Reference: *Biol. Bull.* **223:** 328–336. (December 2012)

Anesthetizing Solar-Powered Sea Slugs for Photobiological Studies

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Abstract. Photosynthetic sea slugs have the ability to "steal" chloroplasts (kleptoplasts) from marine macroalgae and keep them structurally intact and physiologically functional. The photosynthetic activity of these symbioses has been assessed using pulse amplitude modulated (PAM) fluorometry. However, the movement of these sacoglossan slugs can impair specific photobiological studies on kleptoplasts. Thus, immobilizing sacoglossan slugs while not interfering with the photosynthetic activity would be a methodological advance for research in this field. We evaluated the effect of two anesthetics, eugenol and MS-222, on the photosynthetic activity of kleptoplasts and on the behavior of the kleptoplasts-bearing slug *Elysia viridis*. Anesthetics promoted relaxation of sea slug muscle with no touch reaction in about 6 min. Sea slugs immobilized for 120 min completely recovered after anesthetic removal. No significant differences were found on photosynthetic parameters measured immediately (0–1 min) after immobilization. The effective quantum yield of photosystem II of *E. viridis* after 120 min of immobilization was significantly decreased by 12% in the MS-222 treatment, while eugenol promoted no significant effect. Photosynthetic activity assessed by rapid light-response curves (RLC) of relative electron transport rates (rETR) revealed a significant decrease in both initial response to light (–34%) and maximum rETR ($rETR_m$) (–60%), after 120 min of immobilization using MS-222. After 120 min of immobilization with eugenol, the initial response to light significantly decreased 15% and $rETR_m$ decreased 27%. We conclude that, whenever photobiological studies employing PAM fluorometry require immobilization of photosynthetic sea slugs, eugenol can be used as a powerful anesthetic with little impact on the photosynthetic activity of kleptoplasts.

Received 28 March 2012; accepted 22 October 2012.

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Introduction

A range of sacoglossan sea slugs from superfamily Plakobranchoidea have developed the capacity of acquiring phototrophic-mediated carbon. Rather than hosting endosymbiotic microalgae as do nudibranchs (Wägele and Johnsen, 2001), sacoglossans graze on macroalgae and sequester plastids into tubule cells of their digestive diverticula (Kawaguti and Yamasu, 1965), a mechanism often named kleptoplasty or kleptoplastidy (for review, see Johnson, 2011).

Chlorophyll (Chl) fluorescence is a rapid and nonintrusive method widely used to study photosynthesis (Baker and Oxborough, 2004), replacing to a certain extent the use of oxygen evolution or radiolabeled CO_2 fixation in the study of this process. The commercial availability of reasonably cheap, easy-to-use, and portable modulated fluorometers extended the use of Chl fluorescence analysis to a wide range of photosynthetic organisms, including photosynthetic sea slugs (*e.g.,* Evertsen *et al.,* 2007; Vieira *et al.,* 2009; Jesus *et al.,* 2010; Schmitt and Wägele, 2011). However, PAM fluorometry was developed for higher plants and not envisioned for studying motile organisms. To accurately address the photophysiology of kleptoplasts in these "solar-powered" organisms, it is important to maintain the target animal immobilized during measurements. Some authors have immobilized sea slugs by carefully placing the animal in the well of a concavity microscope slide filled

Table 1

Notation used in the text

Parameter	Definition
α	Initial slope parameter of the rETR *vs. E* curve
ΔF	Variable fluorescence ($=F_m' - F_s$) (dimensionless)
$\Delta F/F_m'$	Effective quantum yield of PSII (dimensionless)
E	Spectrally averaged ambient PAR (400-700 nm (μmol photons m^{-2} s^{-1})
rETR	Relative electron transport rate ($= E \times \Delta F/F_m'$) (dimensionless)
$rETR_m$	Maximum relative electron transport rate of the rETR *vs. E* curve (dimensionless)
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/F_m	Maximum quantum yield of PSII of a dark-adapted sample (dimensionless)
LC	Light curves: steady-state rETR *vs. E* curve
NPQ	Non-photochemical quenching of chlorophyll *a* fluorescence [$=(F_m-F_m')/F_m'$] (dimensionless)
RLC	Rapid light-response curves: rETR *vs. E* curve

with seawater and covered with a coverslip (Vieira *et al.,* 2009; Serôdio *et al.,* 2010). Nevertheless, *E. viridis* individuals are still able to move slightly within this limited space. Other authors have preferred to continuously adjust the animal to the fixed PAM fluorometer's optical fiber (Jesus *et al.,* 2010; Schmitt and Wägele, 2011) or place the animal in a small vial (Evertsen *et al.,* 2007).

