Journal of Biotechnology 324S (2020) 100018

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Fluorescence responsiveness of unicellular marine algae *Dunaliella* to stressors under laboratory conditions☆

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ARTICLE INFO

Keywords:
Algae *Dunaliella*
Acidification
Autofluorescence
Cadmium
Flagella region
Photobleaching

ABSTRACT

We examined the responsiveness of unicellular green alga *Dunalliela tertiolecta* to selected stressors employing confocal- and time-resolved imaging of endogenous fluorescence. Our aim was to monitor cell endogenous fluorescence changes under exposure to heavy metal Cd, acidification, as well as light by laser-induced photobleaching. The accumulation of Cd in algae cells was confirmed by the secondary ion mass spectroscopy technique. For the first time, custom-made computational techniques were employed to evaluate separately the fluorescence in the flagella vs. the body region. In the presence of Cd, we recorded increase in the green fluorescence in the flagella region in the form of opacities, without change in the fluorescence lifetimes, suggesting higher availability of the fluorescent molecules. Under acidification, we noted significant rise in the green fluorescence in the flagella region, but associated with longer fluorescence lifetimes, pointing to changes in the algae environment. Photobleaching experiments corroborated gathered observations. Obtained data support a differential responsiveness of the flagella vs. the body region to stressors and enable us to better understand the pathophysiological changes of algal cells in culture under stress conditions.

Introduction

Pollution by heavy metals, acid rains, together with modification of illumination conditions belongs to significant environmental stressors (Huber, 1972; Cimboláková et al., 2019) which consequently affect the algae functioning and their key role in the photosynthesis. Deep understanding of algal cell response is essential to predict how ecosystems may be affected by the climate change and other human activities.

Monitoring rapid responsiveness of the algae fluorescence under environmental stressors presents non-invasive approach in order to understand underlying metabolic changes in stressed living cells which is a prerequisite for understanding their pathophysiological changes. Among the brightest endogenous fluorophores (autofluorescence) inside biological tissue are chlorophylls in plants (Govindjee Papageorgiou and Rabinowitch, 1967) and NADP(H) and flavins in mitochondria (Chorvatova and Chorvat, 2014). These molecules are the main electron donors and acceptors in the biochemical processes such as photosynthesis, or oxidative phosphorylation. Presence of a stressor-sensitive green fluorescence was recorded in *Dunaliella* algae under heavy metal of cadmium (Ivošević DeNardis et al., 2019). It may point to the presence of carotenoids (Dorinde et al., 2010), or lipid accumulation (Minhas et al., 2016). However, contribution of mitochondrial flavins and/or flavonoids cannot be excluded (Chorvatova et Chorvat, 2014). Increase in the autofluorescence related to flavoproteins, derived both from oxidation and synthesis of flavins was described in prokaryotic, as well as eukaryotic cells, mainly when experiencing severe life-threatening stress (Surre et al., 2018).

Time-resolved fluorescence imaging enables evaluation of the sensitivity of the endogenous fluorophores to their environment and thus brings potentially very useful information for examination of changes in the algae responsiveness to stress conditions (Chorvatova and Chorvat, 2014; Becker, 2015). We have previously demonstrated application of time- and spectrally resolved microscopy tools to record the endogenous fluorescence in the *Chlorella sp.* alga (Teplicky et al., 2017). The examination of pH responsiveness in these algae (Marcek Chorvatova et al., 2020) uncovered a decrease in the red endogenous fluorescence with acidification, associated with a shortening of the fluorescence lifetimes, while rise in the green fluorescence accompanied by the fluorescence lifetime prolongation was also observed at low pH. In addition, we discussed potential usability of the endogenous fluorescence

☆ This article was originally published in *Journal of Biotechnology: X. Journal of Biotechnology: X* is now discontinued and the article is republished here for the reader's convenience. For citation purposes, please use the publication details of this article: *Journal of Biotechnology, 324S*.

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http://doi.org/10.1016/j.btecx.2020.100018
Received 28 November 2019; Received in revised form 6 March 2020; Accepted 4 May 2020
Available online 16 May 2020

as a biosensing tool for tracking responsiveness to modulators (Marcek Chorvatova et al., 2018).

Recently, we studied the algal cell response of *Dunalliela tertiolecta* to laboratory-induced stress with heavy metal of Cd using multimethod approach (Ivošević DeNardis et al., 2019). Algal cells shows successful adaptation to toxic concentration of Cd which was manifested through cell shape deterioration, decreased cell motility and increased physiological activity, rise in the cell stiffness and expression of specific proteins, namely of LHC. These effects on cell functioning may be associated with changes in the green fluorescence. During photosynthesis, algae use LHC to capture photons and transfer energy to Photosystem I and II. A subgroup of LHCs is also activated by high light exposure, preventing oxidative stress by binding chlorophylls (Li et al., 2009) and leading to significant bleaching of the red fluorescence. Under such stress conditions (Peers et al., 2009), a nonphotochemical quenching of the chlorophyll fluorescence is triggered (Niyogi and Truong, 2013).

The *Dunaliella* algae are useful and sensitive model organism for evaluation of responsiveness to environmental changes. These halotolerant organisms are found in different aquatic systems (Avron and Ben-Amotz, 1992; Gimmler and Weis, 1992; Borowitzka, 1981). They does not possess cell wall, which enables them to adapt well to various environments in the terms of salinity employing mechanism of glycerol to balance osmotic pressure (Ben-Amotz et al., 2009).

Here, we examine responsiveness of unicellular green alga *Dunalliela tertiolecta* to selected stressors employing spectrally- and time-resolved imaging of endogenous fluorescence and we specifically focus on fluorescence changes in the flagella vs. the body region. Our aim is to monitor cell fluorescence changes under the exposure to heavy metal of Cd, under influence of acidification and/or following changes in illumination conditions using laser-induced photobleaching. The result would enable to better understand pathophysiological response of algal cells in culture under stress.

