SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
CHLOROPLASTS
LIGHT RESPONSES

Received
8 October 2014

Accepted
18 December 2014

Published
20 January 2015

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Photoprotection in sequestered plastids of sea slugs and respective algal sources

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Some sea slugs are capable of retaining functional sequestered chloroplasts (kleptoplasts) for variable periods of time. The mechanisms supporting the maintenance of these organelles in animal hosts are still largely unknown. Non-photochemical quenching (NPQ) and the occurrence of a xanthophyll cycle were investigated in the sea slugs *Elysia viridis* and *E. chlorotica* using chlorophyll fluorescence measurements and pigment analysis. The photoprotective capacity of kleptoplasts was compared to that observed in their respective algal source, *Codium tomentosum* and *Vaucheria litorea*. A functional xanthophyll cycle and a rapidly reversible NPQ component were found in *V. litorea* and *E. chlorotica* but not in *C. tomentosum* and *E. viridis*. To our knowledge, this is the first report of the absence of a functional xanthophyll cycle in a green macroalgae. The absence of a functional xanthophyll cycle in *C. tomentosum* could contribute to the premature loss of photosynthetic activity and relatively short-term retention of kleptoplasts in *E. viridis*. On the contrary, *E. chlorotica* displays one of the longest functional examples of kleptoplasty known so far. We speculate that different efficiencies of photoprotection and repair mechanisms of algal food sources play a role in the longevity of photosynthetic activity in kleptoplasts retained by sea slugs.

The capacity of some sea slugs to retain photosynthetically active chloroplasts from their algal food sources continues to puzzle and attract the attention of researchers. These sacoglossan sea slugs are able to suck the algal cytoplasm and retain intact chloroplasts (kleptoplasts) within the cells of their digestive glands for variable periods of time. The retention time of photosynthetically active kleptoplasts in animals deprived of a food source (starvation) seems to be dependent on the sea slug species, with retention times varying from only a few days (e.g. *Thuridilla* sp., *Elysia atroviridis* and *E. trisinuata*), to some weeks or a few months (*E. viridis*, *E. crispata*, *E. timida*, *Costasiella ocellifera*), or over several months (*E. chlorotica* and *Plakobranchus occellatus*)[1–7]. The longevity of functional kleptoplasts is also dependent on several other conditions such as seasonal differences of wild-collected specimens[8–9], wild *vs.* laboratory breeding origin of specimens and the length of time spent feeding to promote incorporation of the kleptoplasts[10,11], and temperature[1,9,11] and light regimes[12] employed in laboratory protocols for the husbandry of these organisms. Moreover, the algal source of the kleptoplasts can play a key role in the longevity of functional kleptoplasts[13–15].

The mechanisms supporting long-term retention and function of kleptoplasts are still largely unknown. Several chloroplast proteins are either encoded by the nuclear genome or their synthesis requires nuclear-encoded regulatory signals[16]. Mujer et al.[17] described *de novo* transcription and translation of light harvesting components in kleptoplasts of *E. chlorotica* over a period of 8 months. When kleptoplasts are maintained in the animal cells, the algal nucleo-chloroplast communication is disrupted. Horizontal gene transfer from the algal nucleus to the animal cells was hypothesised to explain, to a certain degree, the long-term survival and functioning of *V. litorea* plastids in *E. chlorotica*[18–20]. However, recent works in *E. chlorotica*[21,22] and other sacoglossan sea slugs[23] do not support this hypothesis. The answer to functional long-term kleptoplasty may reside in the plastid robustness itself[15,24,25] and/or "result from a combination of yet-to-be characterized physical and molecular mechanisms"[21].

Oxygen-evolving photosynthetic organisms regulate light harvesting in photosystems (PS) in response to rapid changes in the light environment. Energy is absorbed by light harvesting pigments in antenna proteins and transferred to PSI and PSII reaction centres (RCs) in the thylakoid membrane. The excitation energy is then converted to chemical energy through a charge separation event that begins a chain of electron transport reactions. This ultimately leads to the reduction of $NADP^+$ to NADPH and the production of ATP[26]. When energy absorption exceeds the rate at which energy can be used in the electron transport chain, highly reactive intermediates and by-products will be produced which will potentially cause photo-oxidative damage and inhibit