While the methods described above can be satisfactory when a short saturating light pulse is applied to access, for instance, the maximum quantum yield of photosystem II (PSII) of a dark-adapted sample (F_v/F_m, see Table 1 for notation), the measurement of more complex PAM fluorometry parameters can be compromised by the animal movement. A good example of a photobiological parameter that can be biased by sea slug movement is the characterization of the kinetics of induction and relaxation of Chl *a* fluorescence. This type of measurement is commonly used to study the operation of photoprotective processes and the occurrence of photoinhibition in plants and algae (Niyogi, 1999; Müller *et al.,* 2001). The lowering of fluorescence yield as a result of photoprotective or photoinhibitory processes is quantified by the non-photochemical quenching (NPQ) of Chl *a* fluorescence based on the variation of maximum fluorescence from dark-adapted to light-adapted state (F_m and F_m' respectively; see Table 1 for notation) (Müller *et al.,* 2001). If sea slugs are not fully immobilized, even slight movements of the target animal will cause non-physiological changes in the steady-state fluorescence signal that, as a consequence, will compromise the relation between the maximal fluorescence of dark-adapted and light-adapted samples to be used, for instance, in NPQ calculations.

Steady-state light-response curves (LC) and/or rapid light-response curves (RLC) of relative electron transport rate (rETR, see Table 1 for notation) in photoacclimation studies can also be affected by sea slugs' movement. Both types of light-response curves are constructed by exposing the sample to increasing light steps for a certain period of time in each irradiance level and calculating rETR at each of those levels. rETR is calculated by multiplying the effective quantum yield of photosystem II (PSII) at a certain irradiance by that same given irradiance. However, light-dependent behavior in sea slugs has been reported (Giménez-Casalduero and Muniain, 2008; Jesus *et al.,* 2010; Schmitt and Wägele, 2011), and it is expected that at least some sea slugs will use their lateral body flaps (parapodia) to cover their dorsal surface as a protection from excessive light. Therefore, if the animal is able to move during fluorescence measurements, it will be able to regulate incident light by closing/opening the parapodia, introducing potential sources of error when calculating rETR ($= E \times \Delta F/F_m'$), especially at high light: (1) it is very likely that the effective quantum yield of PSII ($\Delta F/F_m'$) will be overestimated due to the animal's photoprotective behavior; and (2) the irradiance reaching the kleptoplasts will depend on the animal's behavior, with the consequence that the actual light reaching the kleptoplasts is variable and different from that used in the rETR calculation (E).

Given that specific parameters of PAM fluorometry can be significantly biased by animal movements, the use of anesthetics to immobilize *Elysia viridis* individuals was investigated. We hypothesize that a suitable anesthetic keeps sea slugs immobile, yet produces no significant effect on photosynthetic parameters measured using PAM fluorometry in comparison to control treatments (no anesthetic). Hypothermia has been a common method to diminish animal movement, sometimes with little or no regard for the animal's well-being. However, hypothermia would not be suitable in the study of sea slugs bearing kleptoplasts due to the susceptibility of PSII to photoinhibition at low temperature (Falk *et al.,* 1996, and references therein). Magnesium chloride has been used before in sacoglossa (Clark *et al.,* 1981), but it was found to interfere with photosynthetic functions of PSII (Liang *et al.,* 2009) and thus was not tested in the present study. MS-222 (tricaine methanesulphonate) has been for decades one of the most commonly used fish anesthetics (Rombough, 2007; Kiessling *et al.,* 2009), and eugenol, the major constituent of clove oil, has been introduced as an eco-friendly alternative to anesthetize fish (Palić *et al.,* 2006; Ghanawi *et al.,* 2011). For these reasons, the effects of 0.1 ml l^{-1} eugenol and 0.8 g l^{-1} MS-222 on the photosynthetic activity of kleptoplasts in *E. viridis* were tested.

Materials and Methods

Biological material

Adults of the sea slug *Elysia viridis* (Montagu, 1804) and its dietary prey, the macroalga *Codium tomentosum* Stackhouse, 1797, were collected on an intertidal rocky shore in the northwest of Portugal (Aguda beach, 41°02′52.29″N and 8°39′14.43″W). *E. viridis* individuals and *C. tomentosum* were maintained in recirculating seawater under low light (water surface incident light: 20 μmol photons m^{-2} s^{-1}) at 18 °C on a light/dark photoperiod of 14 h:10 h. *C. tomentosum* was replaced every 2 to 4 weeks.

Preliminary test: immobilization and survival of E. viridis *exposed to different concentrations of eugenol and MS-222*

Sea slugs' full length was measured in order to select an experimental size range between 9 and 12 mm (typical size for adult *E. viridis* collected at our sampling site). To select the ideal concentration of each anesthetic, a batch of concentrations from 0.025 to 0.1 ml l^{-1} eugenol (Sigma-Aldrich) and 0.04 to 0.8 g l^{-1} MS-222 (Tris, pH 8) (Sigma-Aldrich) was used to test (i) full immobilization *versus* time of exposure and (ii) post-exposure locomotion recovery. The selected concentrations, 0.1 ml l^{-1} eugenol and 0.8 g l^{-1} MS-222, were chosen based on (i) the rapid immobilization of *E. viridis* individuals in the range size of 9 to 12 mm observed at these concentrations and (ii) full post-exposure motility recovery in a short time. Recovery from anesthetic exposure was done by transferring the sea slugs to a new beaker containing clean seawater (0% anesthetic) and monitoring the time to full recovery. We considered that sea slugs had fully recovered when normal locomotion (*e.g.,* climbing the submerged walls of the beaker without falling) was observed.

Experimental setup

E. viridis adults measuring 9 to 12 mm in full length were selected and placed under low light (20 μmol photons m^{-2} s^{-1}) and room temperature (20 °C) for 30 min. These light and temperature conditions remained the same throughout the experiment. Six sea slugs per treatment were exposed to 0.1 ml l^{-1} eugenol, 0.8 g l^{-1} MS-222, or no anesthetic in 50 ml of seawater. The effective quantum yield of PSII, $\Delta F/F_m'$ (see Table 1 for notations), was measured (i) every 10 min during acclimation to low light and room temperature, (ii) immediately after immobilization or at a similar time for the control treatment, and (iii) during 120 min of the immobilization period. Rapid light-response curves (RLC, see description below and Table 1 for notation) were measured at the following time points: before exposure (BE), immediately (0–1 min) after immobilization (IAI), and 120 min after immobilization (120 AI). For consistency between the control and anesthetized sea slugs, all measurements shown in Figures 1 to 3 were made using a concave slide and a coverslip. More specifically, each sea slug was removed from the respective treatment, placed in the center of the well filled with seawater, and covered with a coverslip. The

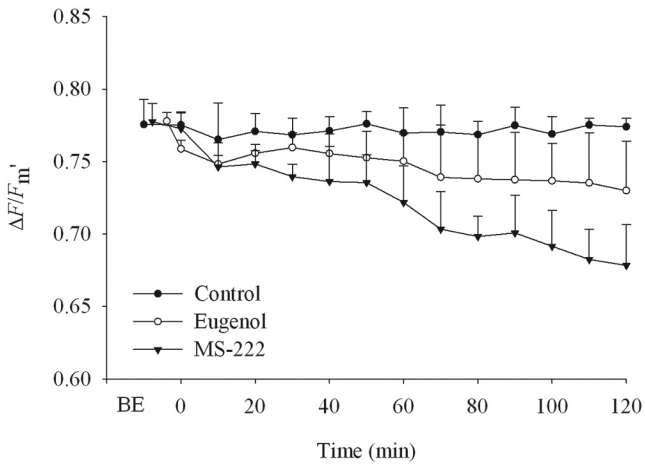

Figure 1. Effects of 0.8 g l^{-1} MS-222, 0.1 ml l^{-1} eugenol in seawater, and 0% anesthetic (control) on the effective quantum yield of PSII ($\Delta F/F_m'$) of kleptoplasts in *Elysia viridis*. Time before exposure (BE) corresponds to the last measurements taken before exposure to different treatments (10 min in control, 3.8 min in eugenol, and 7.7 min in MS-222 treatments); time 0 min corresponds to time immediately (0–1 min) after anesthetic immobilization; remaining time corresponds to exposure time after immobilization of the animal. Control: closed circles; eugenol: open squares; MS-222: closed triangles. Bars represent the standard deviation (n = 6 individuals).

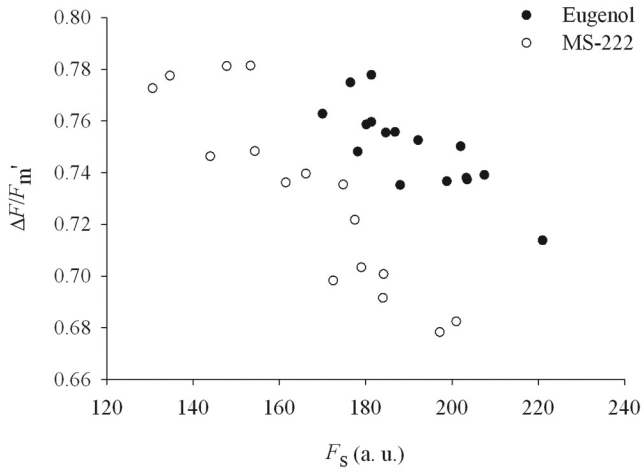

Figure 2. Effective quantum yield of PSII ($\Delta F/F_m'$) and respective minimal fluorescence of light-adapted kleptoplasts (F_s) in *Elysia viridis* exposed to 0.8 g l^{-1} MS-222 and 0.1 ml l^{-1} eugenol at different time points before exposure to different treatments and during 120 min after immobilization. Number of replicas (n): 6 individuals. Maximal standard deviations measured were ± 62 and ± 55 for F_s in eugenol and MS-222, respectively, and ± 0.04 and ± 0.03 for $\Delta F/F_m'$ in eugenol and MS-222, respectively.

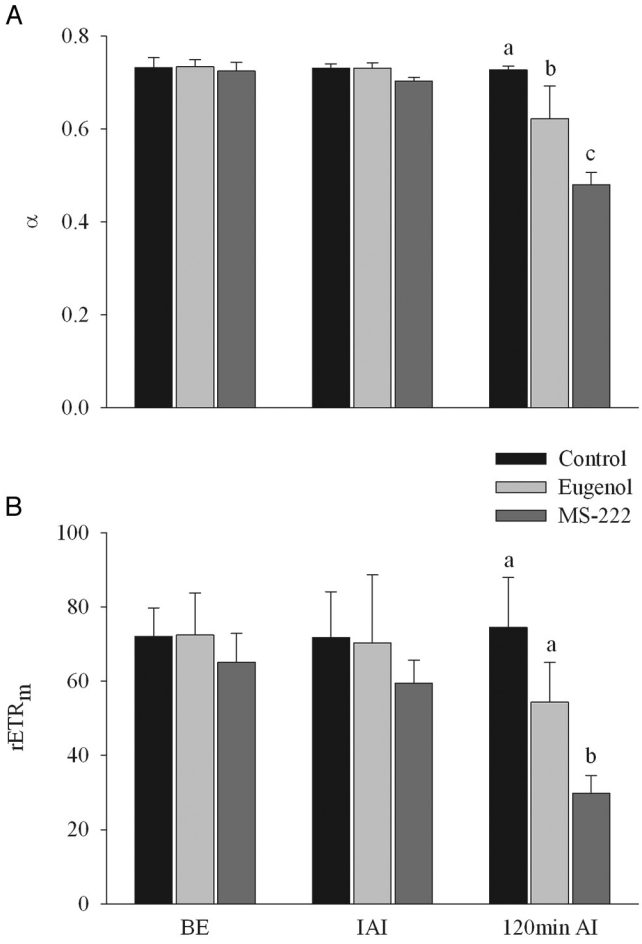

Figure 3. Effects of 0.8 g l^{-1} MS-222, 0.1 ml l^{-1} eugenol in seawater, and no anesthetic (control) on rapid light-response curve (rETR *vs. E* curves) parameters measured in kleptoplasts of *Elysia viridis*. The initial slope (α) (A) and the maximum relative electron transport rate ($rETR_m$) (B) were measured before exposure (BE), immediately after immobilization (IAI), and 120 min after immobilization (120 min AI). Bars represent the standard deviation (n = 6 individuals). Different letters (a, b, and c) indicate significant differences between measurements in that group (see text for details).

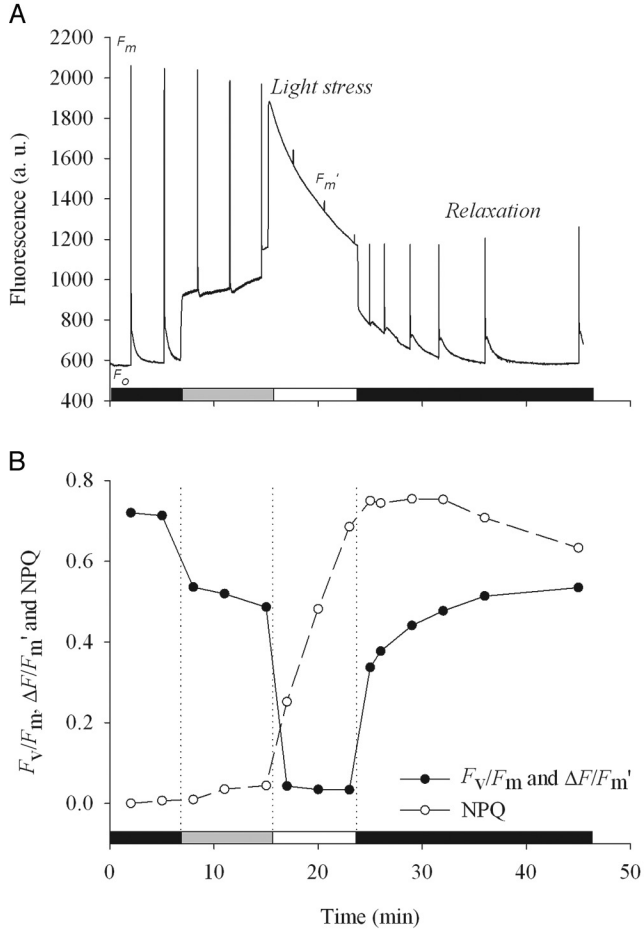

Figure 4. Chlorophyll *a* fluorescence trace from an immobilized *Elysia viridis* individual (A) and respective F_v/F_m, $\Delta F/F_m'$ and non-photochemical quenching (NPQ) (B) using 0.1 ml l^{-1} eugenol in seawater. In the presence of weak measuring light (1st dark bar, 2 data points) the minimal fluorescence of a dark-adapted sample is seen (F_o). When a saturating light pulse is given, the photosynthetic light reactions are saturated and fluorescence reaches a maximum level (F_m). Upon continuous illumination with low light (grey bar: 16 μmol photons m^{-2} s^{-1}, used for activation of light reactions before the light stress, 3 data points) followed by moderately excessive light (white bar: 619 μmol photons m^{-2} s^{-1}, light stress, 3 data points), a combination of non-photochemical processes (*e.g.,* NPQ) lowered the fluorescence yield. NPQ can be seen as the difference between F_m and the measured maximal fluorescence after a saturating light pulse during illumination (F_m'). After switching off the light (2nd dark bar: recovery, 6 data points), recovery of F_m' is expected to reflect relaxation of NPQ components.

same part of the sea slug that was exposed to low light between measurements (sideways with closed parapodia or flat body with open parapodia, depending on the position taken by the sea slug when immobilized) was used to face the optical fiber during the measurements.

For the continuous record of PAM fluorometry measurements shown in Figure 4, the sea slug was placed in a petri dish containing 25 ml of seawater with 0.1 ml l^{-1} eugenol (just enough to cover the sea slug). The optical fiber was placed 1 mm above the water surface and covering most of the sea slug body. Measurements were done continuously with no movement of either the sea slug or the optical fiber. This procedure was necessary to assure that the exact same spot was measured throughout the experiment as required for NPQ calculation (see Table 1 for notation). Since NPQ cannot be determined without full immobilization of the sea slug, this parameter was not measured on individuals trapped between the lamina and the coverslip. Therefore, NPQ was measured only in anesthetized sea slugs placed in a petri dish and 1 mm below the optical fiber.

Fluorescence measurements

Chl *a* fluorescence was measured using a pulse amplitude modulation (PAM) fluorometer (Schreiber *et al.,* 1986)

comprising a computer-operated PAM control unit (Walz, Effeltrich, Germany) and a WATER-EDF-Universal emitter-detector unit (Gademann Instruments GmbH, Würzburg, Germany). Measuring, actinic, and saturating light were provided by a blue LED-lamp (peaking at 450 nm, half-band width of 20 nm), and were emitted at a frequency of 18 Hz when measuring F_o (see Table 1 for notation) or 20 kHz when measuring other fluorescence parameters. The light delivered by the fluorometer and the fluorescence emitted by the sample were conducted by a 6-mm-diameter Fluid Light Guide fiber optics bundle in direct contact with the coverslip covering the sea slugs or at a 1-mm distance from the water surface.

Parameters of rapid light-response curves (RLC)

RLC were constructed by exposing the samples for 10 s to 12 increasing irradiance levels (E, from 12 to 920 μmol photon m^{-2} s^{-1}). For every irradiance level, the relative electron transport rate (rETR = $E \times \Delta F/F_m'$, see Table 1 for notations) was calculated and rETR *versus* E curves were constructed. The initial slope (α) and maximum rETR ($rETR_m$) of RLC were estimated by fitting the Eilers and Peeters (1988) model. The model was fitted iteratively using MS Solver (Microsoft Excel 2007), and the curve fit was very good, with $r > 0.99$ for a total of 54 light curves.

Statistical analyses

Differences in the times for immobilization and post-exposure recovery were tested using one-way analysis of variance (ANOVA). Effective quantum yield ($\Delta F/F_m'$), the initial slope (α) and the maximum rETR ($rETR_m$) of RLC were tested using two-way ANOVA for effects of anesthetic and exposure time. Multiple comparisons among pairs of means were performed using Tukey's HSD. All statistical analyses were performed using the software Statistica 10 (StatSoft Inc., USA).

Results

Immobilization and survival of Elysia viridis *exposed to anesthetics*

There were no significant differences in time for immobilization ($P = 0.063$) and time for post-exposure recovery ($P = 0.542$) between sea slugs anesthetized with 0.1 ml l^{-1} eugenol or 0.8 g l^{-1} MS-222. Anesthetics promoted sea slug muscle relaxation with no touch reaction (here considered the time of immobilization) in 3.8 ± 1.7 and 7.7 ± 4.1 min (average ± SD, $n = 6$ individuals), in eugenol and MS-222, respectively. After 120 min of exposure to the treatments, all sea slugs fully recovered locomotion after 20.0 ± 12.9 and 15.8 ± 9.7 min (average ± SD, $n = 6$ individuals) of post-exposure to eugenol and MS-222, respectively.

Effective quantum yield of PSII ($\Delta F/F_m'$)

After an acclimation to low light (20 μmol photons m^{-2} s^{-1}) and room temperature (20 °C) for 30 min before the experimental treatments, *Elysia viridis* adults exhibited a value of $\Delta F/F_m'$ before exposure (BE) of 0.77 ± 0.02, 0.78 ± 0.01, and 0.77 ± 0.02 (average ± SD, $n = 6$ individuals) for control, eugenol, and MS-222, respectively. Individuals exposed to seawater alone (control treatment) showed no significant difference ($P = 1.000$) in the measured effective quantum yield of PSII ($\Delta F/F_m'$) throughout the duration of experiments (Fig. 1). Also, no significant differences ($P = 1.000$ for control, eugenol, and MS-222 treatments) were found between treatments BE and immediately after immobilization (IAI). At 120 min of immobilization, $\Delta F/F_m'$ had decreased in anesthetized sea slugs to 0.73 ± 0.03 and 0.68 ± 0.03 (average ± SD, $n = 6$ individuals) for eugenol and MS-222, respectively. This decrease was gradual and, at each time of exposure, no significant differences in $\Delta F/F_m'$ were found between control and eugenol treatments ($P = 0.075$ at time 120 min of immobilization; P between 0.175 and 1.000 for any other times). The same result was found for sea slugs immobilized with MS-222 in the first 50 min after immobilization time ($P = 1.000$ at times BE, IAI, and 10 and 20 min after immobilization; $P = 0.894$, 0.629, and 0.255 at times 30, 40, and 50 min after immobilization, respectively). However, at 60 min after immobilization time, a significant difference ($P = 0.023$) in $\Delta F/F_m'$ was found between control and MS-222 treatments. Between 60 and 120 min of immobilization, all differences between control and MS-222 treatments were also significant ($P < 0.001$ for all comparisons). The decrease in $\Delta F/F_m'$ at 120 min of immobilization was 5.4% and 12.1% in eugenol and MS-222 treatments, respectively.

The minimal and maximal fluorescence of light-adapted individuals (F_s and F_m', respectively) used to calculate $\Delta F/F_m'$ (Fig. 1) were plotted against time and showed that F_m' remained reasonably stable (data not shown) while F_s was the main factor decreasing $\Delta F/F_m'$ during exposure time. The latter correlation is represented in Figure 2, where the averaged $\Delta F/F_m'$ values are plotted against the corresponding averaged F_s at each stage of the experiment: (i) before immobilization (3 points: one time point every 10 min between 0 and 30 min of acclimation to low light and room temperature before exposure to anesthetic treatments) and (ii) after immobilization (13 points: one time point every 10 min between 0 and 120 min of the immobilization period). Figure 2 shows that the $\Delta F/F_m'$ decrease was related to the increase in F_s in both eugenol and MS-222 immobilization. In both eugenol and MS-222, F_m' was reasonably stable during the whole period of animal immobilization. In the control treatment both F_s and F_m' were stable throughout the experiment.

Parameters of rapid light-response curves (RLC)

Individuals exposed to seawater alone (control treatment) showed no significant difference ($P = 1.000$) in the RLC parameters estimated ($rETR_m$ and α) throughout the duration of experiments (Fig. 3). When comparing different treatments at times BE and IAI, no significant differences were found in either photosynthetic parameter α ($P = 1.000$ for eugenol treatment; $P = 1.000$ and $P = 0.747$ for times BE and IAI in MS-222, respectively) or rETR ($P = 1.000$ for eugenol treatment; $P = 0.970$ and $P = 0.594$ for times BE and IAI in MS-222, respectively) (Fig. 3).

After 120 min of immobilization, both anesthetics showed a significant effect on the photosynthetic parameter α ($P < 0.001$) with a decrease of 15% in eugenol and 34% in MS-222, relative to control values (Fig. 3A). Regarding $rETR_m$, sea slugs anesthetized for 120 min with eugenol showed a decrease of 27% when compared to control sea slugs (Fig. 3B). This decrease in rETRm was not significant ($P = 0.062$). In contrast, sea slugs anesthetized with MS-222 displayed a significant decrease ($P < 0.001$) in $rETR_m$ values after 120 min of immobilization (59.9%) when compared to control specimens (Fig. 3B).

Example of anesthetic application

Contrary to the maximal and effective PSII quantum yield (F_v/F_m and $\Delta F/F_m'$ respectively), the quantification of non-photochemical quenching (NPQ) requires parameters measured at different stages of an experiment (F_m and F_m', maximal fluorescence emitted by a dark-adapted or light-adapted sample). In this way, NPQ cannot be accurately calculated if the movement of the animal induces a non-physiological change in the minimal fluorescence and, consequently, in the maximal fluorescence. This was often the case in measurements made on non-immobilized specimens of *E. viridis*. As an example of a measurement made possible due to sea slug immobilization, we show a light-stress experiment (Fig. 4) in which it was possible to accurately calculate NPQ in addition to F_v/F_m and $\Delta F/F_m'$ (see Table 1 for notation) at different stages of the experiment: (i) dark-adapted stage; (ii) low-light period for induction of light reaction; (iii) high-light period to induce a light stress; and finally (iv), the recovery when the light stress is over and the sample is transferred to dark condition. For calculation of F_v/F_m and $\Delta F/F_m'$, keeping the sea slug in the exact same position would not be crucial since those parameters derive from a ratio between absolute values. However, the light conditions that reach the sea slug would be different from those desired if the sea slug could hide or change parapodia position. For NPQ calculations, the exact position of the sea slug must be assured to guarantee that ground and maximal fluorescence are comparable at any time of the experiment. Figure 4A shows that, when *E. viridis* individuals are immobilized (with eugenol), Chl *a* fluorescence can be recorded continuously in response to different light treatments, and that any changes in fluorescence result from changes in the physiological state of the kleptoplasts rather than from changes in the position of the sea slug. The photosynthetic parameters F_v/F_m, $\Delta F/F_m'$, and NPQ calculated from the given example of a continuous Chl *a* fluorescence record (Fig. 4A) are presented in Figure 4B. NPQ was zero in dark and low-light conditions and increased to 0.69 in 9 min of exposure to high light (619 μmol photons m^{-2} s^{-1}). After 24 min of relaxation in dark conditions, NPQ decreased by 7.6%, with no decrease observed for the first 14 min. F_v/F_m of the dark-adapted individual was 0.71 and decreased to nearly zero $\Delta F/F_m'$ in 2 min of exposure to high light (619 μmol photons m^{-2} s^{-1}). In the following 24 min of relaxation in dark conditions, F_v/F_m increased to 47.2% and 75.0% of the initial value after 2 and 24 min of post-light-stress, respectively.

Discussion

Choice of anesthetic and respective concentration

Eugenol generally induced anesthesia faster at lower concentrations than MS-222, as shown before for fish (Munday and Wilson, 1997; Keene *et al.,* 1998). In this study we measured similar anesthesia times using 0.1 ml l^{-1} eugenol and 0.8 g l^{-1} MS-222, with a very high variation in sea slugs anesthetized with MS-222. Due to the wide use of eugenol in coral reef ecology, the effects of this anesthetic have been tested on coral health and growth (Frisch *et al.,* 2007; Boyer *et al.,* 2009). Our results were in accordance with those studies, showing that low doses of eugenol had little or no effect on the photosynthetic efficiency of *Elysia viridis*. In the coral *Pocillopora damicornis*, low concentrations of eugenol (0.05 ml l^{-1}) had no effect on color or photosynthetic efficiency, irrespective of exposure time (1–60 min) (Frisch *et al.,* 2007). Higher concentrations (0.5 ml l^{-1}) had variable effects, with 10 min of exposure resulting in bleaching and reduced photosynthetic efficiency, and longer exposure or higher concentrations causing total mortality (Frisch *et al.,* 2007). Boyer and coworkers (2009) found similar results in three other species of corals (*Acropora striata*, *Pacillopora verrucosa,* and *Porites australiensis*), with growth and occurrence of bleaching in 0.7 ml l^{-1} of eugenol in seawater not differing significantly from the control treatment (seawater alone). Higher concentrations (1.4 and 2.8 ml l^{-1}) of eugenol in seawater, as well as the optional dilution of eugenol in ethanol, showed deleterious effects on both coral growth and occurrence of bleaching (Boyer *et al.,* 2009).

Experimental setup and the problematic of a "real" control treatment

Ideally, the control treatment would allow the comparison of photosynthetic parameters between animals exposed

to anesthetics and animals in seawater alone, leaving aside the photobehavior effect so that only the effect of the substances could be tested. The experimental design using a concave slide and a coverslip (Vieira *et al.,* 2009) seemed the best option when trying to immobilize the animals without an anesthetic. Nevertheless, most *E. viridis* individuals would still move within that limited space during measurements of $\Delta F/F_m'$ and RLC construction. Moreover, they were allowed to move their parapodia while exposed to light (20 μmol photons m^{-2} s^{-1}) during the experiments. On the other hand, anesthetized sea slugs always kept the same position (sideways with closed parapodia or flat body with open parapodia, depending on the position taken by the sea slug when immobilized) when continuously exposed to the same low light in between measurements. Thus, the problem of comparing control (reduced motility) and immobilized animals may exist for some parameters, particularly in RLC measurements, and so a cautious approach is recommended.

For the same reasons mentioned above, it would not be possible to perform true steady-state LC in the control treatments due to closing/opening of parapodia or turning of the body of tested individuals during measurements. Since differences in the information given by RLC and LC (Cruz and Serôdio, 2008) were not relevant for the present study, RLC are the best option when comparing electron transport rates between motile and anesthetized *E. viridis* individuals. The use of an anesthetic may allow the construction of LC but, considering the present results, LC must be constructed immediately after immobilization (IAI) and within the shortest period of time. The effects of prolonged immobilization in rETR are further discussed below.

Observations of animal movement during RLC construction indicate that *E. viridis* individuals tried to escape the concave slide trap more actively at higher irradiances, possibly to avoid excessive light, reinforcing the hypothesis that animal behavior could be of photoprotective value (Doonan and Gooday, 1982; Giménez-Casalduero and Muniain, 2008; Jesus *et al.,* 2010; Schmitt and Wägele, 2011).

Effect of eugenol and MS-222 on $\Delta F/F_m'$ *and RLC parameters*

It is important to note that the arrangement of pericardial veins along the parapodia in sacoglossans probably functions as a "negative gill" for uptake of CO_2 and release of O_2 (Clark *et al.,* 1981). These apparent adaptations for gas exchange suggest that CO_2 transport could limit symbiotic photosynthesis; and considering that the lack of movement could reduce carbon availability, it might account for the decrease in the photosynthetic parameters measured in prolonged immobilization of *E. viridis* individuals. However, more data would be necessary to confirm this speculative hypothesis. For instance, uptake of radiolabeled $^{14}CO_2$ in motile *versus* immobilized sea slugs or enhancement of CO_2 concentration in the seawater solution could be used to address the hypothesis that photosynthesis is limited by CO_2 transport, which in turn is influenced by movement of the sea slug and/or carbon utilization.

The absence of photobehavior in immobilized individuals could also account for the observed decrease in photosynthetic parameters. *E. viridis* individuals and respective macroalgae prey, *Codium tomentosum*, were photoacclimated to low-light conditions in the laboratory. Therefore, saturating light pulses and, more importantly, the exposure to excessive light during construction of RLC could be enough to induce a decrease in $\Delta F/F_m'$, α, and $rETR_m$. Although the trapping method with concave slide and coverslip reduces motility in control specimens, they can still move and close parapodia. The slight movement inside the trap could be enough to reduce kleptoplast exposure, or at least alternate which kleptoplasts are exposed to excessive light. It was expected that any effect of exposure to excessive light during RLC construction and saturating light pulses would be dissipated in the measurement interval. However, as the example of a light-stress experiment indicates (Fig. 4), kleptoplasts do not rapidly recover from light-stress photodamage. Therefore, the accumulation of saturating light pulses and RLC construction could account for some of the decrease in the photosynthetic parameters.

RLC can cause some photodamage to kleptoplasts; however, this was not the main factor accounting for a decrease in photosynthetic efficiency. When F_s and F_m' used to calculate $\Delta F/F_m'$ (Fig. 1) were plotted against time (data not shown), it was evident that F_m' remained reasonably stable and that F_s was the main factor decreasing $\Delta F/F_m'$ during exposure time (Fig. 2). If photodamage were the main cause for a reduction in photosynthesis efficiency, this should be seen as a decrease in F_m' relatively to F_m. Since F_s was the main factor responsible for lowering photosynthetic efficiency (Fig. 2), it may be speculated that the reduction of the first electron acceptors in the electron transport chain, and therefore a lower capacity for photochemistry, not photodamage, is the main factor reducing $\Delta F/F_m'$.

$\Delta F/F_m'$ provides an estimate of the effective quantum efficiency of PSII photochemistry in the light-adapted state. The effective rate constant for photochemistry is proportional to the fraction of open PSII reaction centers (Baker and Oxborough, 2004). Therefore, under most non-stress conditions, the effective rate constant for photochemistry is defined by the effective rate constant for Q_A oxidation (Q_A being the first electron acceptor quinone on the photosynthetic electron transfer chain), which in turn is highly dependent on the rate at which carbon assimilation is able to utilize the NADPH and ATP that are produced as the result of photosynthetic electron transfer (Baker and Oxborough, 2004). Since experiments were carried on in individuals adapted to low light, the ability of processes downstream of

PSII to utilize the products of electron transport should be active and, therefore, playing a minor role in defining the PSII operating efficiency. While this seems to hold true for control individuals and for measurements made immediately after immobilization, a gradual decrease in the photosynthetic efficiency (Fig. 1), which is mainly explained by an increase in F_s (Fig. 2), was seen in prolonged immobilization of *E. viridis* individuals. Since RLC depends on rETR values, which in turn depend on $\Delta F/F_m'$ (rETR = $\Delta F/F_m' \times E$), a decrease in the latter will be amplified when multiplied by the irradiance at each light step of the RLC. Consequently, the decrease in RLC parameters at 120 min of immobilization can be explained with the same argument used to explain the gradual decrease observed in $\Delta F/F_m'$: a decrease in carbon availability or other factors limiting the use of products of electron transport induced an accumulation of reduced quinone species, resulting in F_s decrease and lowering of $\Delta F/F_m'$.

Final remarks

In conclusion, eugenol and MS-222 promoted the same muscle relaxation needed for PAM fluorometry measurements in *Elysia viridis*, and no mortality was observed for the concentrations and exposure times tested in the present work. Eugenol showed less effects on the photosynthetic efficiency and appears to be the best eco-friendly option available in the anesthetics market. Therefore, whenever photobiological studies employing PAM fluorometry require immobilization of "solar-powered" sea slugs, we recommend the use of low doses of eugenol. It is important to remember that long immobilization periods (*e.g.,* >120 min) should be avoided, as they can bias experimental results by negatively affecting the photosynthetic parameters of kleptoplasts retained by sacoglossan slugs. Researchers employing this new methodology, particularly when studying different sea slug species, are advised to run preliminary trials to confirm the suitability of the anesthetic product, as well as its dosage.

Acknowledgments

The authors thank Rui Rocha for setting up the recirculating seawater systems for maintenance of *E. viridis* and *C. tomentosum* in the laboratory, and Cláudio Brandão for assisting in the collection of biological material. The first authors, Sónia Cruz and Gisela Dionísio, were supported by Fundação para a Ciência e a Tecnologia (FCT, Portugal) with postdoctoral [SFRH/BPD/74531/2010] and PhD [SFRH/BD/73205/2010] grants, respectively. Finally, the authors are grateful to anonymous reviewers for helpful criticism on the manuscript.

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