal, Canada) inside the algal growth chamber. Algal cultures experienced UV-A intensity of 0.5 w/m^2, UV-B intensity of 0.07 w/m^2 and UV-C intensity of 0.026 w/m^2, measured using an ALMENO 2570 device (Ahlborn, Budapest, Hungary) placed within the Erlenmeyer flask. The UV-B intensity used in this study was selected to replicate the average UV-B radiation encountered at the ocean surface[29]. The UV light-source was operating daily for 14 hours in parallel to the PAR light period, starting one hour after PAR illumination started, and ending one hour before PAR illumination ended. This irradiation regime aimed to mimic a simplified day cycle including dawn and dusk periods.

Algal growth was monitored by a CellStream CS-100496 flow cytometer (Merck, Darmstadt, Germany) using a 561 nm laser and plotting the chlorophyll fluorescence at 702/87 nm against side scatter.

Vitamin D treatment

Cultures were treated with 1 µM of vitamin D_2 or D_3 (Sigma-Aldrich, Burlington, Massachusetts, USA) dissolved in DMSO, as this was the minimal vitamin D concentration in which an effect was observed on algal growth (Fig. S3b). For combined D_2 and D_3 treatment, 0.5 µM of each species was added. The final DMSO concentration in cultures was 0.1%. Control cultures were treated with an equal amount of DMSO.

Vitamin D analysis

Metabolic analysis was conducted following Oberson[77]. Standards for D_2, D_3, ergosterol and 7-dehydrocholesterol were purchased in dry (Sigma-Aldrich) and dissolved in $CHCl_3$. Standard solutions of the different metabolites were combined into a single solution and diluted to create a standard curve. All final standards and samples were spiked with 50 ng of vitamin D2-d_3

(IsoSciences, Ambler, Pennsylvania, USA) serving as an internal standard. Algal samples were centrifuged, lyophilized and stored in -80°C until analysis. Saponification was achieved by resuspending samples in 108 µl 55% KOH, 192 µl ethanol, and 60 µl of 9% NaCl and 7.4% ascorbic acid, followed by homogenization and stirring at room temperature for 18 hours. Samples were then supplemented with 40 µl 10% NaCl and 300 µl of 20% ethyl acetate in heptane, vortexed extensively and centrifuged for 30 minutes. The upper phase was collected, and the process was repeated twice. Samples were evaporated, dissolved in 200 µl of 0.5% isopropanol in hexane and sonicated. Strata SI-silica 55 µm 70 A columns (Phenomenex, Torrance, California, USA) were used for solid phase extraction and were pre-conditioned with 1 ml of 50% $CHCl_3$ in isopropanol, followed by two washes with 1 ml of hexane. Samples were then loaded onto the columns and washed with 0.5 ml of 0.5% isopropanol in hexane which were discarded, and washed again with 2.5 ml of 2.5% isopropanol in hexane which were collected. Samples were evaporated and dissolved in 200 µl of PTAD in acetonitrile, sonicated, stirred at room temperature for 2 hours, centrifuged for 10 minutes and transferred into LC-MS vials. Samples were protected from light as much as possible during the extraction process.

Due to the inconsistency in identification of D_3 in algal samples, several technical adaptations regarding algal growth and sample collection were implemented and evaluated. To examine whether inconsistencies arise due to rapid D_3 enzymatic degradation, algal cultures were immediately placed on ice, centrifuged in a cooled, 4˚C centrifuge, and the supernatant quickly discarded and replaced with 50% methanol in DDW. The samples were then plunged into liquid nitrogen and stored in -80˚C. Later, samples were thawed, evaporated in vacuum to remove the methanol, lyophilized and proceeded to vitamin D extraction. Additional modifications included increasing the intensity of UV-B radiation during algal growth, increasing sample size by combining separate cultures, and using F/2 trace metals instead of L1 trace metals. These attempts did not improve the reproducibility of D_3 detection.

Vitamin D was measured by UPC2-ESI-MS/MS equipped with Acquity UPC2 system (Waters, Milford, Massachusetts, USA). The MS detector (Waters TQ-XS) was equipped with an ESI source. The measurements were performed in the positive ionization mode using MRM. The source and de-solvation temperatures were maintained at 150°C and 500°C, respectively. The capillary voltage was set to 1.5 kV. Nitrogen was used as the de-solvation gas and cone gas at

a flow rate of 700 L h^{-1} and 150 L h^{-1}, respectively. Ionization parameters of ergosterol, 7-dehydrocholesterol, D_2 and D_3 were adjusted by direct infusion of standards. Ionization parameters for other compounds were taken from Oberson[77].