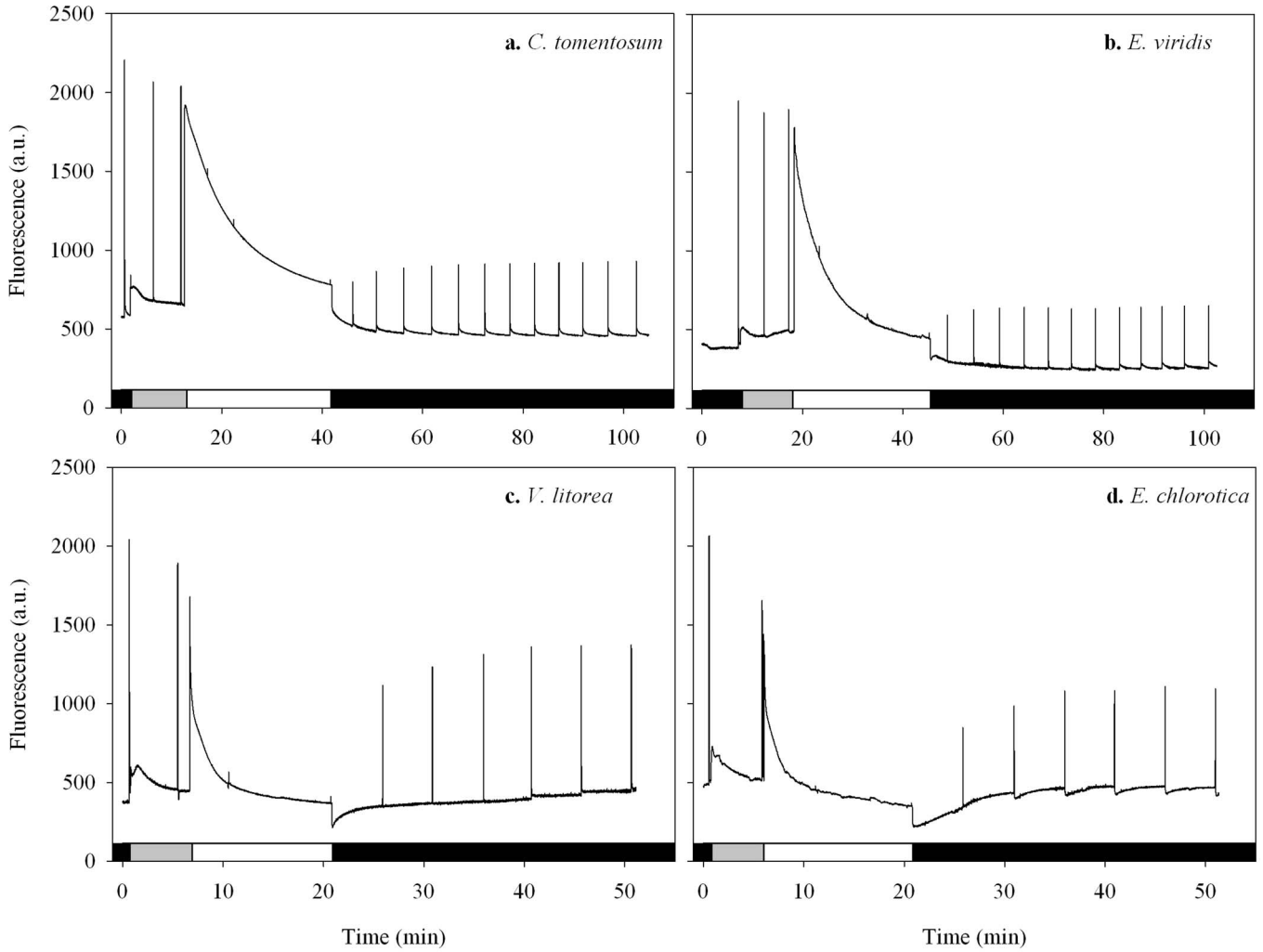

Figure 1 | Representative examples of chlorophyll (Chl) fluorescence measurements as a response to different light conditions in (a) *Codium tomentosum* thallus samples, (b) immobilized *Elysia viridis* individuals, (c) *Vaucheria litorea* filaments and (d) immobilized *E. chlorotica* individuals. Grey bar: 20 μmol photons m^{-2} s^{-1} (low light); White bar: 920 (*C. tomentosum* and *E. viridis*) or 1800 (*V. litorea* and *E. chlorotica*) μmol photons m^{-2} s^{-1} (high-light stress); Dark bar: dark. Samples were dark-adapted for 60 min before the start of Chl fluorescence measurements.

photosynthesis. To reduce photo-oxidative damage, land plants and algae evolved mechanisms for dissipation of excessive energy[27]. When photoprotection mechanisms fail to protect chloroplasts and photodamage occurs, mechanisms that repair damaged proteins are crucial for maintaining photosynthetic performance. Genome-encoded photodamage repair mechanisms could be key to plastid longevity[25]. Therefore, investigating photoprotection mechanisms[28] and photodamage repair capacities[25] of kleptoplasts retained by sea slugs is crucial to understand if such mechanisms play a role in kleptoplast robustness and longevity.

Pulse Amplitude Modulated (PAM) fluorometry has become a key technique for the investigation of photoprotection and photoinhibition mechanisms, collectively referred to as non-photochemical fluorescence quenching (NPQ) because the quenching does not result in the productive storage of energy[29]. In photosynthetic organisms, one of the most important parts of NPQ is the energy-dependent chlorophyll (Chl) fluorescence quenching (qE). Generally, qE requires the irradiance-dependent establishment of a transthylakoidal proton gradient (ΔpH) and the activation of the xanthophyll cycle[30–32]. The xanthophyll cycle can be defined as the interconversion of xanthophylls that involves one or two de-epoxidation reactions occurring in high-light and a reverse epoxidation reaction in limiting light[31]. In the Xanthophycea, such as *Vaucheria* sp., the xanthophyll cycle comprises the de-epoxidation step conversion of diadinoxanthin (Dd) to diatoxanthin (Dt) in high-light and the reverse reaction in dark or dim light[33]. The Dd cycle is equivalent to the violaxanthin (Viola), antheraxanthin (Anth), and zeaxanthin (Zea) xanthophyll cycle present in vascular plants, green and brown algae[30,34]. To date, a functional xanthophyll cycle was only demonstrated to occur in kleptoplasts of *E. timida*[28], but it is still unknown if this photoprotection mechanism remains functional throughout prolonged periods of starvation. In the present work, NPQ and the presence of a functional xanthophyll cycle were investigated in two sacoglossan sea slugs, *E. viridis* and *E. chlorotica*, with distinctly different retention-times of functional kleptoplasts.