Material and methods

Samples and solutions

Algal cell culture

Laboratory monoculture of the unicellular marine algae *Dunaliella tertiolecta* Butcher (Chlorophyceae) was studied. The cells were grown in seawater enriched with f/2 medium (Guillard, 1975) in a batch culture under ambient conditions. Cell density reached up to 2×10^6 cells/mL in the culture after 14 days of growth. The cells were separated by mild centrifugation (1500 g, 5 min) from the growth medium and the loose pellet was washed several times with filtered seawater. Stock suspension contained 1–6 $\times$ 10^7 cells/mL in filtered seawater. All experiments were performed on glass coverslips modified with the polyethylenimine (Sigma-Aldrich, PEI) surfaces (0.2 %) in order to immobilise motile algal cells (Pillet et al., 2019).

Solutions

In the experiments where Cd was the stressor, an aliquot of stock solution of $Cd(NO_3)_2$ was added to cell cultures on the fifth day after the inoculum to reach the total Cd concentration of 1000 μg/L. Algal cells were further characterized in the stationary phase of growth (20 days). Selected concentration is chosen according to the developed methodology based on a generalized mathematical model for the complex formation of a single ligand with several trace metals to predict concentrations of free cadmium (bioavailable form) in culture, published recently (Ivošević DeNardis et al., 2019). The f/2 growth medium contains salts, essential trace metals, ligand, and vitamins. The model calculated that, with the addition of 1000 μg Cd/L, bioavailable cadmium concentration would correspond to 600 μg/L which is around 200 times higher concentration than cadmium concentrations in surface waters of impacted environments according to EPA.

In the acidification experiments, citric acid ($C_6H_8O_7$, Sigma-Aldrich, labelled as CITR, 40 mM) was added to filtered seawater with cells and recorded after 10 min exposure for up to 60 min. This resulted in rapid acidification to pH at 2.34 from pH 7.60 in control conditions, verified by pH meter (Mettler Toledo MP220, USA).

Instrumentation

Absorption and emission spectrofluorimetry. Absorption and fluorescence spectra were recorded on Shimadzu UV-2100 and Horiba-Jobin Yvon SPEX Fluorolog 3–11 spectrometers respectively, with the system's wavelength response correction. The fluorescence spectra were corrected for the wavelength response of the system. Excitation at 375 nm, 488 nm and 633 nm, and emission at 560 nm and 670 nm was employed for measurement of emission and excitation spectra, respectively.

Laser scanning confocal microscopy (LSCM). Confocal microscopy images of autofluorescence were acquired using Axiovert 200 LSM 510 Meta (Carl Zeiss, Germany) equipped with C-Apochromat 40x, 1.2 W corr NA. Individual cells were excited with the 450 nm laser line (Kvant, Slovakia) and recorded with a 16 channel META detector. Channel 1 recorded light with BP 500−550 nm bandpass filter with the pinhole opening of 2.28 AU, while channel 2 detection ranges was 650−710 nm with the pinhole opening of 1.02 AU. Pixel size used was 0.22 $\times$ 0.22 μm. Maximal (100 %) laser power density used for sample excitation was 1580 Wm^{-2} for 450 nm laser line. For standard imaging, the laser was set to 10 % of its maximal intensity, for bleaching experiments 100 % laser power density was employed.

Fluorescence lifetime imaging microscopy (FLIM). FLIM images were recorded using time-correlated single photon counting (TCSPC) technique (Becker, 2015), following excitation with a 473 nm picosecond laser diode (BDL-473, Becker&Hickl, Germany). The laser beam was reflected to the sample through an epifluorescence path of the laser scanning confocal microscope using pixel size scaling 0.29 $\times$ 0.29 μm. The emitted fluorescence was separated from laser excitation using LP500 nm and detected by HPM 100−40 photomultiplier array employing SPC-830 TCSPC board (both Becker&Hickl, Germany). Mean power density at the sample of 473 nm laser used for FLIM experiments was 230 Wm^{-2}, with 20 MHz repetition rate and approx. 60 ps pulse length with $<$ 0.1 pJ/pulse energy.

Secondary ion mass spectrometry (SIMS). Mass spectrometry measurements were performed using a ToF-SIMS IV (ION-TOF, Germany) reflectron type time-of-flight mass spectrometer equipped with a bismuth ion source. Pulsed 25 keV Bi^+ were used as primary ions with a current of 1 pA with total primary ion dose density below the static limit of 10^{13} ions cm^{-2}. Secondary ion mass spectra were measured by scanning over a selected 100 $\times$ 100 μm^2 analysis area. Imaging was performed by selecting masses of interest and recording their intensities with respect to the position of the primary ion beam in the fields of view 250 $\times$ 250 μm^2. Primary ion dose density was 10^{12} ions cm^{-2}. The lateral resolution of measured 2D images was about 1 μm. Measurements were performed in positive and negative polarities but, due to the spectral significance, only positive polarity mass spectra and images are shown. Control sample was prepared as solution of algae in seawater and the sample of interest was prepared with addition of Cd in the concentration of 1000 μg/L. Cd isotopes at masses 106, 108, 110, 111, 112, 113 and 116 mu were identified too as well as cationized Cd with hydrogen as CdH^+ peak at mass 1149 mu. Based on the intensity level, the concentration of Cd may be given in ppm range. The samples for SIMS measurements were prepared by dropping the algae solution on silicon wafer and dried at room temperature.

Irradiation conditions. Illumination conditions were chosen in order to

minimize the effect of laser-induced irradiation on algae during a typical experiment. The LSCM experiments were done using the mean power density of the lasers lower than the power density of solar radiation during daylight at visible wavelengths (400−500 Wm^{-2}). Only during the laser-induced photobleaching, which was induced by consecutive scanning employing 100 % laser power (as opposed to 10 % under classical experimental conditions), the illumination conditions were chosen to affect the algae autofluorescence by excited light. In such case, corresponding photon fluence was 1.4×10^{20} photons m^{-2} s^{-1} for the blue (450 nm) laser running at 100 % of its maximal power. In regard to the use of scanning, we do not expect temperature changes of the sample to occur during photobleaching.

Data analysis

Confocal data were visualized by ZEN 2011 software (Zeiss, Germany). Overall fluorescence intensity from confocal images was analysed using image segmentation method, where only recorded intensities of fluorescence derived from fluorescing algae were measured, without change in the background. FLIM data were were visualized as a map and as a distribution of calculated fluorescence lifetimes. Statistical comparison was done by Origin 6.0 Professional using one-way Anova, with $p < 0.05$ considered as significant.

