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CHARACTERISATION OF ESTUARINE INTERTIDAL MACROALGAE BY LASER-INDUCED FLUORESCENCE

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ABSTRACT

The article reports the application of laser-induced fluorescence (LIF) for the assessment of macroalgae communities of estuarine intertidal areas. The method was applied for the characterisation of fifteen intertidal macroalgae species of the Tagus estuary, Portugal, and adjacent coastal area. Three bands characterised the LIF spectra of red macroalgae with emission maxima in the ranges 577-583 nm, 621-642 nm and 705-731 nm. Green and brown macroalgae showed one emission maximum in the red region (687-690 nm) and/or one in the far-red region (726-732 nm). Characteristics of LIF emission spectra were determined by differences in the main fluorescing pigments: phycoerythrin, phycocyanin and chlorophyll a (Chl a). In the green and brown macroalgae groups, the relative significance of the two emission maxima seems to be related to the thickness of the photosynthetic layer. In thick macroalgae, like Codium tomentosum or Fucus vesiculosus, the contribution of the far-red emission fluorescence peak was more significant, most probably due to re-absorption of the emitted red Chl a fluorescence within the dense photosynthetic layer. Similarly, an increase in the number of layers of the thin-blade green macroalgae Ulva rigida caused a shift to longer wavelengths of the red emission maximum and the development of a fluorescence peak at the far-red region. Water loss from Ulva’s algal tissue also led to a decrease in the red/far-red Chl fluorescence ratio (F<sub>685</sub>/F<sub>735</sub>), indicating an increase in the density of chloroplasts in the shrinking macroalgal tissue during low tide exposure.

**Keywords:** fluorescence; LIF; macroalgae; close-range sensing; water stress
1. INTRODUCTION

Macroalgae (seaweeds) communities are some of the most productive and widespread marine plant communities in the world (Dawes, 1998). In estuarine intertidal environments, macroalgae grow in an exceptionally dynamic environment experiencing stressful environmental conditions with respect to nutrient availability, irradiance, salinity or temperature (Davison and Pearson, 1996). Furthermore, macroalgae of the upper intertidal zone are subjected to frequent and prolonged emersion periods and most species tolerate extreme water loss. Remote sensing techniques are extremely important tools to assay macroalgal community structure due to the large spatio-temporal variability of these communities and the hardly accessible conditions of the estuarine intertidal habitat.

Laser-induced fluorescence (LIF) has been extensively used to infer taxonomic structure of phytoplankton communities (e.g. Babichenko et al., 1993; Barbini et al., 1998), to study changes in intertidal microphytobenthos biomass (Vieira et al., 2011), and to assess different stresses in higher plants (e.g. Subhash and Mohanann, 1997; Lavrov et al., 2012). Environmental monitoring related to the presence of oil slicks and chemical pollutants has also been addressed successfully using LIF technology (Utkin et al., 2002). However, the application of LIF techniques to assay macroalgae communities is scarce and, to the best of our knowledge, only two studies used fluorescence spectral signatures to discriminate between macroalgae groups (Topinka et al., 1990; Kieleck et al., 2001). Topinka et al. (1990) measured fluorescence emission at 685 nm and used fluorescence excitation ratios to distinguish between green, brown and red macroalgae. Kieleck et al. (2001) described a LIF imaging system using two excitation wavelengths to identify various macroalgae groups in the subtidal environment.
The main objective of this work was to evaluate emission fluorescence spectra obtained with a 532 nm pulsed Nd:YAG laser for the three main groups of macroalgae (red, green and brown macroalgae) present in the intertidal areas of the Tagus estuary, Portugal, and adjacent coastal zone. The obtained fluorescence data were related to the different photosynthetic antennae complexes (chlorophyll vs phycobiliproteins) and to the structure of the photosynthetic tissue of the different macroalgae. Furthermore, we investigated the effect of water loss occurring during low tide exposure in the LIF spectral signature of the macroalga Ulva rigida. To our knowledge this is the first application of LIF to the study of intertidal estuarine macroalgae.
2. MATERIAL AND METHODS