Results

Changes in variable Chl fluorescence in response to high-light stress. Chl fluorescence traces showing the responses to high-light stress and the subsequent recovery in darkness (dark-recovery) were generally similar between sea slugs and their respective food source (Figure 1). When comparing the different associations tested, *C. tomentosum* and *E. viridis* (Figures 1a, b), and *V. litorea* and *E. chlorotica* (Figures 1c, d), Chl fluorescence traces showed distinctive trends in the dark-recovery phase. Only a small recovery in F_m' was observed for *C. tomentosum* (Figure 1a) and *E. viridis* (Figure 1b) during the dark-recovery phase, whereas in both *V. litorea* (Figure 1c)

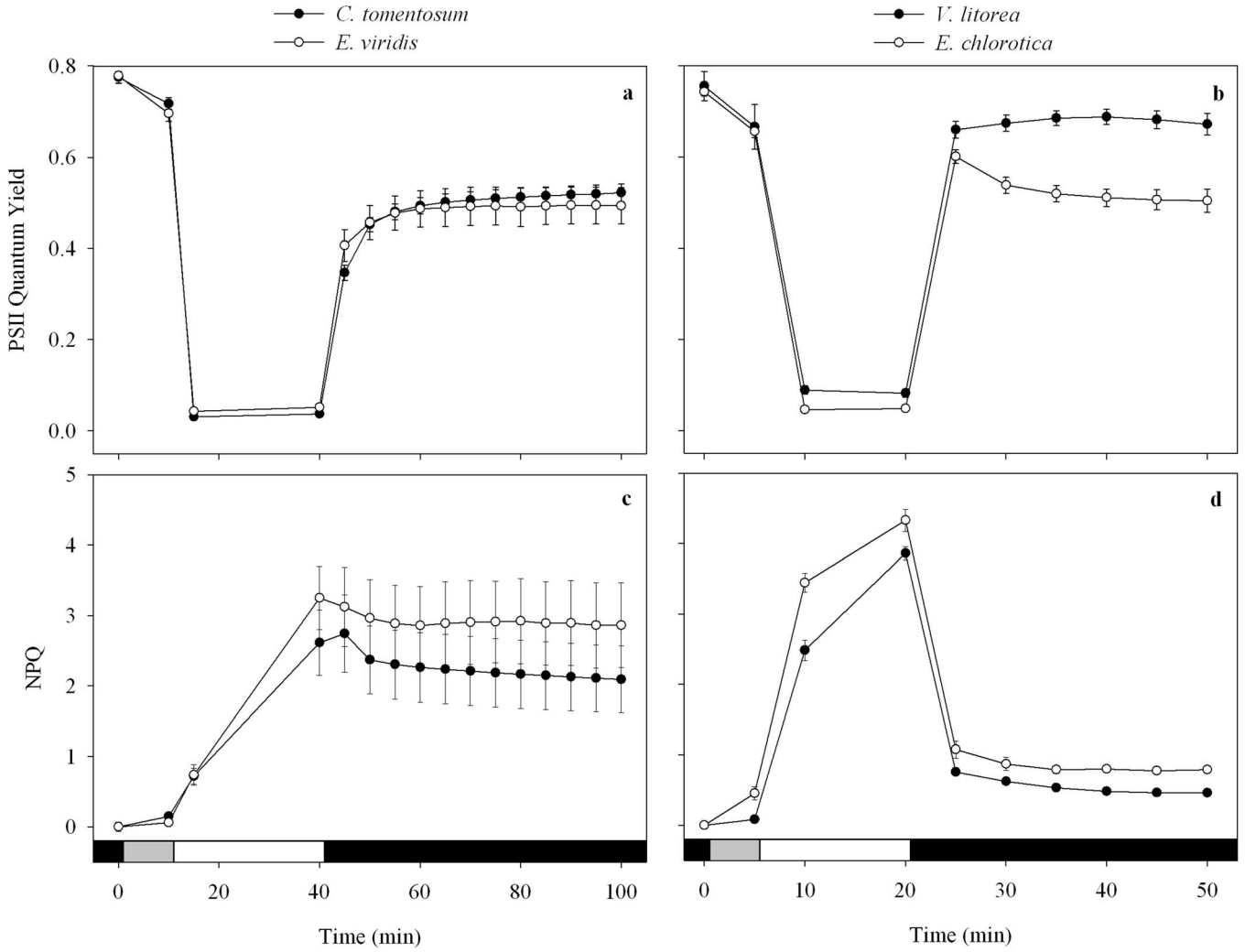

Figure 2 | Chlorophyll (Chl) fluorescence maximum and effective quantum yield of PSII (F_v/F_m or $\Delta F/F_m$', in dark-adapted or light conditions, respectively) and non-photochemical quenching (NPQ) of Chl fluorescence as a response to different light conditions in (a, c) *Codium tomentosum* thallus samples and immobilized *Elysia viridis* individuals and in (b, d) *Vaucheria litorea* filaments and immobilized *E. chlorotica* individuals. Values represent average ± standard error (n = 5). Grey bar: 20 μmol photons m^{-2} s^{-1} (low light); White bar: 920 (*C. tomentosum* and *E. viridis*) or 1800 (*V. litorea* and *E. chlorotica*) μmol photons m^{-2} s^{-1} (high-light exposure, HL); Dark bar: dark. Samples were dark-adapted for 60 min before the start of Chl fluorescence measurements. No significant differences ($P > 0.05$) were found between measurement made on *C. tomentosum* and *E. viridis* at each time point. Significant differences ($P < 0.05$) were found between *V. litorea* and *E. chlorotica* during HL and dark-recovery periods.

and *E. chlorotica* (Figure 1d) the recovery was more significant. In the former organisms, the dark-recovery of the variable fluorescence (F_m' – F_s) was mostly due to a decrease in the minimal fluorescence (F_s) rather than an increase in the maximum fluorescence (F_m'). On the contrary, in both *V. litorea* and *E. chlorotica*, the dark-recovery of variable fluorescence was due to an increase in the F_m' rather than a decrease in F_s.