Analysis of endogenous fluorescence separately in flagella vs. body regions by custom-made computational approach

In fluorescence confocal imaging, to achieve numerical comparison of the mean fluorescence intensities in the individual *Dunaliella* body vs. flagella region, custom computational approach was designed, set up and tested. The fluorescence intensity of each *Dunaliella* cell visible in confocal images for each detection channel was determined by averaging the fluorescence signal from image pixels belonging to the given body or flagella regions. The body's pixels were determined autonomously using an image processing technique that effectively segmented the individual cells in the recorded confocal images. Segmentation technique was based on Canny edge image detector followed by flood fill segmentation. Since the *Dunaliella* cells have shown non-homogenous fluorescence over the cell area, the mean value of fluorescence detected in the area close to the flagella and the fluorescence detected in the remaining area of *Dunaliella* body were determined and compared separately in all measured images.

The area near the flagella root was considered as a region that exibited differnet fluorescence behavior as remaining body of cell. Data has shown that perimeter of this area was typically around 5 μm. The exact shape of this area was depicted for each individual cell in recorded images using following image segmentation approach: As a first step, the individual bodies of *Dunaliella* were segmented from image background applying thresholding filter (Otsu, 1979) and the *Dunaliella* body's contours were next extracted using Canny edge filter. All this image processing was done on bright field image taken in parallel with fluorescence image. The root of flagella was subsequently identified for each body's contour at the pixels in which the contour shows topological crossing. These pixels serve as seeds for flood fill algorithm that grouped all adjacent pixels with similar fluorescene pattern into the being searched area. Based on this approach, in control conditions where the ccomparison was performed in 187 cells (on average 13 per image), the mean circumference of the body region was estimated to 25.6 ± 1.22 μm and the flagella region to 15.1 ± 0.70 μm.This corresponds to the overall algae diameter of about 10−13 μm which is in agreement with previous observations (Ivošević DeNardis et al., 2015; Svetličić et al., 2000).

In FLIM images, to achieve signal separation between the two regions, measured FLIM images were firstly segmented to delineate boundaries and pixels belonging to each flagella vs. body region of the *Dunaliella* cell in the image, comparably to the approach employed for the fluorescence evaluation. Segmentation was done using simple Otsu thresholding approach (Otsu, 1979) on the fluorescence intensity image that was built by summation of fluorescence signal across all time channels of corresponding FLIM image. Segmented intensity image was further used to construct a binary image mask that enables to sum decay signals corresponding to pixels of individual *Dunaliella* flagella or body region for each cell object in the image. Similar to analysis of fluorescence images, the lifetimes were also analyzed in the context of distance from the flagellum root. In the FLIM images, however, direct identification of the flagellum root position was not possible, so the region was identified indirectly by flood fill algorithm as the region near the borderline pixel of segmented *Dunaliella* region with the lowest cumulative fluorescence intensity.

Final summed decay signals were then analyzed with a two component multi-exponential model separately for each *Dunaliella* object in the image and results were presented as average values from all the cells found in image. The choice of the number of fluorescence lifetimes was based on the best χ^2 in most of the recorded experimental conditions, but it was not an easy issue taking into consideration very small difference between tau1 and tau2 in control conditions. At the same time, due to rise in the tau2 in acidic conditions, the necessity to employ more than a single exponential model was confirmed in the stress conditions. Data fitting was done using custom software exploiting open source NLOPT library (Johnson, 2020). As an instrument response function in data fitting procedure, the synthetic IRF function produced by SPCImage software (Becker Hickl, Germany) was used. Chosen approach allowed automatization of the procedure in varying experimental conditions, as well as separation between the two regions.

Results

Fluorescence spectral recordings of **Dunaliella** *cells in suspension*

Absorption spectra, recorded in algal cell suspension using spectrophotometer, uncovered an increased absorption in the UV region (up to 500 nm with a second peak at 670 nm; Fig. 1A), corresponding to the presence of chlorophylls. Excitation spectra at emission 670 nm confirmed increased intensity under 500 nm and over 600 nm (shown on Fig. 1B), which is in agreement with the absorption measurements. At all tested excitations, the algae emission presented clear peak at 680 nm, as expected for the chlorophyll. Increased fluorescence emission between 420−540 nm can be associated with flavins, flavonoids or carotenoids (Fig. 1C).

To study naturally emitted fluorescence in *Dunaliella* algae in suspension, LSCM was employed. Based on the spectrometry measurement, two emission windows were chosen: first recording window was in the green spectral region, 500−550 nm, and the second one in the red spectral region, 650−710 nm (Fig. 1D), following excitation at 450 nm. Intensity in the red spectral region was higher than that in the green one, as expected for the major contribution of chlorophylls. Effects on *Dunaliella* autofluorescence were therefore evaluated from multichannel confocal images, while comparing the intensities captured in the two channels covering the green and the red regions of the visible spectrum separately. Using this approach, gathered data confirmed a recordable autofluorescence at both spectral channels in control conditions (Fig. 1D).

Effects of cadmium on the cell autofluorescence

Presence of cadmium in *Dunaliella* algae was recorded using the SIMS technique (Fig. 2). In the positive polarity SIMS spectra, basic Cd ion peak was identified at mass 113.9 mu and intensity of 387 counts. In the control sample, Cd was not identified, as shown in Supplement Fig. 1. SIMS images, shown in Fig. 2, represent the two-dimensional distributions of selected elements and the molecular fragment CH_3^+ in the positive polarity for algae sample with addition of Cd. The single algae cell estimated from SIMS images is represented by Mg^+ ion as the constituent