2.1. Sampling

Fifteen macroalgae species (see Table 1) were collected at the Tagus Estuary and adjacent coastal area on several occasions between December 2012 and April 2013 (Fig. 1). The Tagus estuary is located on the west coast of Portugal (38.75°N, 09.15°W) and has a broad shallow bay (10 m mean depth) covering an area of about 320 km². The estuary has extensive intertidal flats covering an area of approximately 100 Km² (Brotas and Catarino, 1995), ranging from very fine muddy sediment to sand and oyster banks. Three sites in the Tagus Estuary were sampled: Alcochete, Banco do Cavalo and Ponta do Destrói. Alcochete sampling site is located at a small fishing harbour characterised by a muddy substrate and the latter two sites are oyster banks. Rocky and sandy shores characterise Tagus estuary adjacent costal area. Two adjacent coastal area sites were sampled: Cabo Raso and Avencas. The first is a rocky zone, while the latter is a sandy beach with intertidal rock pools. Sampling was always carried out during low tide periods, and macroalgae species were collected at different sediment and substrata types. After sampling, all collected species were taken immediately to the laboratory, stored at in situ temperature, and frequently hydrated with collected water from each site. All experiments and measurements were carried out on the sampling day or the following day. Laser-induced fluorescence (LIF) spectra were determined for all macroalgae species in healthy and non-reproductive material. Each macroalgae LIF spectral signature was obtained from triplicate measurements in three different individuals per species.

2.2. Laser-induced fluorescence (LIF)

The LIF detector was developed on the basis of a commercial palm-size spectrometer Ocean Optics USB4000 and a frequency-doubled Nd:YAG laser, manufactured by
Quantel (model Ultra 532 30 20 H N). The laser provides 30 mJ, 7 ns radiation pulses at 532 nm, with a pulse repetition rate up to 20 Hz and spot diameter at the sample location of ~3 mm (about 1 m from the laser output aperture). The pulse energy is sufficiently low to prevent disturbing effects that could result from reaction centre closure and excitation annihilation (Rosema et al., 1998). A part of the fluorescence emission, reflected back to the instrument, is collected by a light gathering optical train based on low-cost components provided by Thorlabs, assembled within the Ø1-in lens mounting tube SM1L30.

The train comprises an optical filter and a telescopic system, positioned and centred using three retaining rings SM1RR. The long pass optical filter FEL0550, with the cut off wavelength of 550 nm and transmission of ~80% in the region of 650-730 nm, protects the spectrometer from strong retroreflected laser light. The SMA fiber optics collimation package F810SMA 635 is installed immediately after the filter. Usually being intended for collimating a laser beam propagating from the tip of an SMA-connectorized fiber, here it operates in the reciprocal mode, playing a role of a principal light gathering element, which collects the fluorescence radiation over a Ø21 mm input pupil and transmits it into an optical fiber. This fiber transports the optical signal to the spectrometer optical bench, the USB4000-f/4 asymmetrical crossed Czerny Turner configuration with the diffraction grating Ocean Optics #9, providing nearly uniform efficiency at wavelengths from 450 to 800 nm.

The spectrometer was tuned and calibrated with the help of the mercury argon calibration source CAL 2000, demonstrating the sensitivity of ~55 photons per count, and resolution of 0.19 nm per channel in the spectral range of interest (650 to 730 nm). Spectrometer synchronization with the laser pulse enabled the signal to be measured...
with minimum permissible exposure, which reduces the influence of the background radiation, allowing carrying out measurements in the daylight conditions.

2.3. Effects of the thickness of the photosynthetic layer on macroalgae laser-induced fluorescence spectra

The green macroalgae *Ulva rigida* morphology consists of a very thin single sheet-like blade that can be flat or ruffled. *U. rigida* LIF spectra were measured in single sheet or in folded tissue comprising two or four layers.

2.4. Effects of water loss on macroalgae laser-induced fluorescence spectra

*U. rigida* was used to examine the stability of the fluorescence spectra under varying stages of water loss, similar to that occurring during low tide exposure. The *U. rigida* samples were placed at room temperature (20 °C) with no addition of water. During the drying period (total of 3 hours), fresh weight was measured using an analytical balance (Mettler Toledo MS204S/01) with a 0.1 mg precision. Fluorescence spectral analysis was performed every 60 min until macroalgae were desiccated. The percentage of fresh weight water loss at $t_i$ was calculated as the $(FW_i \times 100 \%) / TW$ where $TW$ and $FW_i$ are the sample fully turgid weight (after floating then in water until saturation) and its fresh weight at $t = t_i$, respectively.
3. RESULTS

Analysed algal species showed characteristic fluorescence emission maxima. Chlorophyta and Phaeophyta macroalgae species (green and brown algae, respectively) revealed similar laser-induced fluorescence spectra, with one or two fluorescence peak maxima (Figs. 2a and 2b). A red region maximum was observed between 687 and 690 nm and a far-red region maximum between 726 and 732 nm. The Ulva species (*U. rigida* and *U. intestinalis*), the green algae *Bryopsis plumosa* and the brown algae *Colpomenia peregrina* revealed a similar fluorescence emission with a maximum between 687 and 690 nm. In addition, the last two species showed a second lower peak at the far-red region (731-732 nm). The LIF spectra of *Codium tomentosum* and *Fucus vesiculosus* showed similar fluorescence spectra with a maximum at 726 nm.