The capacity of the chloroplasts in the macroalgae and kleptoplasts in the sea slugs to recover from a high-light stress was evaluated by measuring Chl fluorescence PSII quantum yield and NPQ (Figure 2). As a result of exposure to high-light, the PSII quantum yield significantly decreased followed by a significant increase (in all cases $P < 0.001$) in the dark-recovery phase in both macroalgae and sea slugs (Figures 2a, b). In *C. tomentosum*, 60 min in darkness was not sufficient for a full recovery from the high-light exposure (PSII quantum yield was 67% of the F_v/F_m after 60 min in dark-recovery). Similar results were found for *E. viridis* with no significant differences ($P = 0.862$) in the PSII quantum yield found between this species and *C. tomentosum* (Figure 2a). Generally, a similar trend occurred in *V. litorea* and *E. chlorotica*. In *V. litorea*, within 5 min of dark-recovery, 88% of the PSII quantum yield was recovered. In *E. chlorotica* PSII quantum yield recovered to 81% of the maximal capacity within the first 5 min, although it then decreased to 68% in the following 25 min (Figure 2b).

Generally, NPQ reached higher values in the sea slugs then in their corresponding algal food source (Figures 2c, d), although differences were only significant ($P < 0.001$) between *V. litorea* and *E. chlorotica* (Figure 2d). In both macroalgae and sea slugs, NPQ significantly increased (in all cases $P < 0.001$) in response to exposure to high-light. Surprisingly, in *C. tomentosum* and *E. viridis* NPQ was still sustained after 60 min in darkness following exposure to high-light (Figure 2c). On the contrary, in *V. litorea* and *E. chlorotica* NPQ significantly decreased ($P < 0.001$) when organisms were transferred to dark conditions (Figure 2d).

Changes in pigment composition in response to light-stress and dark-recovery experiments. Samples collected at the different stages of the light-stress and dark-recovery experiments (dark-adapted state, after exposure to high-light, and after recovery in darkness) were analysed for pigment content. In *C. tomentosum* and *E. viridis*

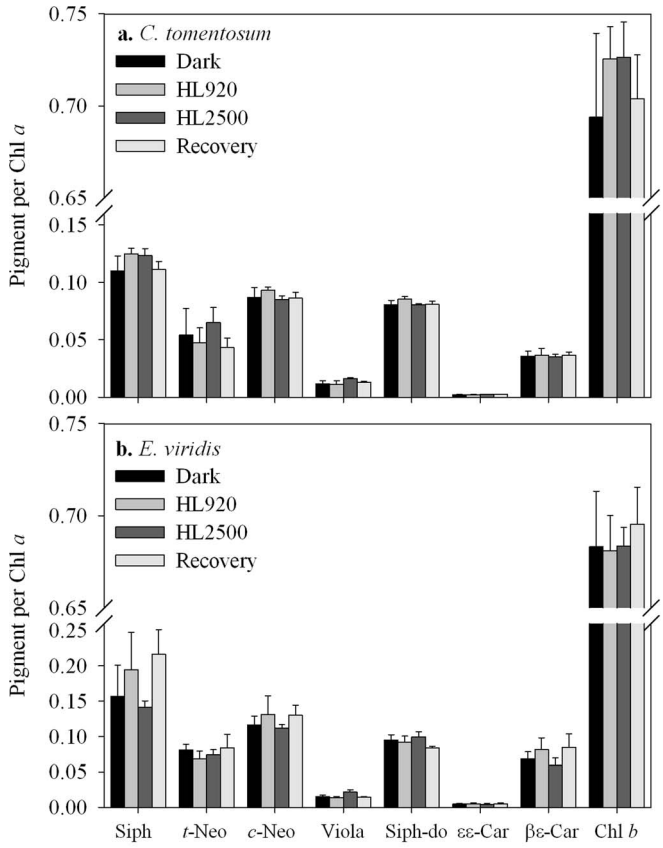

Figure 3 | Pigment per chlorophyll (Chl) *a* ratios under different light conditions in (a) *Codium tomentosum* thallus and (b) immobilized *Elysia viridis* individuals. Siphonaxanthin (Siph), all-*trans*-Neoxanthin (*t*-Neo), 9′-*cis*-Neoxanthin (*c*-Neo), Violaxanthin (Viola), Siphonaxanthin dodecenoate (Sipho-do), ε,ε-Carotene (εε-Car) and β,ε-Carotene (βε-Car), Chl *b* and Chl *a* were quantified using HPLC (average ± standard deviation; n = 5). Samples for pigment analyses were freeze-dried after 60 min of dark-adaptation ("Dark"), after 30 min of a high-light (HL) exposure ("HL920" and "HL2500": 920 and 2500 µmol photons m^{-2} s^{-1}, respectively), and after 60 min of recovery in the dark following the 920 µmol photons m^{-2} s^{-1} HL exposure ("Recovery"). No significant differences ($P > 0.05$) were found between pigment ratios in the different light treatments in both *C. tomentosum* and *E. viridis*.

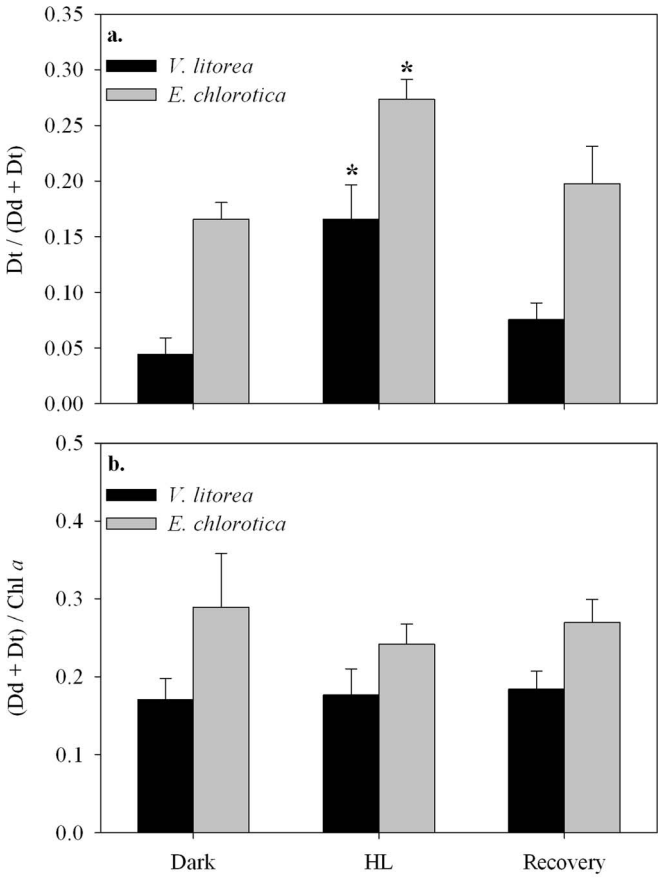

Figure 4 | Xanthophyll cycle pigment ratios (average ± standard deviation; n = 5) in *Vaucheria litorea* and *Elysia chlorotica* in response to different light conditions: (i) "Dark", dark-adapted samples for 60 min before the start of chlorophyll (Chl) fluorescence measurements; (ii) "HL", high-light stress correspondent to exposure to 1800 µmol photons m^{-2} s^{-1} for 15 min; and (iii) "Recovery", exposure to dark conditions during 30 min following the HL stress. (**a**) Dt/(Dt + Dd) ratios (content of diatoxanthin [Dt] relative to the total pool of pigments of the xanthophyll cycle Dt + diadinoxanthin [Dd]). (**b**) Total pool of pigments of the xanthophyll cycle per Chl *a*. * indicates significant differences ($P < 0.05$) between light treatments (Dark, HL and Recovery).

the pigments Anth and Zea were not present in all four treatments, while Viola was found in trace amounts (Figure 3). Thus, it was concluded that the typical xanthophyll cycle pigments found in green algae as a response to high-light (conversion of Viola into Anth and Zea) were not present in *C. tomentosum*. Lutein, another pigment commonly involved in photoprotection from high-light, was also absent in both *C. tomentosum* and *E. viridis* (Figure 3). We tested if decapitation of sea slugs interfered with pigment composition, but again no significant differences (in all cases $P > 0.05$) were found between pigments extracted from motile and immobilized sea slugs exposed to the higher light level tested (2500 µmol photons m^{-2} s^{-1}; Figure S2).

On the other hand, the xanthophyll cycle pigments Dd and Dt (equivalent to Viola and Zea in green algae) were found in both *V. litorea* and *E. chlorotica* (Figure S3). Using the Dd and Dt pigment concentrations, the de-epoxidation state (DES; changes in Dt/[Dt + Dd] ratio) was evaluated. A significant ($P < 0.001$) de-epoxidation of Dd into Dt was observed in response to exposure to high-light that was reversed in the dark-recovery phase (Figure 4a). A considerable amount of Dt was already present in dark-adapted *V. litorea* and *E. chlorotica*, with a significantly higher ($P < 0.001$) Dt/[Dt + Dd] ratio in *E. chlorotica* (Figure 4a). *De novo* synthesis of Dd was investigated by quantifying the total pool of pigments of the xanthophyll cycle ([Dd + Dt]/Chl *a*), with no significant differences ($P = 0.460$) found in response to changes in the light conditions (Figure 4b).

Discussion

Immobilization of sea slugs for photobiology studies. A prerequisite for evaluation of fluorescence quenching during illumination is reliable determination of the minimal and maximal fluorescence after dark adaptation, F_o and F_m, respectively. These values serve as reference for the evaluation of photochemical and non-photochemical quenching in an illuminated sample by the saturation pulse method. To address Chl fluorescence quenching in kleptoplasts of sea slugs it is, therefore, crucial to immobilize individuals during measurements[35]. Since sea slugs used in Chl fluorescence measurements would be sacrificed for pigment analysis, rapid decapitation was the preferred immobilization method applied in this study. This methodology did not significantly affect the PSII quantum yield of the kleptoplasts (Figure S1), therefore, it can be assumed that NPQ measurements performed in both *E. viridis* and *E. chlorotica* sea slugs were also not affected by animal decapitation.

Comparing responses of different organisms to light exposure. The fraction of incident light absorbed will drastically differ in cells displaying different optical properties[36]. Factors such as species-specific morphology will affect the Chl-specific *in vivo*-absorption coefficient. For instance, lateral light transfer in coral tissues affects the irradiance reaching the algal symbionts, with consequences at the photophysiology level[37]. Chloroplasts in the algae may not be in the same optical environment as kleptoplasts in the sea slugs. Thus, even when the same photon flux is applied, responses of chloroplasts and kleptoplasts must be compared with care[38,39]. Moreover, pigments determine light absorption of a cell because only photons absorbed by pigments can work photochemically[40]. This implies that, without determination of the specific light absorption profile, the comparison of PSII quantum yield and NPQ may be biased by differences in the composition of light harvesting complexes of the different sea slugs and macroalgae.

NPQ and xanthophyll cycle functionality. In both *V. litorea* and *E. chlorotica*, Chl fluorescence dark-recovery after high-light exposure suggested the presence of typical reversible NPQ[41,42]. A functional xanthophyll cycle was also found, as demonstrated by the de-epoxidation of Dd to Dt during high-light exposure and the reversed reaction in dark conditions. Contrary to *V. litorea* and *E. chlorotica*, in the Chl fluorescence traces observed for *C. tomentosum* and *E. viridis*, F_m' did not increase significantly in the recovery phase but a pronounced decay of F_o was recorded. This translated into significant recovery of PSII quantum yield after high-light exposure, while NPQ evolving during that period was sustained in the dark-recovery phase.

Similar Chl fluorescence traces to those observed in *C. tomentosum* and *E. viridis* were found in the diatom *Phaeodactylum tricornutum*[43]. However, in this diatom, NPQ in the dark was sustained due to the presence of the xanthophyll Dt. Surprisingly, neither Anth or Zea (Dt equivalent in the xanthophyll cycle of green algae) were found in high-light exposed *C. tomentosum* or *E. viridis*, and Viola was only a minor pigment. This indicates that one of the most important photoprotection mechanisms in plant cells, the xanthophyll cycle, was absent in chloroplasts of *C. tomentosum*. Consequently, no functional xanthophyll cycle was recorded in kleptoplasts of *E. viridis*. Lutein is also known to be involved in quenching of excessive energy and to have a photoprotective function[32,34]. However, in the present work, no lutein was detected in *C. tomentosum* and *E. viridis*. Furthermore, Raniello et al.[44] showed that lutein and siphonaxanthin varied inversely through the day and suggested a photoprotective role for the interconversion of these two pigments. In search of a similar photoprotective role of siphonaxanthin in *C. tomentosum*, the interconversion between siphonaxanthin and siphonaxanthin-dodecenoate (also known as siphonein) was investigated with no significant changes between these pigments being found throughout the experimental trials. In order to exclude a potential artefact, promoted by the immobilization method employed (e.g., in the enzyme activity of the xanthophyll cycle), we performed additional pigment analysis in motile and immobilized animals exposed to high-light conditions and found no differences between them (Figure S2).

The absence of a xanthophyll cycle is known in cyanobacteria and its presence is questionable in red algae[30]. An absent xanthophyll cycle in a macroalgae from the Chlorophyte lineage is here reported, to our knowledge, for the first time. Previous works have reported the absence of the xanthophyll Zea in *Codium* sp.[45–47], but none have investigated the interconversion between the xanthophylls Viola, Anth and Zea as a photoprotection mechanism. NPQ and PSII quantum yield in *C. tomentosum* and *E. viridis* were similar to that observed in a mutant of *Arabidopsis thaliana* defective in the gene encoding Viola de-epoxidase (*npq1* mutant)[48] and a mutant of *A. thaliana* lacking PsbS protein (*npq4* mutant)[49–51]. In vascular plants, the presence of activated PsbS (member of the LHC superfamily that seems to have appeared exclusively in the green lineage)[31] together with a de-epoxidised xanthophyll in PSII seems essential for the formation of qE[26]. We speculate that, similar to the *npq1* mutant of *A. thaliana*, *C. tomentosum* is defective in the Viola de-epoxidase gene, but further investigations are needed to confirm this assumption.

It is not possible to conclude from the present experimental data if the sustained NPQ in the dark-recovery phase observed for *C. tomentosum* and *E. viridis* is caused by photoinhibition, or is simply indicative of a sustained and possibly protective quenching mechanism[52]. Recent works have demonstrated that there are slower components in photoprotective quenching which are impossible to distinguish from true photoinhibitory quenching using analysis of reversible NPQ upon light treatment[53,54]. To overcome this issue, a new method allowing one to verify during the light treatment the amount of protective NPQ (pNPQ)[55–57] and the point of the onset of the inactivation of the PSII reaction centres, should be applied.

Photoprotection mechanisms may enhance functional kleptoplast longevity. How the kleptoplasts remain functional for several months inside the animal cells is still not well understood. *E. viridis* has been shown to survive up to 3 months without feeding[1,8,12,46], while *E. chlorotica* can maintain functional kleptoplasts for eight to ten months[2,17]. However, retention times of functional kleptoplasts in sacoglossan sea slugs can drastically change depending on factors such as husbandry conditions in the laboratory, life-history traits prior to their collection from the wild, or the algal source of kleptoplasts[38]. Therefore, the often-used definition of "short-" or "long-term" retention times must be applied with caution. In addition, different photoprotection capacity and/or repair mechanisms of algal food sources will have implications in the longevity of photosynthetic activity in kleptoplasts retained by the sea slugs. In the particular case of *E. viridis* feeding on *C. tomentosum*, the absence of a functional xanthophyll cycle in *C. tomentosum* could relate to the shorter longevity of kleptoplasts in *E. viridis* when compared to other sea slugs such as *E. timida*[28] or *E. chlorotica*. For instance, Vieira and co-workers[12] clearly showed that when *E. viridis* was deprived of a food source, exposure to higher light regimes resulted in a drastic reduction in the time kleptoplasts remained functional, indicating potential permanent damage due to photoinhibition limited kleptoplasty longevity.

Conclusion. Photoprotection capacities of algal food sources may largely determine the longevity of sequestered chloroplasts and it is important to further understand if such mechanisms are maintained in kleptoplasts isolated from their algal nucleus. For instance, although two recently fed sea slug species have been shown to possess a functional xanthophyll cycle (*Elysia timida*[28] and *E. chorotica* [present work]), it is unknown if this ability is maintained throughout a prolonged starvation period. Genes for the xanthophyll cycle enzymes are nuclear encoded in many organisms. It is therefore possible that a functional xanthophyll cycle in kleptoplasts is limited by the abundance and lifetime of enzymes (co-factors must also be considered) present at the time the chloroplasts were sequestered. de Vries et al.[25] suggests that the presence of a specific gene (*ftsH*, a D1 quality control protease that is essential for photosystem II repair) in the chloroplast genomes of *Acetabularia acetabulum* and *V. litorea* (sole food source of two "long-term" sacoglossa species, *E. timida* and *E. chlorotica*, respectively) might be a key to "long-term" plastid activity in sea slugs, stressing the importance of PSII maintenance for kleptoplast longevity. Algae possess multiple photoprotective mechanisms including adjustment of light-harvesting antenna size, synthesis of stress proteins, accumulation of lipophilic and water-soluble antioxidant molecules, enhancement of scavenging enzymatic systems and accumulation of sunscreen[58]. In this way, further investigation of those processes is required to gain an in-depth knowledge on how light harvesting, photoprotection and photodamage repair can condition

Table 1 | Notations for chlorophyll fluorescence measurements

F_o, F_m	minimum and maximum fluorescence emitted by a dark-adapted sample (arbitrary units)
F_s, F_m'	steady-state and maximum fluorescence emitted by a light-adapted sample (arbitrary units)
F_v, ΔF	variable fluorescence: $F_v = F_m - F_o$; $\Delta F = F_m' - F_s$ (dimensionless)
$\Delta F/F_m'$	effective quantum yield of PSII (dimensionless)
F_v/F_m	maximum quantum yield of PSII of a dark-adapted sample (dimensionless)
NPQ	Non-Photochemical Quenching of chlorophyll *a* fluorescence [$=(F_m-F_m')/F_m'$] (dimensionless)

the long-term retention of functional kleptoplasts in sacoglossan sea slugs.

Methods

Biological material: collection and maintenance. Adults of the sea slug *E. viridis* Montagu 1804 and specimens of the macroalgae *C. tomentosum* Stackhouse 1797 were collected in the intertidal rocky area of Barra beach, Aveiro, Portugal (40° 38' 37.67" N, 8° 44' 56.75" W). The sea slugs and the macroalgae were maintained in a recirculated life support system operated with artificial seawater (prepared by mixing Tropic Marin© salts with freshwater purified by reverse osmosis) at salinity 35 and 18°C temperature. Photoperiod was 14 h light:10 h dark at an irradiance of approximately 20 µmol photons m^{-2} s^{-1} (all light intensities measured at air-water interface). *E. chlorotica* Gould 1870 were raised in the laboratory at the University of Connecticut, USA, as described by Pelletreau et al.[11]. Specimens were originally collected from wild populations from Martha's Vineyard, MA (USA). Adults of the sea slug *E. chlorotica* were maintained in artificial seawater (prepared by mixing Instant Ocean© salts with freshwater purified by reverse osmosis) at salinity 32 and 10°C temperature on a 12 h light:12 h dark photoperiod at 10 µmol photons m^{-2} s^{-1}. Unialgal cultures of the filamentous alga *V. litorea* Agardh 1823, originating from a collection from Martha's Vineyard, were cultured in modified f/2 media ($Na_2SiO_3 \cdot 9H_2O$ was omitted; concentrations of vitamins in final media were as follows: biotin 0.16 µM; thiamine HCl 0.118 µM; vitamin B12 0.029 µM). *V. litorea* cultures were maintained at 24°C on a 12 h light:12 h dark photoperiod and at a light intensity of 23 µmol photons m^{-2} s^{-1}.

Chl fluorescence measurements. Variable fluorescence of *C. tomentosum* and *E. viridis* was measured at room temperature using a PAM fluorometer comprising a computer-operated PAM-Control Unit (Walz GmbH, Effeltrich, Germany) and a WATER-EDF-Universal emitter-detector unit with a 6 mm diameter Fluid Light Guide fiberoptics bundle (Gademann Instruments GmbH, Würzburg, Germany). This fluorometer uses a modulated blue light (LED-lamp, emission maximum at 450 nm and a half-bandwidth of 20 nm) as source for measuring, actinic and saturating light, emitted at a frequency of 18 Hz when measuring F_o or 20 kHz when measuring other fluorescence parameters (see notation in Table 1). Variable fluorescence of *V. litorea* and *E. chlorotica* was measured at room temperature using a mini-PAM coupled with a 5.5 mm mini-PAM/F fiberoptics (Walz GmbH, Effeltrich, Germany). This fluorometer uses a modulated red light (LED-lamp, emission maximum at 650 nm) as source for measuring light, emitted at a frequency of 0.6 Hz when measuring F_o or 20 kHz when measuring other fluorescence parameters, and a halogen lamp (8 V/20 W blue enriched, $\lambda < 710$ nm) as source for actinic and saturating light. Both fluorometers use fiberoptics to guide measuring, actinic and saturating light to the sample and to collect fluorescence. Variable fluorescence was measured by recording F_o and F_s (dark and light adapted samples, Table 1) in the presence of a weak measuring-light and applying a saturating-light pulse (SP) to obtain F_m and F_m' (dark and light adapted, Table 1).

Thalli of *C. tomentosum* measuring 6 mm in length, filaments of *V. litorea*, and motile or immobilized *E. viridis* and *E. chlorotica* sea slugs were individually placed on a well of a 96-well plate filled with water and covered with a coverslip for subsequent measurements of Chl fluorescence. In the case of *C. tomentosum* and both sea slugs, the well was partially filled with polystyrene foam (up to 2 mm from the top). Chl fluorescence fiberoptics where placed directly on top of the coverslip covering the sample. Samples were previously dark-adapted for 60 min in a Petri dish before being transferred to the well plate for Chl fluorescence measurements.

Immobilization methodology. To measure Chl fluorescence in the sea slugs and enable accurate NPQ calculations, individuals were immobilized using rapid decapitation. The effect of decapitation of sea slugs on Chl fluorescence quantum yield of PSII (F_v/F_m and $\Delta F/F_m'$, in dark and light conditions respectively) was tested using *E. viridis* as described in the supplementary material (Figure S1). No differences were found between measurements taken in motile or immobilized sea slugs.

Light-stress and dark-recovery experiments. The effect of a high-light exposure and recovery in darkness was tested as follows: Chl fluorescence was measured in 15 samples of *C. tomentosum* and *V. litorea*, and in 15 specimens of immobilized *E. viridis* and *E. chlorotica* as described above. All samples were dark-adapted for 60 min in a petri dish before being transferred to the well plate. After transfer to the well, a SP was applied to determine F_v/F_m (see notations in Table 1). Subsequently, samples were exposed to low light (LL; 20 µmol photons m^{-2} s^{-1} for 10 min in *C. tomentosum* and *E. viridis* or 5 min in *V. litorea* and *E. chlorotica*) followed by a high-light exposure (HL; 920 µmol photons m^{-2} s^{-1} for 30 min in *C. tomentosum* and *E. viridis* or 1800 µmol photons m^{-2} s^{-1} for 15 min in *V. litorea* and *E. chlorotica*). Following the exposure to high-light, samples were allowed to recover in the dark for 60 or 30 min (*C. tomentosum* and *E. viridis* or *V. litorea* and *E. chlorotica*, respectively). A SP was applied every 10 or 5 min (*C. tomentosum* and *E. viridis* or *V. litorea* and *E. chlorotica*, respectively) for calculation of $\Delta F/F_m'$ and NPQ (see notations in Table 1). Differences in the protocols applied to the different organisms were due to PAM fluorometer equipment-specific limitations. Preliminary tests (data not shown) allowed us to determine the level of high-light exposure that would return similar levels of NPQ and PS II quantum yield efficiencies between both associations. From each group of macroalgae or sea slug specimens, five replicates were collected and immediately frozen in liquid nitrogen for pigment analysis at the following steps: (i) after F_v/F_m determination; (ii) at the end of the high-light exposure and (iii) at the end of the dark-recovery period. In order (i) to test an alternative light source and intensity that could induce de-epoxidation of Viola to Anth and Zea in *C. tomentosum* and *E. viridis*, and (ii) to exclude a possible negative effect of decapitation of *E. viridis* in the xanthophyll cycle activation, the following experiment was performed: five samples of *C. tomentosum*, five specimens of immobilized *E. viridis* and five specimens of motile *E. viridis* were exposed to 2500 µmol photons m^{-2} s^{-1} for 30 min using a halogen lamp coupled to a heat absorbing glass. At the end of exposure to high-light, each sample was immediately frozen in liquid nitrogen for pigment analysis.

Pigment analysis. Sea slugs and macroalgal samples were freeze-dried and pigments extracted in 95% cold buffered methanol (2% ammonium acetate). Samples were ground with a glass rod, sonicated for 30 s and briefly vortexed. Samples were then transferred to −20°C for 20 min in the dark. Extracts were filtered through 0.2 µm Fluoropore membrane filters (Millipore, Billerica, MA, USA) and immediately injected into an HPLC system (Shimadzu, Kyoto, Japan) with a photodiode array detector (SPD-M10AVP). Chromatographic separation was carried out using a Supelcosil C18 column (25 cm length; 4.6 mm diameter; 5 µm particles; Sigma-Aldrich, St. Louis, MO, USA) for reverse phase chromatography and a 35 min elution programme. The solvent gradient followed Kraay et al.[59], with an injection volume of 100 µl and a flow rate of 0.6 ml min^{-1}. Pigments were identified from absorbance spectra and retention times and concentrations calculated from the signals in the photodiode array detector in comparison with pure crystalline standards from DHI (Hørsolm, Denmark). The fucoxanthin standard was used for the quantification of siphonaxanthin and siphonaxanthin dodecenoate because no purified standard or specific absorption coefficients were available[60].

Statistical analysis. Differences in PSII quantum yield and NPQ between sea slugs and respective food source during the light stress experiments were tested using repeated measures one-way analysis of variance (ANOVA). Differences in pigment ratios were tested using two-way ANOVA for effects of light treatments (dark, high-light and dark-recovery) and organisms (sea slugs and respective algal food source). Pairwise comparisons were performed using the Tukey HSD test. Statistical analyses were carried out using Statistica 10 (StatSoft Inc., USA).

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Acknowledgments

The authors thank Susan Devine and Gregory Mirando for help in the laboratory during the experimental work at the University of Connecticut (USA) and for growing and maintaining *E. chlorotica* specimens and the alga *V. litorea* used in this work. Sónia Cruz and Gisela Dionísio were supported by the Fundação para a Ciência e a Tecnologia, through the postdoctoral grant SFRH/BPD/74531/2010 (S. Cruz) and doctoral grant SFRH/BD/73205/2010 (G. Dionísio). This work was funded by the FP7 Marie Curie Career Integration grant PCIG11-GA-2012-322349 (S. Cruz) and by National Science Foundation grant IOS-0726178 (M. Rumpho).

Author contributions

S.C., P.C., R.C., J.S., K.N.P. and M.E.R. conceived and designed research. S.C., P.C., G.D. and R.N. conducted experiments. S.C. and P.C. analyzed data. S.C. wrote the manuscript. All authors read, commented and approved the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Cruz, S. *et al*. Photoprotection in sequestered plastids of sea slugs and respective algal sources. *Sci. Rep.* **5**, 7904; DOI:10.1038/srep07904 (2015).