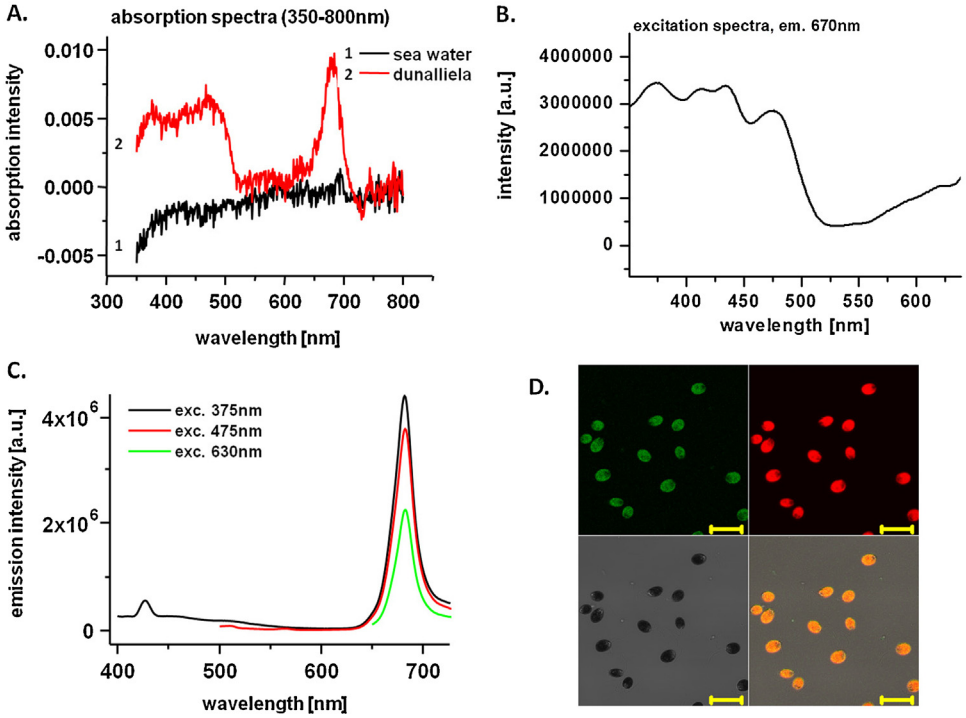

Fig. 1. Endogenous fluorescence of *Dunaliella tertiolecta* cells. A) Absorption spectra recorded at 350-750 nm in *Dunaliella* (2) vs. in seawater (1). B) Excitation spectrum recorded at emission 670 nm. C) Emission spectra measured following excitation 375 nm, 450 nm and 630 nm. D) Green (top left) and red {top right} fluorescence of algal cell recorded in control sample under laboratory conditions. Images recorded by laser scanning confocal microscopy, excitation 450 nm, emission channel 1 (green): 500-550 nm, channel 2 (red): 650-710 nm, channel 3 (grey): transmission image, channel 4: overlay channels 1-3, scale 20 μm. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

of chlorophyl molecule. The single cells contours are clearly visible in intensive yellow color. Cd^{+} ion distribution is correlated with Mg^{+} distribution (see principal component analysis at Supplement Fig. 2) which confirms the Cd absorption by algae cells from environment.

Further, in the presence of Cd, we evaluated changes in the fluorescence of the flagella vs. the body region separately (Fig. 3), employing the segmentation method described in Material and Methods. We observed that, in the presence of the stressor, the green fluorescence raised in the form of opacities in the flagella region (Fig. 3B) when compared to control conditions (Fig. 3A). Statistical comparison revealed significant rise in the green fluorescence in the flagella, but not in the body region in the presence of Cd (Fig. 3E). The red fluorescence, on the other hand, presented rather a decreasing tendency that did not reach significance (Fig. 3F). This result clearly indicates that the flagella region plays different role when compared to the body region in the responsiveness to Cd.

Effect of acidification on the cell autofluorescence

Next, we evaluated the responsiveness of *Dunaliella* cells to acidification (Fig. 3). Addition by citric acid (CITR, 40 mM) was accompanied by fast accumulation of the green fluorescence into flagella region of the algae (Fig. 3C). The accumulation of the fluorescence was in the forms of opacities fluorescing in green. When compared among cells, this had for effect a significant rise in the green fluorescence in the flagella region (Fig. 3E flagella), while no significant change was identified neither in the body region (Fig. 3E body), nor in the red fluorescence (Fig. 3F).

Acidification had a comparable effect in the presence of Cd as in control conditions: we noted significant rise in the green fluorescence in the flagella but not in the body region when compared to Cd alone (Fig. 3D, E). Red fluorescence has tendency to decrease in acidic conditions in the presence of Cd, but this trend remained non significant (Fig. 3F). In addition, in the presence of Cd, acidification also lead to shortening of the mean circumference of the flagella region of cells to 12.8 ± 0.7 μm, corresponding to the decrease in the cells diameter of about 1.5 μm; indicating possible osmotic change.

Effects of stressors on time-resolved fluorescence

The effect of stressors on time-resolved fluorescence, examined by recording FLIM images of autofluorescence using TCSPC, is summarized at Fig. 4. Excitation by 473 nm picoseconds laser, employing the LP500 filter, enabled to record the fluorescence lifetimes of both, the red and the green fluorescence (Fig. 4A-D). Algal fluorescence responsiveness was examined in the two (flagella vs. body) regions separately. In control conditions, the cell body region presented fluorescence lifetime tau 1 at 0.41 ± 0.02 ns and tau 2 at 0.54 ± 0.12 ns. In the flagella region, the fluorescence reached tau1 of 0.48 ± 0.04 ns and tau 2 remained at 0.55 ± 0.12 ns. The weight of the components is listed in Supplement Table 1.

In the presence of Cd, we observed no change in the fluorescence lifetimes (Figs. 4E-G). Increase in the fluorescence in the green region therefore cannot be explained by change in the fluorescence lifetimes but rather by an increase in the number of fluorescent molecules. On the other hand, in the presence of citric acid, we observed a clear prolongation in the tau2 in the flagella, but not in the body region (Fig. 4E-G). Such prolongation can explain significant rise in the fluorescence in the flagella region. Cd ions did not affect the responsiveness of the cells to acidification, suggesting presence of distinct mechanisms between the rise in the green fluorescence in the presence of Cd and acidification.

Effects of stressors on laser-induced photobleaching of endogenous fluorescence in **Dunaliella** *cells*

Responsiveness to high light exposure by inducing experimental photobleaching was also tested as an additional stressor. It was chosen to evaluate the capacity of the two regions to respond selectively. The cell responsiveness in control conditions to laser-induced photobleaching was achieved by successive scanning of images while exciting cells with 100 % laser power (Fig. 5A). In control conditions, we have observed clear differences in the way how the cell regions responded to photobleaching (Fig. 5B). Consecutive scanning lead to an immediate reaction of the flagella region: the red fluorescence presented a quasi-linear decrease (Fig. 5B), reaching complete attenuation of the red signal at around 450 s. In the body region, the bleaching of the red fluorescence followed after a gap of about 70 s (3rd scan) and complete bleaching was achieved after

560 s. On the other hand, photobleaching of the green fluorescence presented a different pattern: small lowering of the fluorescence in the

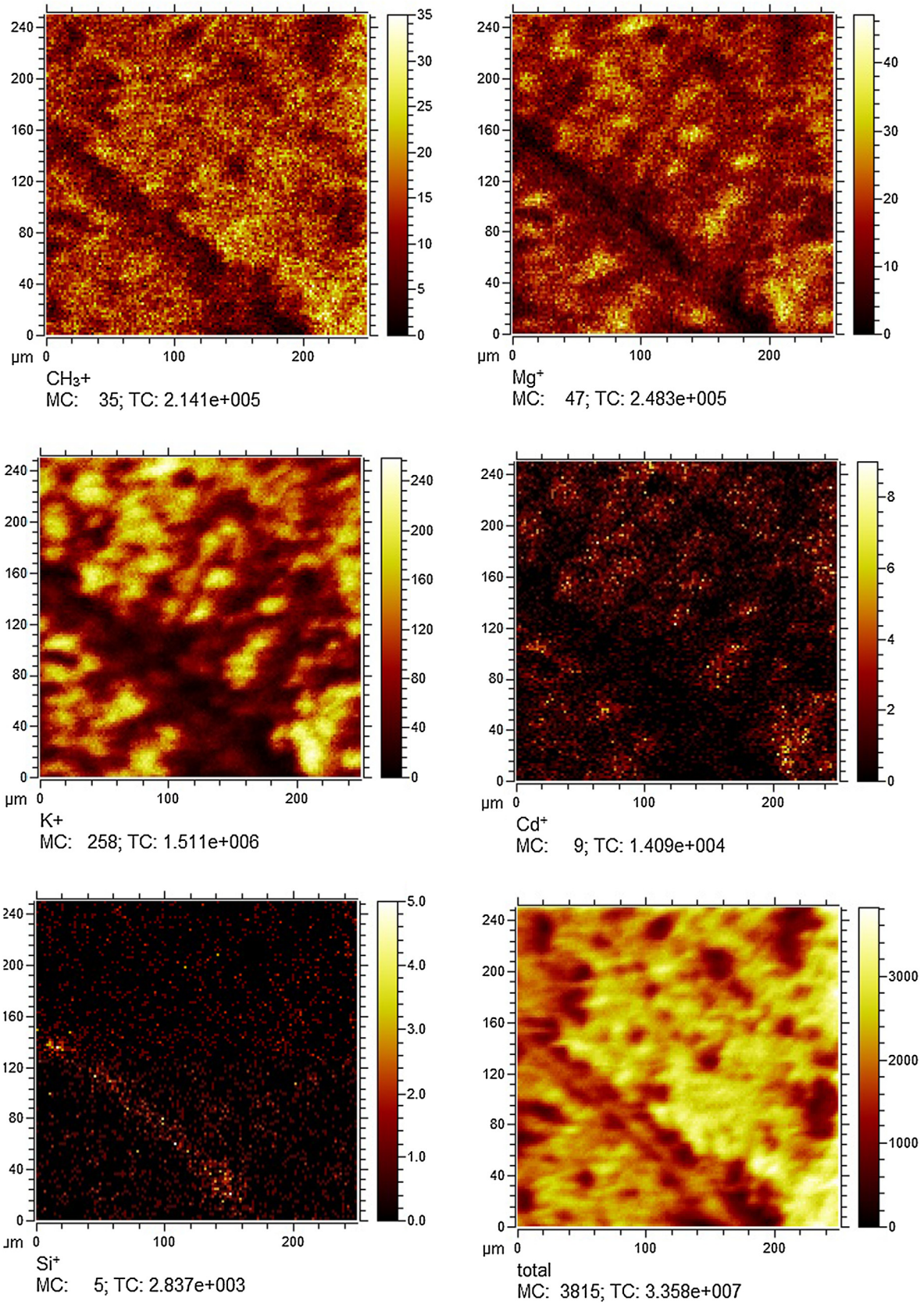

Fig. 2. SIMS spectra. Selected regions of mass spectrum for algae sample with Cd in a positive polarity. Two dimensional images of positive ions on algae sample with Cd surface. Abbreviation mc means the highest intensity of specified ion in single shot measurement and tc in means an overall intensity of selected ion defined by total counts.

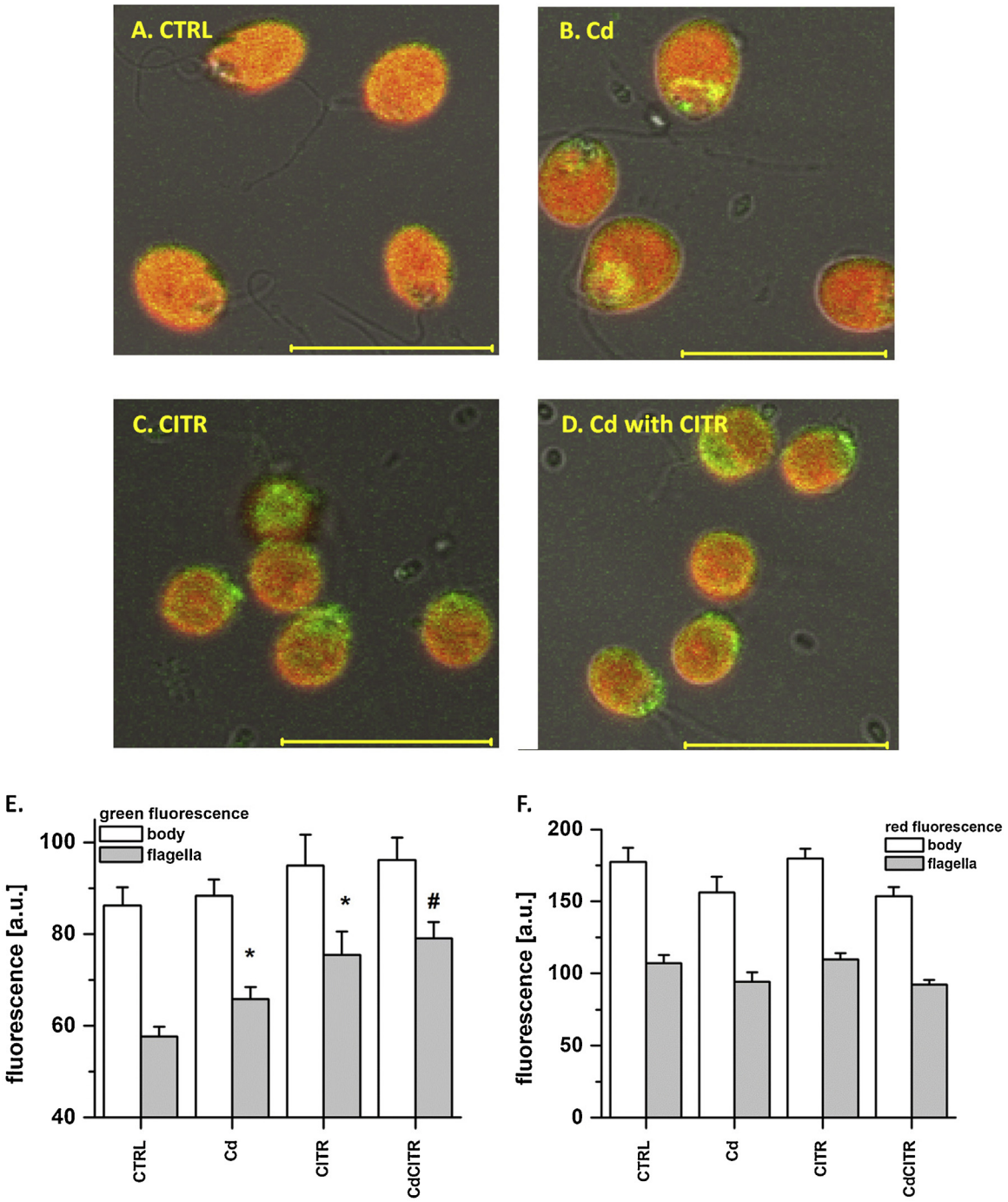

Fig. 3. Green and red fluorescence in the body vs. the flagellar region under stress. Comparison of the effects A) in control conditions (CTRL) vs. in the presence of B) Cd, C) citric acid (CITR) D) Cd with CITR. E) Statistical comparison of the effect in the flagella region vs. in the body region on E) the overall green fluorescence at channel 1 (500-550 nm), F) the overall red fluorescence at channel 2 (650-710 nm). *$p < 0.05$ vs. CTRL, #$p < 0.05$ vs. Cd. CTRL (n = 187/14 cells/images), Cd (n = 365/19), CITR (111/11), Cd with CITR (199/19). Scale: 20 μm. (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

first 240−250 s was followed by a transient rise in the green fluorescence. This increase was faster in the flagella region, where it reached maximum at 480 s and more pronounced in the body region, where it peaked at 600 s in control conditions.

In the presence of Cd, faster bleaching of the red fluorescence was noted in both, the flagella and the body regions (Fig. 5C). This effect was accompanied by faster and more pronounced transient rise in the green fluorescence, peaking at 300 s and 410 s in the flagella and body regions respectively (Fig. 5C). Such stronger response is in agreement with bigger availability of the fluorescing molecules in the presence of the Cd stressor.

Acidification, on the other hand, fastened but attenuated the responsiveness of the cells to photobleaching in control conditions. It also obliterated differences between the flagella and the body regions: the red fluorescence photobleached at a comparable rate in both regions and the rise in the green fluorescence was attenuated. The green fluorescence following photobleaching peaked faster in the two regions, at 300 s, while the differences between the two regions disappeared (Fig. 5D). This result points to a possible role of acidification in the observed differences of responsiveness between the two regions. It also corroborate with the finding that, during acidification, a change in the environment of the fluorescing molecules take place.

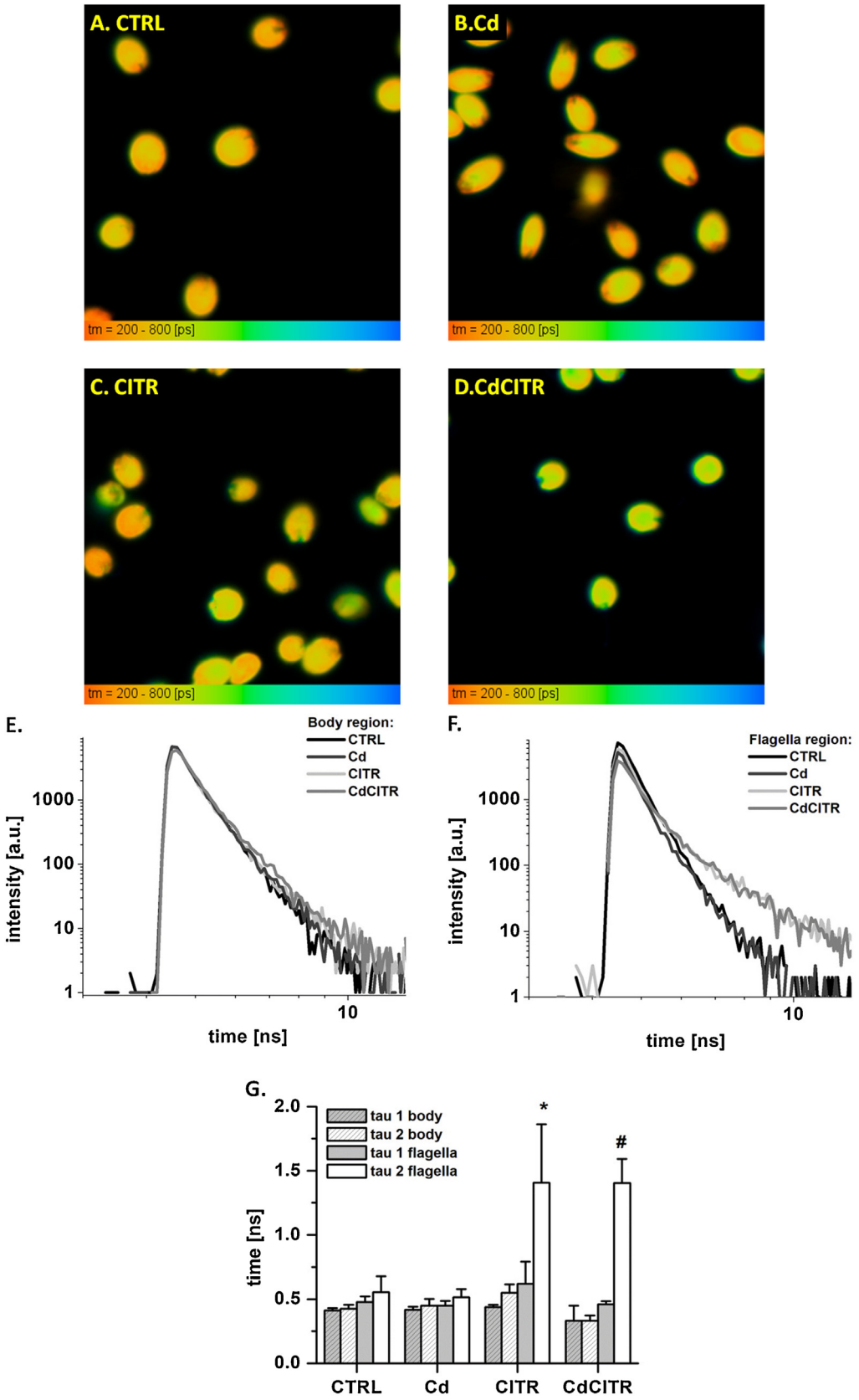

Fig. 4. Time-resolved fluorescence the body vs. the flagellar region. FLIM images of endogenous fluorescence of *Dunaliella* algae following excitation by 473 nm picosecond laser, LP 500 nm. Lifetime distribution is showed in the range 250-800 ps, A) control, B) Cd, C) CITR, D) Cd with CITR. Original traces from FLIM recordings in E) the body region and F) in the flagella region. G) Statistical comparison of tau1 and tau2 in different regions under recorded conditions. CTRL (n = 47 cells), Cd (107), CITR (34), Cd with CITR (74).

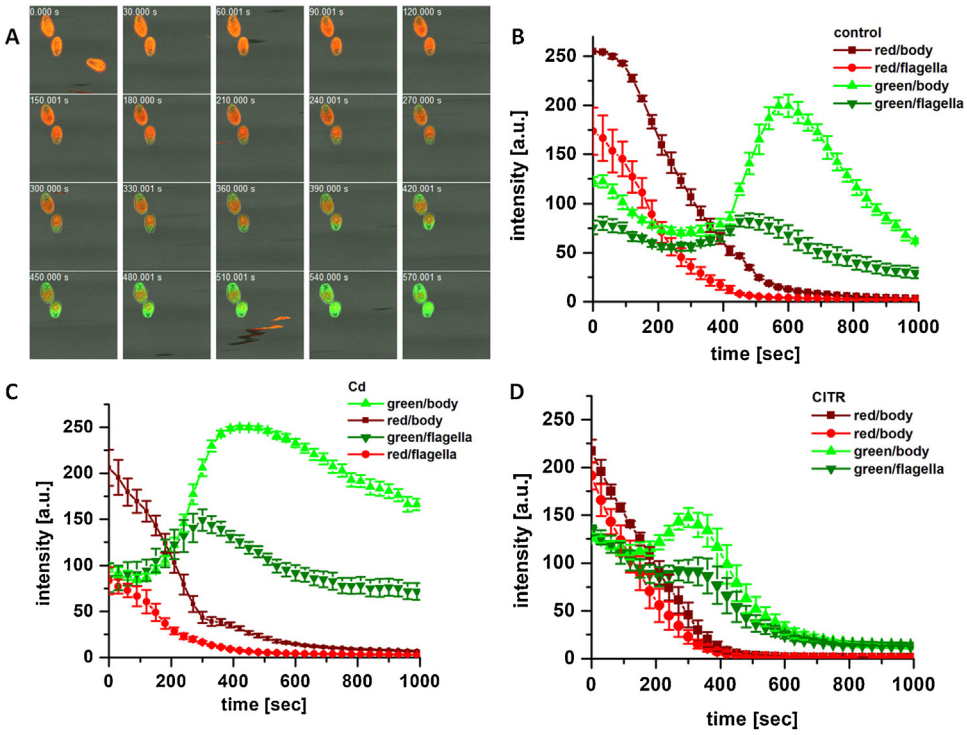

Fig. 5. Laser-induced photobleaching the body vs. the flagellar region. Bleaching induced by successive scanning at 100 % laser power every 30 s for 10 min. A) Original recordings. Effect of the photobleaching on the green vs. red spectral channels in the flagella vs. body region in B) control (n = 5 cells), C) Cd (4) and D) CITR (6). (For interpretation of the references to colour in the Figure, the reader is referred to the web version of this article).

Discussion

Gathered results demonstrate novel findings: for the first time, differential responsiveness of endogenous fluorescence of marine algae *Dunaliella* to selected environmental stressors is shown in the flagella vs. the body region separately. Custom-made computational techniques were employed to evaluate responsiveness to heavy metal Cd, acidification, and photobleaching. All stressors induced rise in the green fluorescence in the flagella region. At the same time, while change in pH resulted in the prolongation in the fluorescence decays, Cd rather involved increase in the number of the fluorescent species. Bleaching experiments corroborated the gathered findings. In addition, for the first time, transient rise in the green fluorescence in response to photobleaching was described.

In control sample, the recorded light absorption of *Dunaliella* algae is in agreement with reported data and main absorption between 400−500 nm and lesser one around 650−700 nm (Nordlund and Knox, 1981; Sosik and Mitchell, 1991). The emission at 680 nm corresponds well to the one of chlorophyll a (Sosik and Mitchell, 1991).

In cell stressed with Cd, in order to confirm the presence of heavy metals in the solution with algae, we have employed the SIMS technique that provides chemical lateral information directly from the surface of the cells or tissues in native form. Among the methods providing the images of biological samples, currently, there is a possibility to investigate not only tissue samples but also single cells with high mass and lateral resolution (Jerigova et al., 2010). Relevant informations are determined from mass spectra by identification of elements and molecular fragments and consequently from 2D images as their distributions with sub-micron lateral resolution. SIMS technique confirmed the capability of algae to accumulate the Cd from the local environment and pathway for entering into the cell.

Gathered results shed a new light on the possible way how these algae respond to exposure of various stressors. Our study revealed rise in the yellow/green fluorescence of the *Dunaliella* algae in the presence of both, Cd and citric acid. We previously reported (Ivosević DeNardis et al., 2019) that the green fluorescence, present in control sample was most pronounced between 510−550 nm, reaching maximum at 520 nm. In the presence of Cd or acidification, appearance of fluorescence opacities leads to rise in the fluorescence in the green spectral region with maximum between 560−580 nm. Contrary to the green fluorescence, the red one was not significantly affected by the application of the stressors. This differs from our observations in the fresh water algae *Chlorella sp.* (Marcek Chorvatova et al., 2020), where the rise in the green fluorescence was observed in severe acidification, but accompanied by lowering of the red chlorophyll fluorescence. The difference can be related to the fact that *Dunaliella* species are capable to adapt to very harsh environmental conditions, namely to wide range of salinities (Avron and Ben-Amotz, 1992). *Dunaliella acidophila*, for example, is one of the most extreme acidophiles, capable to survive pH under 3 (Gimmler and Weis, 1992, Uri Pick, 1999, Schmuck et al., 1999).

In the aim to better understand mechanisms underlying rise in the green fluorescence in response to stressors, we employed measurements of fluorescence lifetimes. FLIM method allows to determine changes in the environment of the fluorescent molecule (Becker, 2015). To record FLIM images, we have therefore chosen to use the LP500 filter in order to block the excitation laser of 473 nm and, at the same time, to accumulate a maximum of photons. Fluorescence lifetimes of chlorophyll were identified to be around 700−1000 ps in algae (Singhal and Rabinowitch, 1969; Moya et al., 2001). When analysed by double exponential decay, two fluorescence lifetimes, 0.262 and 0.728 ns, were found in green algae (Kristoffersen et al., 2016). In *Chlorella sp.* algae, we have identified fluorescence lifetimes between 0.4−0.6 ms in control conditions (Marcek Chorvatova et al., 2020); this range of values thus corresponds to values estimated in *Dunaliella* algae. In our setting, while most of the fluorescence recorded in control FLIM images is derived from photons with wavelength between 650−750 nm, corresponding to chlorophylls (namely chlorophyll a), in conditions when the red fluorescence is inhibited and/or rise in the green fluorescence occurs, the green fluorescence can also contribute to the resolved lifetimes in FLIM images.

Responsiveness to change in the illumination conditions was also tested by laser-induced photobleaching. Photobleaching is a dynamic process in which fluorochrome molecules undergo photoinduced chemical destruction upon exposure to excitation light and thus lose their ability to emit fluorescence (Song et al., 1995). Chlorophyll a is known to undergo fast photobleaching (Andreeva et al., 2007). Indeed, in control conditions, red fluorescence bleached fast in the flagella region, accompanied shortly after by bleaching in the body region. Carotenoids, luteins and anthocyanins are also capable to photobleach rapidly (Andreeva et al., 2007; Sweeny et al., 1981). Flavonoids, on the other hand, were showed to overcome the problem of anthocyanin

photobleaching when used as co-pigments (Sweeny et al., 1981). Transient increase in the green fluorescence during photobleaching, observed in the *Dunaliella* algae, may therefore suggest presence of some kind of protective mechanisms. Gathered results revealed potentiation of the responsiveness to bleaching in the presence of Cd. Also, the effect of photobleaching uncovered differential responsiveness in the presence of Cd vs. acidification. In the presence of Cd, the transient rise in the green fluorescence was stronger and faster, while the bleaching of the red fluorescence was quicker. Acidification, on the other hand, attenuated the response to photobleaching.

For the first time, we have noted differential effects of stressors on the endogeneous fluorescence in the body vs. the flagella region. Cd exposure induced no change in the fluorescence lifetimes, indicating that observed changes in the green fluorescence are related to increase in the number of the fluorescing molecules rather than to modification in their environment. In another study, where we examined the effect of pH on the endogenous fluorescence in *Chlorella sp.*, we also recorded rise in the green fluorescence in pH 2–3, associated with the fluorescence lifetime prolongation (Marcek Chorvatova et al., 2020). The rise in the green fluorescence was, however, accompanied by decrease in the red fluorescence due to shortening of the tau1. The difference can be related to distinct structural properties and/or physiological mechanisms in the two algae (Thiagarajan et al., 2019). Acidification, on the other hand, increased the tau2 fluorescence lifetime, pointing to the role of the changing environment in this action. The green, rather than the red fluorescence is expected to contribute to this action. Such conclusion also corroborate clear prolongation of the fluorescence lifetimes following photobleaching of the red chlorophyll fluorescence (data not shown). Harshley described the flagella region to play role in sensing, namely mechanosensing and chemosensing (Harshey, 2016). In our work, we noted differential responsiveness of the fluorescence in the flagella vs. body region of *Dunaliella* alga to all applied stressors. The flagella region responded by rise in the green fluorescence to Cd, but also to acidification of the cell environment. Increase in the green fluorescence in the flagella region also preceded that in the body region during photobleaching. It is not clear what is the reason behind such differential responsiveness. We can hypothesize that the body region is designed to maintain constant rate of photosynthesis under each condition as long as possible, while the flagella region responded faster (possibly due to smaller surface area or different photoreceptors), acting as the cells antena. Nevertheless, further data is necessary to fully understand complex metabolic changes in algal cell when exposed to stress condition. In the future, pigment fingerprint analysis may be of help to better link changes in the green fluorescence as a part of the cell adaptation response.

Conclusions

We explored response in *Dunaliella* cells in terms of autofluorescence under the influence of selected stressors (heavy metal, acidification, high light exposure) by employing direct and non disruptive approach combining confocal imaging and FLIM. For the first time, we have specifically evaluated and demonstrated differences in the fluorescence intensity between the cell body and the flagella regions. Our data revealed differential responsiveness of *Dunaliella* fluorescence in the flagella vs. the body region under the presence of stressors in laboratory conditions. Autofluorescence responded to all applied stressors by significant accumulation of the green fluorescence in the flagella region only. Gathered observations will contribute to understanding of the patho-physiological cell responsiveness to environmental stress.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no 871124 Laserlab-Europe (the Integrated Initiative of European Laser Infrastructures LASERLAB-EUROPE V). