

**Rapid colour changes in *Euglena sanguinea* (Euglenophyceae) caused by internal lipid globule migration.**

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**Running title:** Reddening in *E. sanguinea*

## Abstract

The accumulation of red pigments, frequently carotenoids, under chronic stress is a response observed in diverse kinds of eukaryotic photoautotrophs. It is thought that red pigments protect the chlorophyll located underneath by a light-shielding mechanism. However, the synthesis or degradation of carotenoids is a slow process and this response is usually only observed when the stress is maintained over long periods of time. In contrast, rapid colour changes have been reported in the euglenophyte *Euglena sanguinea*. Here we study the ecophysiological process behind this phenomenon through chlorophyll fluorescence, and pigment, colour and ultrastructural analyses. Reddening in *E. sanguinea* was due to the presence of a large amount of free and esterified astaxanthin (representing 80% of the carotenoid pool). The process was highly dynamic, shifting from green to red in 8 min (and *vice-versa* in 20 min). This change was not due to *de novo* carotenogenesis, but to the relocation of cytoplasmic lipid globules where astaxanthin accumulates. Thus, red globules were observed to migrate from the centre of the cell to peripheral locations when exposed to high light. Globule migration seems to be so efficient that other classical mechanisms are not operative in this species. Despite the presence and operation of the diadino-diatoxanthin cycle, non-photochemical quenching was almost undetectable. Since *E. sanguinea* forms extensive floating colonies, reddening can be observed at a much greater scale than at the cellular level and the mechanism described here is one of the fastest and most dramatic colour changes attributable to photosynthetic organisms at cell and landscape level. In conclusion *E. sanguinea* shows an extremely dynamic and efficient photoprotective mechanism, based on more on organelle migration than on carotenoid

biosynthesis, which prevents excess light absorption by chlorophylls reducing the need for other protective processes related to energy dissipation.

**Key words:** Astaxanthin, chlorophyll fluorescence, diadinoxanthin, neuston, photoprotection, reddening

## Introduction

There is a general consensus that chloroplasts of euglenophytes (Kingdom Excavata) derive from a green alga through a secondary endosymbiotic event (Koziol *et al.*, 2007; Archibald, 2009). However, the evolution of this plastid is particularly puzzling and it is the only group that contains the pigment markers of green algae (chlorophyll *b* and neoxanthin) together with the xanthophylls of the diadinoxanthin (Ddx/Dtx) cycle (Casper-Lindley & Björkman, 1998) not found in green algae species. The violaxanthin cycle is ubiquitous in the green clade (subkingdom Viridiplantae) but controversial in euglenophytes, with some authors describing the presence of the carotenoids violaxanthin or at least zeaxanthin (Grung & Liaaen-Jensen, 1993; Roach *et al.*, 2015) while others do not. Neither of the cycles is mutually exclusive and it has been shown that both xanthophyll cycles can operate simultaneously in diatoms (Lohr & Wilhem, 1999). However, in euglenophytes the mechanism of non-photochemical quenching (NPQ) seems to be independent of the operation of xanthophyll cycles (Doege *et al.*, 2000). In this group, photoprotective movements (migration up or down in the water column) may be more important than other protective mechanisms (Häder & Iseki, 2017).

Some euglenophyte species have been reported to be red or to have the ability to become red, with *Euglena sanguinea* Ehrenberg being the most commonly cited. *E. sanguinea* is a microalga that inhabits eutrophic lentic freshwater habitats (Wołowski, 2011) throughout the world (Guiry, 2017). It was originally described from a blood-coloured water sample from Silesia, although it was noted that cells were at first green and then red (Ehrenberg, 1831). In this species, large amounts of the red xanthophyll astaxanthin (Ast) have been reported to occur (Grung & Liaaen-Jensen, 1993; Gerber &

Häder, 1994; Frassanito *et al.*, 2008). However, other authors have not detected Ast in this species (Deli *et al.*, 2014).

Astaxanthin (Ast) is a keto-carotenoid present in different microalgal lineages such as chlorophytes, dinoflagellates, prasinophytes or euglenophytes. It is also present in animals such as fishes, crustaceans and birds, giving them a characteristic pink or red colouration (Maoka, 2011). Because of its nutraceutical importance, as it is involved in the prevention of several diseases and disorders, over recent years great efforts have been made to improve Ast production by microalgae in culture (Varela *et al.*, 2015). In plant and animal tissues, Ast may be free or form monoesters and diesters, and this differing degree of esterification alters hydrophobicity, adjusting its content to the polarity of storage compartments. In algae, the biosynthesis, regulation and biotechnological production of Ast has been best characterised for the chlorophyte *Haematococcus lacustris* (Gir.-Chantr.) Rostaf. (as *H. pluvialis* Flotow; Lemoine & Schoefs, 2010). In this species the presence of Ast is not constitutive, being induced by environmental stresses such as nitrogen starvation, salinity or high light (Lemoine & Schoefs, 2010). In fact, the expression of genes that encode enzymes for the synthesis of Ast is mostly triggered by reactive oxygen species (ROS) (Li *et al.*, 2009). Once synthesised Ast can be incorporated into chloroplasts (thylakoids, plastoglobules or proteins) where it can replace other carotenoids, as has been shown in transgenic tobacco plants expressing *Chlamydomonas* genes for Ast biosynthesis (Röding *et al.*, 2015) and in red cysts of *H. lacustris* (Mascia *et al.*, 2017). Astaxanthin can be also synthesised out of the chloroplasts from  $\beta$ -carotene molecules that are exported to the cytoplasm and incorporated into extraplastidial lipid globules (Lemoine & Schoefs, 2010). Given the relationship between Ast biosynthesis and unfavourable environmental

conditions (Lemoine & Schoefs, 2010) the most probable function of its accumulation in algae is stress protection. Among its protective functions, chloroplast protection from light excess light through a shielding effect has been proposed for Ast in *H. lacustris* (Torzillo *et al.*, 2005; Wang *et al.*, 2003) and *Chlamydomonas nivalis* (Remias *et al.*, 2005). Other functions such as antioxidant (Dambeck & Sandmann, 2014) or carbon storage (Remias *et al.*, 2005) have been ruled out. In addition to Ast production under stress conditions, light-induced peripheral migration of pigmented globules was described in *H. lacustris* (Peled *et al.*, 2012). There is reference to migration of pigmented globules in microalgae in old observations on *Euglena sanguinea* (Heidt, 1934). Analogous observations have been made in animal cells, such as in the chromatophores of crustaceans, where the mechanism of pigmented bodies transport has been better studied (Boyle & McNamara, 2005; Fuhrmann *et al.*, 2011; Milograna *et al.*, 2016). Moreover, Ast has been reported to be the pigment responsible for the colour in Antarctic krill (Auerswald *et al.*, 2008).

In *E. sanguinea*, the pool of free and esterified Ast represents the most abundant pigment, accounting for 75% of the total carotenoid pool (Grung & Liaaen-Jensen, 1993). This high Ast pool gives this species its characteristic red colouration from which its name is derived (*sanguinea* = bloody). As a result, blooms of *E. sanguinea* turn water a bloody colour, which led Ehrenberg (1831), when describing the species, to speculate that this organism gave rise to legends of waters turning into blood, as may have happened in Egypt at the time of Moses.

In the present study we have described the ecophysiological process behind the extraordinary colour changes observed in *E. sanguinea* in response to environmental triggers. For the first time the reddening mechanism has been studied in *E. sanguinea*

from macroscopic to microscopic levels integrating chlorophyll *a* fluorescence, pigment, colour and ultrastructural analyses. We propose a photoprotective role for the reddening of *E. sanguinea*, with Ast being the main pigment involved in this process. We have also compared this reddening process with other potential photoprotective mechanisms such as de-epoxidation of xanthophyll cycles and development of thermal dissipation of excess energy in the photosynthetic apparatus.

## **Materials and methods**

### *Species studied, field sampling, strains isolation and culture conditions*

After some weeks of unusually warm, dry and sunny weather during April and May 2017 (Euskalmet, 2017), the formation of a reddish neuston scum could be observed on some areas of Lertutxe pond. This is a small (2.18 Ha) reservoir (43°19'48"N 2°58'08"W) where a layer of aquatic plants (*Ceratophyllum demersum* L., *Egeria densa* Planch.) and filamentous green algae cover the water sub-surface from spring to autumn. The reservoir is included in the Wetlands Inventory of the Basque Government under the code EB18 ([http://opendata.euskadi.eus/contenidos/ds\\_geograficos/inventario\\_humedales/es\\_opendata/adjuntos/memoria\\_Grupo3\\_PTSHum\\_2011\\_05.pdf](http://opendata.euskadi.eus/contenidos/ds_geograficos/inventario_humedales/es_opendata/adjuntos/memoria_Grupo3_PTSHum_2011_05.pdf)). The neuston was observed under the microscope and it was found to be formed by the palmelloid or colonial form of a euglenophyte identified as *Euglena sanguinea*. Euglenoids are typically flagellated, but many *Euglena* species can form non-motile mucilaginous colonies developing a neustonic stage (Hindák et al., 2000). In some papers, those spherical cells that have lost flagella are referred to as cysts. In others, the term palmella or palmelloid stage has been used. Here, “palmelloid” has been preferred in order to avoid confusion with the

resistance forms (cysts) that many microalgae form in stress conditions. Floating palmelloid colonies of *Euglena sanguinea* were sampled with a glass tray from the surface of the pond.

Clonal non-axenic strains (Eg07EHU and Eg08EHU) were isolated from a field-collected sample and cultures established on a liquid medium. A small portion of a neustonic colony was re-suspended in filtered water. Single cells detached from the colony were captured with a glass capillary under an inverted microscope and inoculated in 24-well tissue culture plates (Iwaki Microplate, Japan) filled with 1 ml of medium. When cell growth was observed and the concentration was sufficient, the cells were transferred to Nunclon culture flasks containing 20 ml of medium. The culture medium was prepared with filtered (0.22  $\mu\text{m}$ , Millipore) water from the reservoir and enriched with nitrates, phosphates, trace elements, vitamins and soil extract (Supplementary table S1). Cultures were maintained in a growth chamber at 22°C and PFD 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  under a 14:10 h light-dark cycle with a white fluorescent light. Cultured strains did not form the neustonic colonies as observed in the field, but instead grew as motile cells and as loose aggregates of palmelloid cells embedded in a mucilaginous matrix loosely attached to flask walls. For studies on colour changes and associated pigment dynamics, floating palmelloid colonies were collected from the reservoir water surface, transferred to moist filter paper and studied immediately.

#### *Pigment and tocopherol analysis*

Lipophilic antioxidant composition (pigments and tocopherol) was analysed in red and green colonies. A red colony of approximately 2  $\text{cm}^2$  was taken from the field. One



portion was extracted in its red form and another portion was extracted after one hour in darkness, after which it had turned green. The analysis was performed by reverse phase HPLC as described previously (García-Plazaola & Becerril, 1999) with some modifications (García-Plazaola & Becerril, 2001). Essentially, 15 mg of freeze-dried sample were extracted with 0.5 ml of acetone (95%) in a mortar and pestle. Samples were then centrifuged at 13200 rpm for 20 min at 4°C. Supernatant was preserved and a second extraction in 0.5 ml acetone (100%) and consecutive centrifugation were conducted. Both supernatants were combined and syringe-filtered through a 0.22 µm PTFE filter (Whatman, Maidstone, UK). Acetone extraction media contained 0.5 g l<sup>-1</sup> of CaCO<sub>3</sub>, in order to avoid acid traces that might change pigment composition. Extracts were injected (15 µl) in a Waters (Milford, Massachusetts, USA) HPLC system following the García-Plazaola & Becerril (1999) protocol. Photosynthetic pigments were measured with a PDA detector (Waters model 996) in the range 400–700 nm. Peaks were detected and integrated at 445 nm for carotenoid and chlorophyll content. Pigments were identified by comparing spectral characteristics obtained by the PDA detector and retention times with those of standard materials (Sigma-Aldrich for Ast, and DHI, Hørsholm, Denmark for the rest of the pigments). Retention times and conversion factors for pigments were the same as those described by García-Plazaola & Becerril (1999, 2001). For tocopherols, detection was carried out with a fluorescence detector (Waters model 474) set to  $\lambda_{\text{exc}} = 295$  nm and  $\lambda_{\text{em}} = 340$  nm and calibrated with tocopherol standards (Calbiochem, San Diego, California).

### *Image analysis*

Macroscopic photographs of neustonic palmelloid colonies were taken using a digital camera (Nikon Coolpix 4500) with a flashlight. Microscopic photographs were taken under a Leica<sup>TM</sup> DMRB light microscope equipped with a Nikon DS-U2 camera with a NIS-Elements D 2.30 image acquisition software (Nikon Instruments Europe, Badhoevedorp, the Netherlands). A small portion of a neustonic colony in its green form was mounted on a slide and the colour change which was triggered by the microscope's high light illumination was monitored. Photographs were taken at 1 min intervals during the first 5 minutes and then at 2 min intervals until 15 minutes. The image processing software ImageJ (Abramoff *et al.*, 2004) was used to extract information from the red (R), blue (B) and green (G) channels. Several chromatic indexes were used to quantify the degree of greenness/redness: red chromatic coordinate (rCC), green chromatic coordinate (gCC), normalised difference index (NDI) and excess green index (ExG) (Junker & Ensminger, 2016). These indexes were calculated as:

$$\text{NDI} = (G - R) / (G + R)$$

$$\text{gCC} = G / (R + G + B)$$

$$\text{rCC} = R / (R + G + B)$$

$$\text{ExG} = 2G - (R + B)$$

#### *Transmission electron microscopy*

To study the ultrastructural differences of green and red forms, thin sections were observed under transmission electron microscopy. Neustonic colony portions of approximately 2 cm<sup>2</sup> were immediately fixed *in situ* in 2.5% glutaraldehyde in Sorensen's buffer. One sample was fixed early in the afternoon on a sunny day when it

looked red and another was fixed the same day at dusk when it looked green. Samples were left fixing overnight in a refrigerator (4°C). Then, samples were centrifuged at 10,000 g for 3 min. The pellets were then rinsed in the same buffer without fixative, postfixed in 1% OsO<sub>4</sub> for 1 h, rinsed again in the buffer and dehydrated in a graded ethanol series. Pellets were then embedded in EPON Polarbed 812 resin in a series of steps that went from propylene to pure resin, and polymerised in an oven at 55°C for two days. The sections from the blocks were 50 nm thick and were placed on copper mesh. They were stained with uranyl acetate and contrasted with lead citrate. Sections were viewed with a JEOL JEM-1400Plus transmission electron microscope (JEOL Ltd., Tokyo, Japan).

#### *Chlorophyll a fluorescence*

To study chlorophyll a fluorescence, three different systems were employed:

i) on floating palmelloid colonies, kinetic-induction and light response curves were measured by a PAM2500 fluorometer (Walz, Efflricht, Germany). The maximum chlorophyll fluorescence yield ( $F_m$ ) was induced with a saturating pulse (8000  $\mu$  mol photons  $m^{-2} s^{-1}$ ) while minimum fluorescence ( $F_o$ ) was recorded at low measuring light intensities. Light ramps were performed over the range of photon irradiance 2–1300  $\mu$ mol photons  $m^{-2} s^{-1}$  and kinetic responses were studied on colonies illuminated with a photon irradiance of 300  $\mu$ mol photons  $m^{-2} s^{-1}$ . The maximum chlorophyll fluorescence yield in illuminated samples ( $F_m'$ ) was induced with a saturating pulse (8000  $\mu$  mol photons  $m^{-2} s^{-1}$ ).

ii) in liquid cultures of motile forms of strain Eg07EHU, a Water PAM (Walz, Efflricht, Germany) was used instead. The maximum chlorophyll fluorescence yield

( $F_m$ ) induced with a saturating pulse ( $>6000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and kinetic responses were studied with an actinic light with a photon irradiance of  $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

iii) chlorophyll fluorescence in individual cells was studied with an Imaging PAM microscope, version (Walz, Efflricht, Germany), coupled to an Axiostar plus microscope (Carl Zeiss, Gottingen, Germany) with the WinControl programme for image processing software used to control the timing, settings and trigger signals for the saturating pulse light resources and special detector-ocular microscope. The epi-illuminator features contain a MICRO-head blue single Luxeon LED measuring light source with a peak emission at 470 nm, with short-pass filter, diachronic beam splitter and collimators optic which provides pulse-modulated measuring light and actinic and saturation pulse illumination. It is integrated with a CCD camera Imag-K4 (1392 x 1040 pixel) and Zeiss fluor objective 20X/0.75. Fluorescence was detected by the photomultiplier attached to the photo port of the Axiostar plus microscope. To process the images captured, false colour images of leaf chlorophyll fluorescence yield ( $F_v/F_m$ ) were established for each individual cell.

## **Results**

*Daily changes in the colour of water surface are caused by E. sanguinea.*

At the study site (Lertutxe reservoir), the reddening of the water surface was conspicuous on sunny days, particularly in summer, but also in late spring and early autumn (Figs 1–4), as the result of a neuston algae layer. When samples were taken in the field and brought to the laboratory, a colour change from the initial red to green was observed over a timescale of hours (Figs 5, 6). The same colour change was also observed at a landscape scale (Figs 1, 2). When observed under the microscope, the

main component of these floating mats was colonies of *E. sanguinea*. When cultured, we were able to observe that isolated *E. sanguinea* cells maintain the ability to redden (Figs 5–8) when exposed to high irradiance.

*Colour changes are fast and reversible in E. sanguinea.*

Dark-adapted colonies suddenly exposed to the high light of the microscope illumination system only required 8 minutes to reach the maximum level of redness, and the four chromatic indexes used to characterise redness showed the same trend (Fig. 9, Supplementary video 1). Among these, the green chromatic coordinate (gCC) was chosen as the most accurate to quantify colour changes as it showed the highest degree of variation for the experimental conditions of the present study. Thus, for the following results, gCC is the only parameter shown. Conversely, when entire red colonies were transferred to a dim light environment, these only required twenty minutes to become green when transferred to dim light (Fig. 10). When chromatic characteristics were analysed under the microscope, the gCC of each individual cell correlated positively with Fv/Fm (Fig. 11), implying that red cells were more photoinhibited than green ones.

*Pigment composition was almost stable except for the operation of the diadinoxanthin-diatoxanthin cycle.*

Fig. 12 shows the overlaid chromatograms of a red mat of *E. sanguinea* (insert A), and its green form (insert B), after one hour in darkness. Despite the change in colour, the chromatograms were almost identical except for the inter-conversions of the pigments involved in the Ddx/Dtx cycle (third and fifth peaks). In red colonies, diatoxanthin (Dtx) represented 30% of the total pool of diadinoxanthin (Ddx) + Dtx (bottom panels),

while in the green stage Dtx was almost negligible. In the same transition from red to green, total Chl (a+b) and total Ast (free and sterified forms) increased slightly. In both red and green cells, Ast was the main pigment representing 80% of the total carotenoid pool and a molar ratio of Ast to Chl of approximately two.

*Colour changes are likely caused by lipid globule migration*

In the light microscope, *E. sanguinea* cells exhibited red-coloured grains aggregated to the cell centre or more dispersed (Supplementary fig 1), and their movement was also observed in living cells. Free and sterified Ast was mainly contained in the red grains, not in the chloroplasts which appeared green under the light microscope. A more detailed study of transmission electron microscopy (TEM) images showed that in the red colonies the globules appeared in the cell periphery, whereas in the green colonies, osmiophilic globules were aggregated in the centre of the cells, meaning that the globules had migrated (Figs 13, 15). Furthermore, this migration had a polar component and the peripheral accumulation occurred in the part of the cell most exposed to light. Higher magnification showed that lipid bodies were surrounded by a membrane-like structure (Figs 14, 16). These osmiophilic globules were presumably the same as those red pigmented grains observed under the light microscope.

*Other photoprotection mechanisms in E. sanguinea.*

Light responses of chlorophyll fluorescence were studied to characterise photoprotective responses in *E. sanguinea*. When both free-living and palmelloid (colonial) forms of *E. sanguinea* were illuminated under photon irradiance  $300 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for at least 250 s, no evidence of Fm' quenching was observed (Fig. 17),

irrespectively of the initial state of light or dark-acclimation. Furthermore, in spite of being quenched,  $F_m'$  even increased with illumination, particularly in dark-adapted palmelloid cells.

Average contents of  $\alpha$ -tocopherol,  $\beta$ -+ $\gamma$ -tocopherol,  $\delta$ -tocopherol and tocotrienols (expressed as  $\text{mmol mol}^{-1}$  Chl a+b) were relatively high: 48, 85 and 271, respectively (data not shown).

## Discussion

The accumulation of large amounts of red metabolites (anthocyanins, betacyanins, carotenoids) is a frequent stress response observed in a wide variety of photosynthetic organisms (Hughes, 2011). Among these substances, secondary red carotenoids, such as Ast, rhodoxanthin or eschscholtzxanthin are frequently found in different algae and plant groups under stress conditions (Díaz *et al.*, 1990; Hormaetxe *et al.*, 2004; Lemoine & Schoefs, 2010). In plants and algae, these carotenoids typically accumulate in extraplastidial lipid bodies inside the cytoplasm or to form plastoglobules in plastids (Moriyama *et al.*, 2018). The former are in essence spherical lipid droplets surrounded by a phospholipid monolayer with coating proteins (Pyc *et al.*, 2017). This is probably the case for the osmiophilic globules that appear to be surrounded by a membrane-like structure in *E. sanguinea*, resembling similar structures found in *Haematococcus lacustris* (as *H. pluvialis*; Wang *et al.*, 2003). This complex structure deserves further research as it may confirm a fundamental difference in globule biogenesis and mechanisms of migration between *E. sanguinea* and others. Moreover, given the light-induced migration of these globules inside the cell (Figs 13, 15), and the high proportion of free and esterified Ast (80% of total carotenoids) (Fig. 12) it is very

probable that most, if not all, Ast in the cell is contained within these globules, through a process of direct metabolic transfer from the chloroplast to the lipid body (Moriyama *et al.*, 2018). Moreover, the Ast-containing organelles observed in *E. sanguinea* were structurally very similar to the globules or grains that make up the stigma or eyespots in euglenids (Walne & Arnott, 1967; Kivic & Vesik, 1972). The stigma grains are red-orange coloured and have been reported to contain  $\beta$ -carotene, Dtx and Ddx (Heelis *et al.*, 1979).

The presence of pigmented globules seems to be a permanent trait in the local *E. sanguinea* strains Eg07EHU and Eg08EHU. Cells maintained the bulk of red globules after months of growing the cultures without high light (data not shown), when the cells persist in their green form (i.e. with the globules aggregated in the cell centre). This is in contrast to observations of *Haematococcus lacustris*, where cells lacking red lipid globules can form them after exposure to high irradiance (Wang *et al.*, 2003), causing reddening of the cells. The reddening phenomenon in *E. sanguinea* reported here is caused by the relocation of red globules within the cell, not by their *de novo* production. This mechanism of colour change is analogous to the physiological colour change observed in the chromophores of some crustaceans (Auerswald *et al.*, 2008; Fuhrmann *et al.*, 2011). In the redescription of *Euglena sanguinea* by Karnkowska-Ishikawa *et al.* (2013), the presence of red pigmented globules was not described nor can they be observed in the micrographs. The possibility that the constitutive or facultative presence of the globules is a strain-dependent trait within the species, or if the specimens reported here containing the red globules and those studied by Karnkowska-Ishikawa *et al.* (2013) are separate species, remains to be studied.



The process of carotenoid accumulation in photosynthetic tissues, called the carotenogenic response (CR), is considered a safe way to achieve a sustained increase in photoprotective capacity. Compared to primary carotenoids, secondary carotenoids are remarkably stable (Solovchenko & Neverov, 2017), thus providing a metabolically inexpensive and durable mechanism of photoprotection, particularly under conditions of prolonged stress. Several methods of Chl protection by red pigments have been proposed (Hughes, 2011), with optical masking of photodynamic chlorophyll being among the most probable. However, light shielding by red carotenoids implies an unavoidable reduction in light absorption by Chl, and consequently, photosynthetic efficiency. The drawback of such a strategy is that this mechanism is only reversible on a time scale of days or weeks (Solovchenko & Neverov, 2017), therefore being much less dynamic than the classical NPQ or antioxidant systems observed in most phototrophs, which typically activate or deactivate in minutes or even seconds (Ruban, 2016). This is probably why the light shielding mechanism is only observed under conditions of sustained stress (Solovchenko & Neverov, 2017). In the case of *E. sanguinea*, the process of light-induced lipid globule migration represents a solution to the trade-off between the accumulation of protective red pigments and sustained losses of photosynthetic efficiency. The rapid relocation of red grains to the periphery or the centre of the cell provides a fast adjustment of the level of light shielding in response to the photoprotective demand, without involving a slow and expensive carotenoid biosynthesis. This mechanism represents an adaptive advantage especially for a microalga that, when adopting a neustonic habit, occupies the water surface which is the most potentially light-stressed environment in a water-habitat. This mechanism allows *E. sanguinea* neustonic colonies to deal with daily changes in irradiance but also with

more rapid variations caused by cloudiness or shading by riparian vegetation. Although similar mechanisms of intracellular pigmented organelles migration have been studied in specialised colour changing cells (chromatophores or melanophores) in animals such as amphibians, reptiles or shrimps (Immerstrand *et al.*, 2007; Milograna *et al.*, 2016), to our knowledge in phototrophs, a similar process has been only described in *H. lacustris*, where these globules can perform light-induced peripheral migrations that reduce rapidly the amount of light reaching the chloroplasts (Peled *et al.*, 2012). In experiments with inhibitors Peled *et al.* (2012) showed that in *H. lacustris* globule migration is regulated by the reduction state of the electron transport chain. We have also shown that the reddening process in *E. sanguinea* correlates negatively with Fv/Fm (Fig. 11), suggesting the existence of a similar coordination with photosynthetic activity.

In view of the above-mentioned observations, it seems likely that the protective efficiency of Ast-globule migration may contribute to withstanding light stress, i.e. that it plays a relevant role in photoprotection. In fact, *Euglena sanguinea* seems to be deficient in some of the “traditional” photoprotection mechanisms. Photoprotection mechanisms generally operate at three levels: decreasing light absorption (avoidance), increasing the dissipation of absorbed energy and repairing the oxidative damage caused by the excess of light (Valladares *et al.*, 2012). In the case of *E. sanguinea*, unlike other *Euglena* species, light-avoidance movements are absent, and in fact this species only orientates by positive phototaxis (Gerber & Häder, 1994), even at high irradiance. We also show that *E. sanguinea* was defective in thermal energy dissipation mechanisms (Fig. 17). Thus, unlike other *Euglena* species where two protective xanthophyll cycles, the VAZ and the Ddx/Dtx, operate in response to illumination (Roach *et al.*, 2015), in *E. sanguinea* only the latter was in operation (Fig. 12). However, its activity did not

appear to be dependent on the regulation of thermal energy dissipation, the so-called “NPQ” (Fig. 17). Thus, in contrast to the general response observed in phototrophs where  $F_m$  (maximal fluorescence in the dark) is quenched upon illumination by the development of NPQ, the absence of changes in  $F_m'$  (maximal fluorescence in the light shown in Fig. 17) suggests the absence of a proton-gradient-related rapid component of NPQ (termed  $qE$ ) (Jahns & Holzwarth, 2012). In fact, the increase of  $F_m'$  in dark-adapted cells would suggest the rapid activation of a state transition process ( $qT$ ) with the migration of antennae from PSI to PSII (transition from state II to state I) (Roach & Na, 2017). In contrast to the absence of the afore-mentioned defences, the antioxidant potential is remarkably high in *E. sanguinea*, and as has been described in *E. gracilis* (Ruggeri *et al.*, 1985), *E. sanguinea* also possesses a remarkable set of tocopherols with antioxidant function. However, these tocopherol molecules are not necessarily located in the chloroplasts, as has been shown in a mutant of *E. gracilis* lacking plastids (Kusmic *et al.*, 1999), therefore its presence does not imply a direct photoprotective role.

Overall, the mechanism in the floating colonies of *E. sanguinea* is probably one of the fastest, if not the fastest, colour change observed in photosynthetic organisms. In fact, it can be even detected at landscape level on the eutrophic ponds where *E. sanguinea* grows, perhaps giving rise to some of the old myths of water becoming blood. Ast-globule migration seems to be so efficient in protective terms, that most other photoprotection mechanisms are inactive in *E. sanguinea*. These results together with previous evidence available in the literature highlight *Euglena* as an interesting model genus for the study of diverse physiological solutions to the changes in light.

## Acknowledgements

This work was supported by the Basque Government [UPV/EHU-GV IT-1018-16] [UPV/EHU PPG17/67 – GV IT-1040-16], and by the Spanish Ministry of Economy and Competitiveness (MINECO) and the European Research and Development Foundation (FEDER) through (i) [CTM2014-53902-C2-2-P] national grant and (ii) a “Juan de la Cierva-Incorporación” postdoctoral grant [IJCI-2014-22489] to BFM. We also thank to technical and human support provided by SGIker (UPV/EHU, MICINN, GV/EJ, ESF) and to Brian Webster for language revision.

## Author Contributions

### SUPPLEMENTARY INFORMATION

The following supplementary material is accessible via the Supplementary Content tab on the article’s online page at <http://xxx>

**Supplementary table 1.** Composition of the liquid culture medium employed to isolate and grow *Euglena sanguinea*.<sup>a</sup> water from Lertutxe reservoir.

**Supplementary table 2.** Summary of the ecophysiological processes observed in *Euglena sanguinea* in relation to rapid acclimation to light environment, within this work.

**Supplementary fig. 1.**

**Supplementary video 1.**

## References

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### Legend to figures

**Figs 1–8.** Colour shifts in *Euglena sanguinea* mats at different spatial scales. **Figs 1, 2.** Pond surface at the early morning or at noon (July 28<sup>th</sup>), respectively. **Fig. 3.** Detail of the surface on a cloudy day (November 4<sup>th</sup>). **Fig. 4.** The same place on the previous (sunny) day (November 3<sup>rd</sup>). **Figs 5, 6.** *E. sanguinea* culture (strain Eg07EHU) before and 15 min after exposure to direct sunlight, respectively. **Figs 7, 8.** Colour change induced by 10 minutes of illumination under the microscope light on palmelloid cells.

**Fig. 9.** Time course of changes in chromatic indexes red chromatic coordinate (rCC), green chromatic coordinate (gCC), normalized difference index (NDI) and excess green index (ExG) during 15 min of high light exposure from the illumination system of the microscope on *Euglena sanguinea* (see also Figs 7, 8).

**Fig. 10** Time course of changes in green chromatic coordinate (gCC) in red colonies during 20 minutes of dim light after transfer of *Euglena sanguinea* from full sunlight. Pictures above plot show representative images of a colony at different time points.

**Fig. 11.** Relationship between green chromatic coordinate (gCC) and Fv/Fm of individual cells of *Euglena sanguinea*. A representative example is shown on to the right of the plot.

**Fig. 12.** Upper panel: Overlaid chromatograms (at  $\lambda_{445 \text{ nm}}$ ) of floating *Euglena sanguinea* mats immediately after collection in the field (June 27<sup>th</sup>) (plate A, chromatogram black line) and after one hour in low light (plate B, chromatogram blue

line). Peak identification is based on comparison with standards, absorption spectra and retention times. Bottom panel: Changes in the epoxidation state of the Ddx cycle, total Chl content and total Ast (including free form, mono- and diesters) during the transition from red to green form. Letters above bars denote significant differences among colour stages at  $P < 0.05$  (ANOVA test with Duncan *post-hoc*).

**Figs13–16.** TEM images showing Fig. 13. The structure of a red *Euglena sanguinea* cell showing accumulation of lipid bodies (*lb*) in the periphery (note the polar distribution); Fig. 14. Detail of the lipid bodies of the same cell and their outer envelope (arrows); Fig. 15. The structure of a green cell with lipid bodies located in the centre; Fig. 16. Fine structure of a lipid body from the same cell. *c*: chloroplast; *pm*: paramylon granules; *pp*: periplast

**Fig.17.** Illumination induced changes of  $Fm'$  (expressed as normalized to  $Fm$  in darkness) in motile (strain Eg07EHU) and palmelloid forms (field sample) of *Euglena sanguinea*, when cells were light-acclimated (empty symbols) or dark-acclimated (filled symbols).