UPC2 system: mobile phase A consisted of CO_2, and mobile phase B consisted of 98% MeOH, 2% DDW and 10mM ammonium formate. Make up solvent was 1% formic acid in 90% MeOH and 10% DDW at a flow rate of 0.4 ml min^{-1}. The column (WATERS Acquity CSH FluoroPhenyl 1.7 µm, 3.0x100 mm, cat. 186006573) was maintained at 45˚C, injection volume was 3 µl. At the first 0.5 min of injection, 99.5% of mobile phase B, and 0.5% of mobile phase A were run at flow rate of 2.0 ml min^{-1}. Then, mobile phase A was gradually reduced to 92% at 6 min, and further decreased to 70% at a flow rate of 1.75 ml min^{-1} at 6.5 min. This composition of mobile phase and flow rate were kept until 7 min, followed by increase in mobile phase A to 99.5% at 7.8 min, and then increase in flow rate to 2 ml min^{-1} at 8.5 min, and running at those conditions until 9 min.

RNA extraction

Algal cultures were harvested for RNA extraction by centrifugation at 4000 rpm for 5 min at 18°C. RNA was extracted using the Isolate II RNA mini kit (Meridian Bioscience, London, UK) according to manufacturer instructions. Cells were ruptured in RLT buffer containing 1% β-mercapto-ethanol by bead beating for 5 min at 30 mHz. RNA was then treated with 3 µl Turbo DNAse (ThermoFisher, Waltham, MA, USA) in a 50 µl reaction volume, followed by a cleaning step using RNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, CA, USA) according to manufacturer instructions. RNA was used for generating transcriptomic data and qRT-PCR analysis.

Transcriptomic analysis

Transcriptomic data was generated using the MARS-seq library preparation protocol[91], and analyzed with the UTAP pipeline[92]. As part of the pipeline, read counts for each gene were normalized using the DESeq2's median of ratios method[93]. Differential expression (DE) between treatments was calculated using the following thresholds: mean number of normalized reads across all samples ≥ 5, adjusted p-value ≤ 0.05, Log2 fold change ≤ -0.7 or ≥ 0.7. The previously generated *E. huxleyi* CCMP3226 synthetic genome (sGenome) and annotation file was used as

372 reference for the UTAP pipeline[94]. Briefly, the *E. huxleyi* CCMP3226 sGenome was generated
373 by *de novo* transcriptome assembly of short-reads and long-reads. The assembled *E. huxleyi*
374 CCMP3226 transcripts were then mapped to the *E. huxleyi* CCMP1516 reference genome[95] to
375 define gene loci. For the current work, functional gene annotations were manually curated by
376 identifying open reading frames in assembled transcripts using the ORF finder tool
377 (www.ncbi.nlm.nih.gov/orffinder; transcript accessions are given in Table S1) and analyzing
378 protein domains in the translated sequences using InterProScan 5[96]. Additionally, transcript
379 sequences were searched against the swissprot database using NCBI blastx[97], and the hit with
380 the highest E-value taken. Specifically, the gene loci analyzed using blastx were G18590,
381 sharing highest similarity to *Chlamydomonas reinhardtii* LHCSR (NCBI accession P93664.1)
382 with E-value of 1e-26 and nucleotide identity of 57%; G25467, sharing highest similarity to rat
383 *cyp1a2* (NCBI accession P04799.2) with E-value of 1e-31 and nucleotide identity of 27%;
384 G14502, sharing highest similarity to *Arabidopsis thaliana* Calmodulin-like protein 12 (NCBI
385 accession P25071.3) with E-value of 6-e5 and nucleotide identity of 22.6%. The putative Ca-
386 binding activity of G26534 was assessed by identifying *bona fide* Ca-binding domains using
387 InterProScan 5. Specifically, we performed blastx[97] and focused on the highest hit that contained
388 an identifiable protein domain using InterProScan 5[96], resulting in the identification of an EF-
389 hand family protein in *Chrysochromulina tobinii* that harbors three EF-hand domain pairs (NCBI
390 accession KOO34173.1, with E-value of 8e-13 and nucleotide identity of 32%).

391 Quantitative real time PCR (qRT-PCR)

392 Algal cultures were treated with vitamin D as described earlier. UV-treated cultures were
393 exposed to the same UV intensities as described above and treated with equal amounts of
394 DMSO. All treatments lasted 1 hour. Equal concentrations of RNA taken from 10 days old
395 cultures were utilized for cDNA synthesis using Superscript IV (ThermoFisher), according to
396 manufacturer instructions. qPCR was conducted in 384 well plates using SensiFAST SYBR Lo-
397 ROX Kit (Meridian Bioscience, Cincinnati, OH, USA) in a QuantStudio 5 qPCR cycler (Applied
398 Biosystems, Foster City, CA, USA). The qPCR program ran according to enzyme requirements
399 for 40 cycles. Samples were normalized using three housekeeping genes: *alpha-tubulin*, *beta-*
400 *tubulin* and *ribosomal protein l13 (rpl13)*. DNA contamination was assessed by applying the
401 same program on RNA samples that were not reverse transcribed (omitting the Superscript IV

enzyme in the reverse transcription reaction mix). Gene expression ratios were analyzed according to Vandesompele[98] by geometric averaging of housekeeping genes. Relative gene expression levels were compared to control samples. Primer efficiencies were determined using the QuantStudio 5 software, by qPCR amplification of serially diluted cDNA. All primers had a measured efficiency between 80-120%. Primer sequences are given in Table S2.

Chlorophyll fluorescence

Algal cultures were divided into four subcultures that were subjected to one of four treatments: 1000 PAR with or without vitamin D (as described earlier) and 130 PAR with or without vitamin D. Chlorophyll A fluorescence parameters were estimated following 2 hours of treatment. Parameters were estimated by pulse amplitude-modulated fluorometry using WATER-PAM II (Heinz Walz GmbH, Effeltrich, Germany). Maximum photosystem II quantum yield (F_v/F_m) was calculated as $F_v/F_m = (F_m - F_0) / F_m$[64], where F_o is the baseline fluorescence under a measuring light of 160 PAR and F_m is the maximum fluorescence measured with a 0.9 s saturating light pulse of 6000 µmol photons m^{-2} s^{-1}. Non-photochemical quenching (NPQ) was calculated as $NPQ = (F_m - F'_m) / F'_m$[63], where F_m was the maximum fluorescence yield after dark adaptation and F'_m the maximum fluorescence yield under actinic light of 1150 PAR. Prior to analysis, algal samples were dark-adapted for 5 minutes. No major difference in F_v/F_m was observed between algae that were dark-adapted for 5 or 30 minutes following exposure to regular light (Fig. S6), suggesting that a darkness period of 5 minutes sufficiently relaxed PSII reaction centers.

Intracellular reactive oxygen species (ROS) measurements

Algal cultures were divided into subcultures and treated as described under 'chlorophyll fluorescence'. Vitamin D-treated and control cultures were placed in a growth chamber and exposed to either regular light conditions (130 PAR) or to high-light conditions (1000 PAR) for 5 hours. For intracellular ROS assessment, cultures were stained with 0.5 µM of H_2DCFDA (ThermoFisher) in DMSO. Staining was performed 2 hours after the start of the treatment, and cultures were left in the dark for 20 minutes before they were introduced back into the growth chamber for the remaining 3 hours. Samples were measured using CellStream CS-100496, excited at 488 nm and the signal was collected at 528/46 nm. The algal population was gated by plotting chlorophyll fluorescence (excitation-emission 561-702/87 nm) against side scatter.

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Authors Contributions

O.E. and E.S. designed the study. O.E., S.M. and I.P. performed and analyzed experiments. O.E., E. F. and M.S. performed computational analyses. O.E. and E.S. wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

Competing interests

The authors declare no competing interests.

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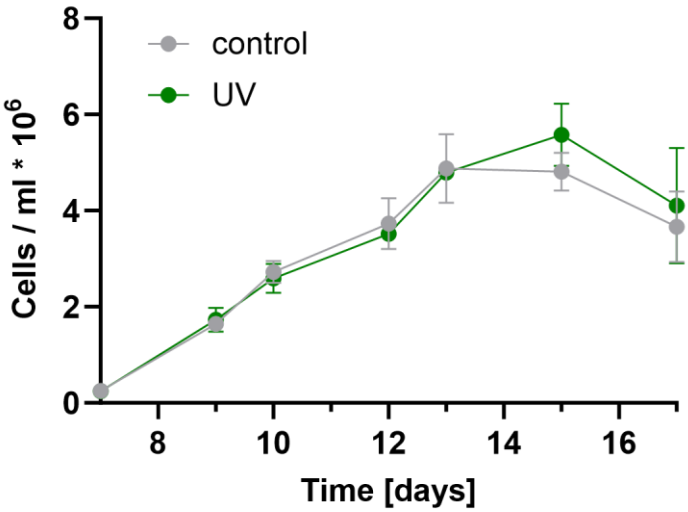

Figure S1. Continuous UV irradiation during the light period did not affect algal growth. Growth curves of the algal cultures that were grown under control and UV regime and were used for generating the transcriptomic data. Cell density was measured from day 7. Error bars indicate standard deviation based on 3 biological replicates.

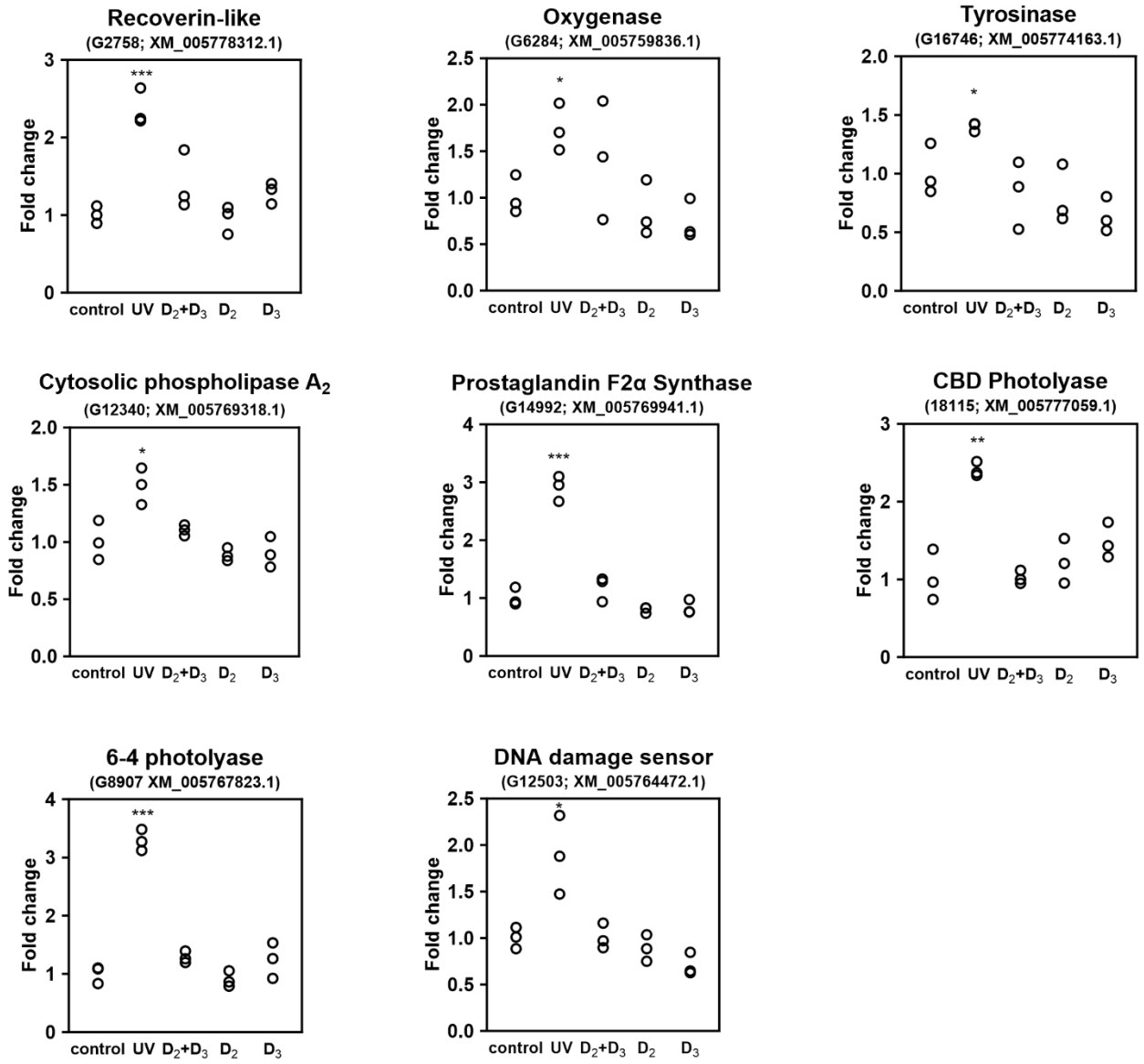

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Figure S2. UV radiation causes upregulation of various signaling and stress-response mechanisms. qRT-PCR analysis of genes under 1 hour of UV exposure or vitamin D treatments. Top title denotes gene products. In brackets: gene identifier in *E. huxleyi*; matching gene in *E. huxleyi* CCMP1516 reference genome[95]. Statistical significance compared to control was calculated using two-tailed t-test assuming equal variances. One, two or three asterisks indicate $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

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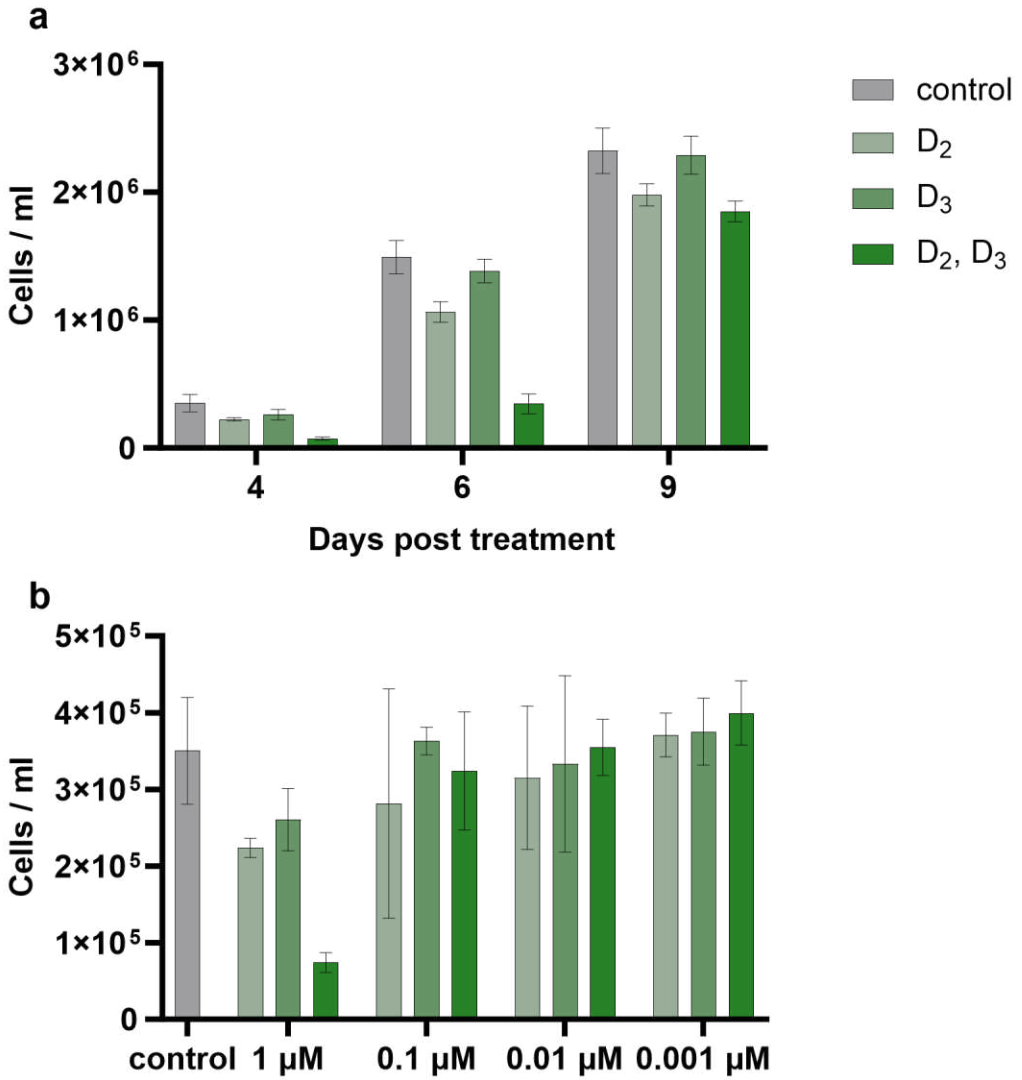

Figure S3. The combined treatment of vitamin D_2 and D_3 has a synergistic effect on algal growth, outperforming individual D_2 or D_3 application. (a) Growth dynamics of algal cultures treated with 1µM of vitamin D species. (b) Variation in algal cell densities after 4 days of treatment with different concentrations of vitamin D_2, D_3, and both (indicated by the green shades in the figure legend). Error bars indicate standard deviation from 3 biological replicates.

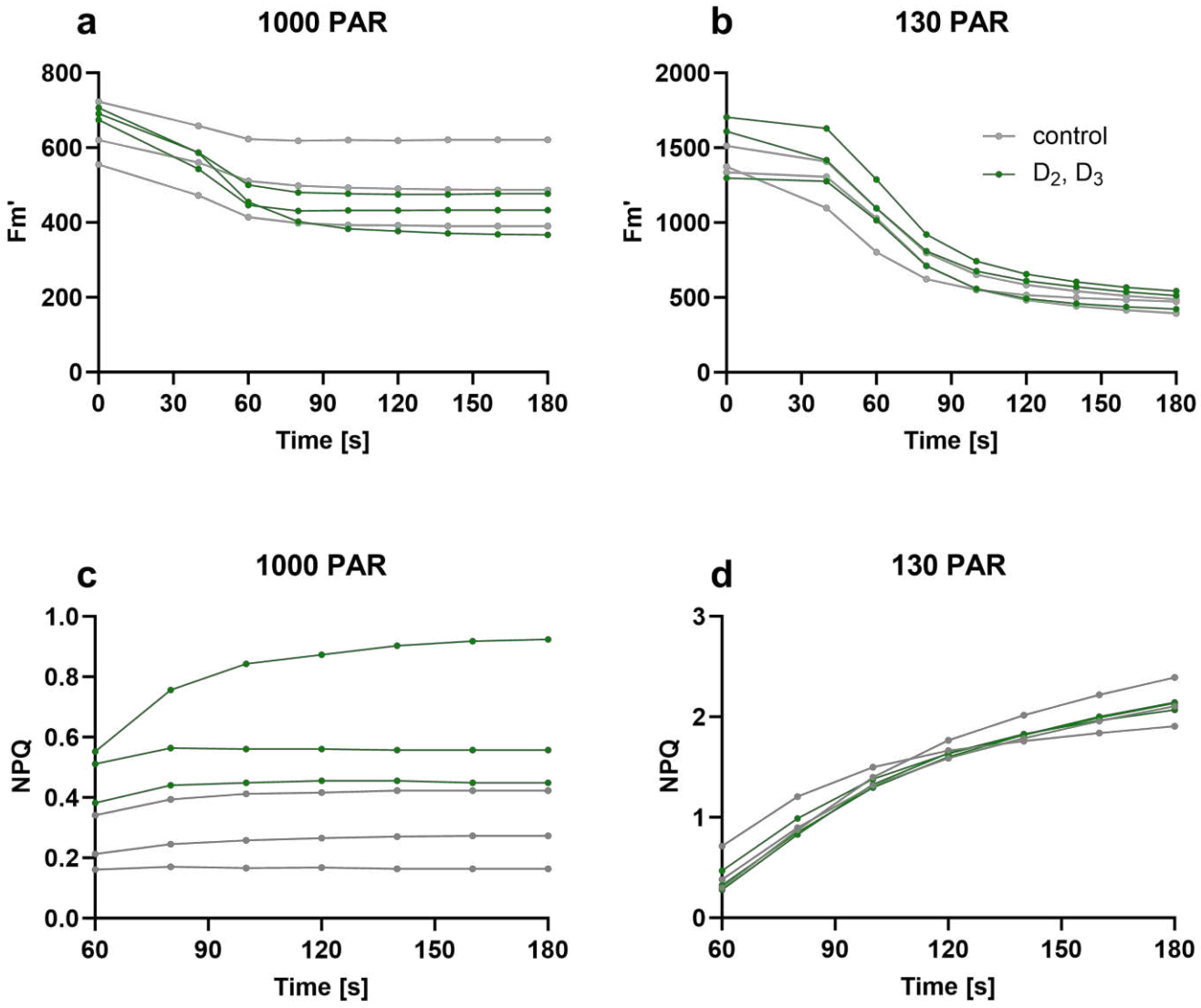

Figure S4. Temporal dynamics in NPQ and Fm' values of vitamin D-treated algae, exposed to regular or excess light. Temporal dynamics in Fm' and NPQ of both control and vitamin D-treated algae exposed to regular light levels (130 PAR) or excess light levels (1000 PAR) for 2 hours prior to analysis. The timing of saturating pulses is represented by dots. NPQ values were measurable from t=60s onwards. Each line represents a single biological replicate.

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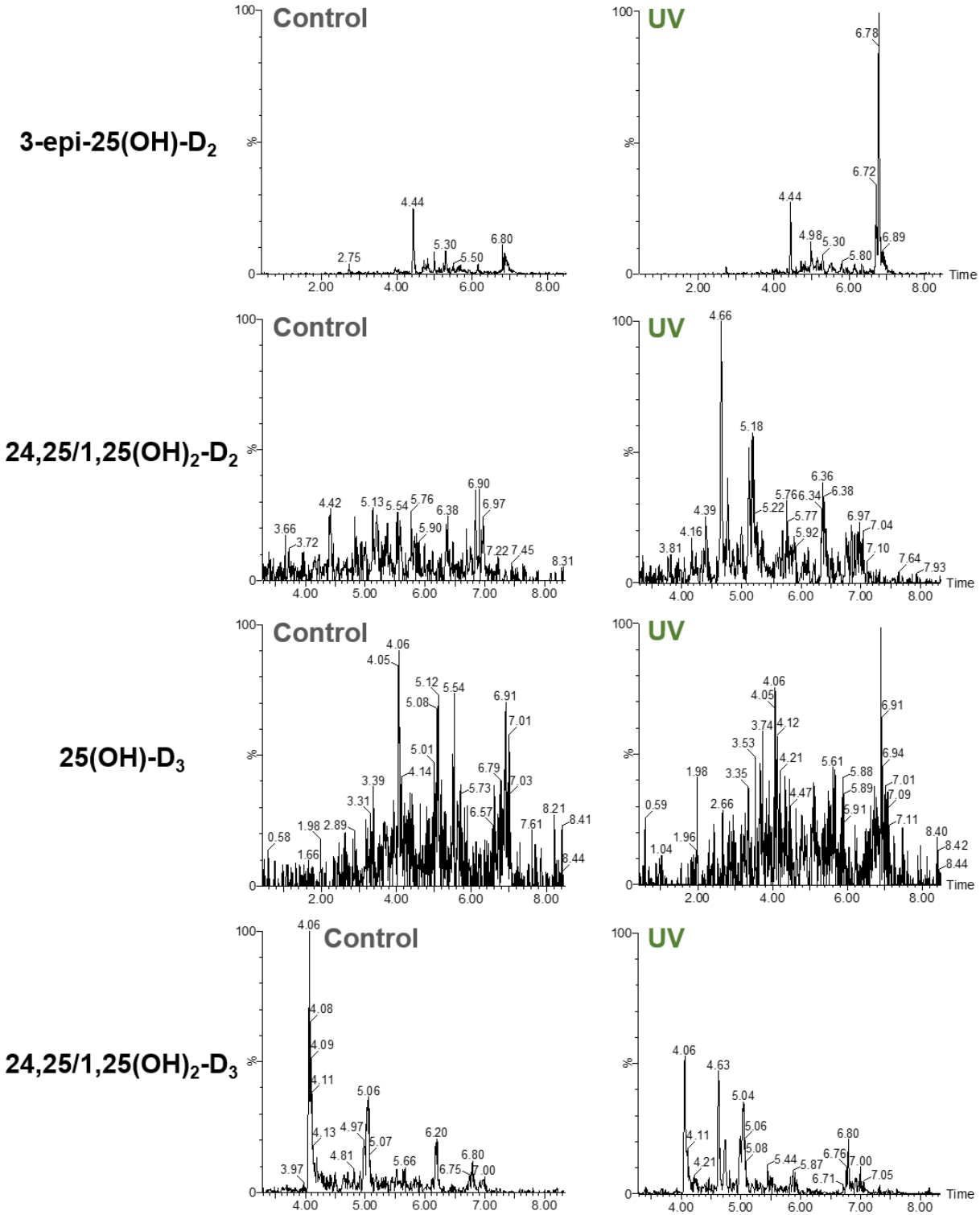

Figure S5. *E. huxleyi* algae produce putative hydroxylated vitamin D species. Representative chromatograms of hydroxylated vitamin D species detected in UV-treated and control cultures. The range of the Y axis in chromatograms of the same vitamin D species are identical and therefore comparable. The identification of metabolites is putative according to MRMs taken from Oberson et al.[77]. Further analysis against standards is needed in order to verify the identification of the hydroxylated species.

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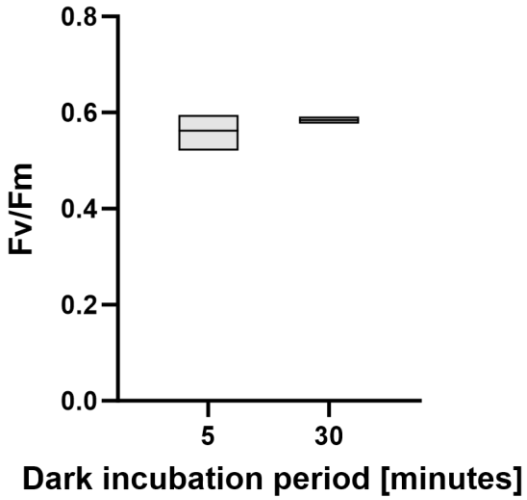

Figure S6. Dark adaptation of 5 minutes sufficiently relaxes PSII reaction centers. Comparison of maximum PSII quantum yields (F_v/F_m) between algae that were dark-adapted for 5 or 30 minutes, calculated based on three biological replicates.

Gene	Forward	Reverse
G6284	GCCCTACCGGGTGTTTATCC	CTCGACTTGGTTGAGAACTTGC
G18115	CACCGAGCCGACCCAAAAT	TTCATCTCGGTCGTTGACAGG
G18590	CTCCTCGCGATGCAGAACAA	CCCTTTCTGCCAACGTGATCT
G25467	TACGAGAATCGGCTGCTACG	CCCGTCGGACCTTAAGACAG
G12503	TCTCGGTGGAAATGGCGAC	ATTGTCATCGAGGCCGCAAA
G93	GTCACGCCGGCGACAAA	GCGATGTGCGGGTGTATCT
G12340	AACCTGCTTGCCGACATGAT	GGTTGAATCAGCATTGACCCC
G14992	GCGGGCTCTACTGAATCCG	GGGTCCTCGTAGAAGGTGTG
G16746	TCGAAGATCCGGACGACGAT	AGCGCGAGACGAAAATAACG
G2511	TTGTCGCGTCGCTCTACTTT	GCCGAAGTAGTACGCCATGT
G2758	ATGGACCTAGACTCGGACGG	ACAGCCCTCAAGCTCACATC
G8907	CTCTCGTGCTCGTGCTTCTT	TCGTAGATGTACTTTGCCGGG
G26534	CTGGAAGATCGAGGCAACGG	TATGGCGTCGCCGTCAAAG
G26797 *alpha-tubulin*	CGAGAAGGCGTACCACGAG	CTTCGTCTTGATGGTGGCGA
G28192 *beta-tubulin*	CAACATGAAGTGCGCCATCT	CCTCGGTGAACTCCATCTCG
G1895 *rpl13*	ACCAGCACTTCCACAAGACG	TGCCGCAGCTTGTAGTTGTA

Table S2. Primers used in this study. Gene identifiers in *E. huxleyi* CCMP3266[94]. Primers were used to generate qRT-PCR data using *E. huxleyi* CCMP3266 cDNA.