Rhodophyta macroalgae species (red algae) showed typical laser-induced fluorescence spectra characterised by three peaks (Fig. 2c). The first peak was found between 577 and 583 nm, the second peak in the range 621-642 nm, and the third peak from 705 to 731 nm. Table 1 summarizes the typical wavelength emission maxima of the LIF spectra recorded for each studied macroalgae species.

Figure 3 illustrates the influence of the number of *U. rigida* sheet-like blades on the fluorescence emission spectra. Significant change in the LIF emission spectrum was observed with the increase in the number of sheet-like blades analysed. The increase in the thickness of the photosynthetic layer led to an augment of the peak area, a shift to longer wavelengths of the red emission maximum and the development of a fluorescence peak at the far-red region (Fig. 3).

Effects of water loss on *U. rigida* LIF spectra are depicted in Figures 4 and 5. Fluorescence spectrum analysis during water loss revealed a notable shift of the fluorescence peak maxima at the red and far-red regions. A shift from a red emission to
a far-red emission maximum was persistently observed during water loss, reaching 732 nm under extreme desiccation (Fig. 4). The red/far-red fluorescence ratio ($F_{685}/F_{735}$) noticeably decreased during the drying process. Fresh weight loss around 50% caused a significant change in $F_{685}/F_{735}$ (from 9.4 to 1.2), and subsequent ~75% fresh weight loss led to further decrease of $F_{685}/F_{735}$ down to ca. 0.5 (Fig. 5).
4. DISCUSSION

LIF spectra of green and brown macroalgae usually possessed a dominant fluorescence peak in the red region (maximum at ca. 688 nm) and a shoulder at the far-red region (maximum at ca. 730 nm) attributable to Chl $a$, as the accessory pigments (other chlorophylls and carotenoids) do not emit their own fluorescence. It is commonly established that \textit{in vivo} Chl $a$ fluorescence at room temperature consists of two main emission bands, one in the red ($\lambda_{\text{max}} \sim 685$-690 nm) and the other at the far-red spectral region ($\lambda_{\text{max}} \sim 730$-740 nm) (Buschmann, 2007). Most of this fluorescence (approx. 90%) originates from PSII reaction centers (Govindjee, 1995), in which the red fluorescence band arises from the main electronic transitions and the far-red band arises from vibrational energy sublevels whose relative intensities increase through self-absorption in the shorter wavelength region (Franck et al., 2002).

Contrary to the other macroalgae species of the green and brown groups, \textit{Codium tomentosum} and \textit{Fucus vesiculosus} had a dominant fluorescence emission peak at the far-red region (726 nm), while the red emission fluorescence was very low. These two macroalgae species present a much thicker photosynthetic layer when compared to the other studied macroalgae. Green excitation light is not readily absorbed, penetrating deep in the macroalgal tissue. Therefore, Chl $a$ fluorescence is emitted deep within the tissue and on its path to the surface is strongly re-absorbed. Since the red Chl $a$ fluorescence is more strongly affected by re-absorption than the long-wavelength maximum in the far-red region, the relative magnitude of the latter peak increases. It has been previously described that increased re-absorption of red Chl $a$ fluorescence with increasing Chl $a$ content in plant leaves leads to the development of the long-wavelength Chl $a$ fluorescence shoulder and the establishment of a new fluorescence maximum around 735 nm (Gitelson et al., 1998; Franck et al., 2002;
Buschmann, 2007). Similarly, an increase in the number of layers of the thin-blade green macroalgae *Ulva rigida* caused a shift to longer wavelengths of the red emission maximum and the development of a fluorescence peak at the far-red region.

Discrimination between green and brown macroalgae using a monochromatic radiation (532 nm), even invoking complex analysis of the complete spectra, was not possible due to the fact that the detected LIF spectra (i) are of the same generic type for these two groups, (ii) demonstrate evident variation for different species of the same group, and (iii) vary in a noticeable way within the same species for specimens under specific conditions (e.g. water stress). Seemingly, reliable distinction between Chlorophyta and Phaeophyta requires the application of at least two wavelengths of the excitation radiation, one to secure the direct excitation of Chl *a* and the other its excitation through the accessory pigments (chlorophyll *b* and *c*, carotenoids), as reported by Topinka et al. (1990) and Kieleck et al. (2001). The red macroalgae species showed a similar LIF spectra fingerprint, which was significantly different from those observed for green and brown macroalgae. Contrary to the latter macroalgae groups, the red macroalgae possess phycobiliproteins as primarily PSII photosynthetic antennae complexes (Grossman et al., 1993). The presence of highly fluorescent phycobilin pigments (phycoerythrin and phycocyanins) dictates their distinctive emission spectra characteristics: i) an emission maximum at ca. 580 nm corresponding to phycoerythrin; and ii) an emission band in the range 621-642 nm corresponding to phycocyanins. Similar fluorescence emission characteristics have been described for several red macroalgae (Kieleck et al., 2001) and cyanobacteria (Ikeya et al., 1994; Babichenko et al., 1995; Barbini et al., 1998). Phycobiliproteins provide a powerful light-harvesting system in the region of the spectrum where Chls or carotenoids absorb poorly (Toole and Allnutt, 2003). Curiously, red macroalgae did not show the characteristic Chl *a*
fluorescence emission maximum at ca. 685 nm, presenting a peak at the far-red region between 705-731 nm. The absence of the 685 nm peak could be related to the reduced energy efficiency transfer of phycocyanin to Chl $a$ as previously observed for the blue-green algae *Anacystis nidulans* (Ghosh and Govindjee, 1966).

Under lower irradiance levels macroalgae have been shown to require greater amounts of light harvesting molecules to drive photosynthesis, whereas under higher light pigment levels are decreased to avoid excessive excitation and reactive oxygen species (ROS) formation (Sampath-Wiley et al., 2008). Daylength has been shown to affect total pigment content and the ratio of chlorophyll and phycobilins in the red macroalgae *Palmaria decipiens* (Lüder et al., 2001). Although fluorescence data were extremely consistent between different specimens of the same species, a seasonal effect on LIF spectra characteristics cannot be ruled out.

In higher plants, the red/far red Chl $a$ fluorescence ratio is primarily determined by the Chl content of leaves (Buschmann, 2007). Therefore, longer-term stresses that are able to decreased the cell tissue Chl $a$ content can be monitored by an increase of the $F_{685}/F_{735}$ ratio. This ratio has been used to infer the effect of longer-term stress events, e.g. nutrient stress in sunflower (45 days; Subhash and Mohanan, 1997) and water stress in cork oak and maritime pine (11 days; Lavrov et al., 2012). Contrary to the previous studies, the decrease in $F_{685}/F_{735}$ ratio was observed in response to water stress in the intertidal macroalgae *U. rigida*. This can be explained by the fact that shorter-term desiccation stress caused by exposure during a low tide period (~3 hours) is not sufficient to significantly affect the Chl $a$ content of the macroalgae. Rather, the main effect is the shrinking of the macroalgal tissue and the increase in the density of chloroplasts during desiccation. This causes an increased re-absorption of the red fluorescence, leading to a shift of the position of the fluorescence maximum towards
longer wavelengths and a decrease of the $F_{685}/F_{735}$ ratio. A closer grouping of chloroplasts caused by water loss in leaves of the moss *Rhizomnium punctatum* has been previously shown to increase re-absorption and decrease the $F_{685}/F_{735}$ ratio during desiccation (Bartošková et al., 1999). Response of macroalgae to water stress has been shown to vary significantly between high and low intertidal macroalgal species, depending on the capacity to attenuate ROS produced during desiccation (Flores-Molina et al., 2014).
5. CONCLUSIONS

This work shows that LIF spectra characteristics of intertidal macroalgae are related to the different organizations of the light harvesting complexes and to structural differences of the macroalgae tissue (i.e. tallus thickness). Red macroalgae species exhibit fluorescence emission spectra that are significantly distinct from those of green and brown macroalgae, due to the presence of phycobilin pigments (phycoerythrin and phycocyanins). Therefore, differences in fluorescence emission spectra can be used to partially characterise the taxonomic structure of macroalgal intertidal communities using LIF with a single excitation wavelength. Furthermore, the $F_{685}/F_{735}$ emission ratio can be used to assess desiccation stress in exposed macroalgae during low tide. Recent development of compact, robust, and low-cost detectors and lasers for LIF-based sensors adapted to field applications (e.g. Utkin et al., 2013, 2014) makes this technique promising for the assessment of macroalgae communities of estuarine intertidal areas and their response to the environment.

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6. LITERATURE CITED


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determination via laser-induced fluorescence. Marine Ecology Progress Series 432:45-52. doi:10.3354/meps09157
FIGURES CAPTIONS:

Figure 1: Map of Portugal with enlarged zone of the Tagus estuary and adjacent coastal area. Black circles indicate the location of the sampling sites and the corresponding collected macroalgae number (see Table 1).

Figure 2: Characteristic laser-induced fluorescence spectral signature of (a) three Chlorophyta macroalgae (Codium tomentosum, Bryopsis plumosa and Ulva rigida) (b) two Phaeophyta macroalgae (Fucus vesiculosus and Colpomenia peregrina), and (c) three Rhodophyta macroalgae (Gracillaria gracilis, Gelidium comeum and Lithophyllum incrustans) found at Tagus estuary and the adjacent coastal area (fluorescence emission maxima shown in Table 1).

Figure 3: Shift in laser-induced fluorescence spectra caused by the change in the number of sheet-like blades (layers) of Ulva rigida.

Figure 4: Changes in laser-induced relative fluorescence spectra of Ulva rigida with desiccation time (0, 1, 2 and 3 h).

Figure 5: Fresh weight loss (%) and changes in red/far-red chlorophyll fluorescence ratio ($F_{665}/F_{735}$) of Ulva rigida with desiccation time (0, 1, 2 and 3 h).
Table 1: Classification of studied algal species and corresponding sampling site, date of collection and laser-induced fluorescence emission maxima

<table>
<thead>
<tr>
<th>Algal group/species</th>
<th>Site</th>
<th>Date (month)</th>
<th>Fluorescence λ_{max} (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHLOROPHYTA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Green algae)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Bryopsis plumosa</td>
<td>Raso</td>
<td>Apr</td>
<td>-</td>
</tr>
<tr>
<td>2 Codium tomentosum</td>
<td>Raso</td>
<td>Apr</td>
<td>-</td>
</tr>
<tr>
<td>3 Ulva intestinalis</td>
<td>Avencas/B. Cavalo</td>
<td>Mar</td>
<td>-</td>
</tr>
<tr>
<td>4 Ulva rigida</td>
<td>P. Destrói</td>
<td>Dec</td>
<td>-</td>
</tr>
<tr>
<td><strong>PHAEOPHYTA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Brown algae)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Colpomenia peregrina</td>
<td>Raso</td>
<td>Apr</td>
<td>-</td>
</tr>
<tr>
<td>6 Fucus vesiculosus</td>
<td>Alcochete</td>
<td>Dec</td>
<td>-</td>
</tr>
<tr>
<td><strong>RHODOPHYTA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Red algae)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Calliblepharis jubata</td>
<td>Raso</td>
<td>Apr</td>
<td>582</td>
</tr>
<tr>
<td>8 Corallina officinalis</td>
<td>Raso</td>
<td>Apr</td>
<td>583</td>
</tr>
<tr>
<td>9 Cryptopleura ramosa</td>
<td>Raso/P. Destrói</td>
<td>Apr</td>
<td>580</td>
</tr>
<tr>
<td>10 Gelidium corneum</td>
<td>P. Destrói</td>
<td>Feb</td>
<td>582</td>
</tr>
<tr>
<td>11 Gracilaria gracilis</td>
<td>B. Cavalo</td>
<td>Feb</td>
<td>582</td>
</tr>
<tr>
<td>12 Lithophyllum incrustans</td>
<td>Raso</td>
<td>Apr</td>
<td>583</td>
</tr>
<tr>
<td>13 Lithophyllum tortuosum</td>
<td>Raso</td>
<td>Apr</td>
<td>581</td>
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<td>14 Osmundea pinnatifida</td>
<td>Raso</td>
<td>Apr</td>
<td>577</td>
</tr>
<tr>
<td>15 Plocamium cartilagineum</td>
<td>Raso</td>
<td>Apr</td>
<td>582</td>
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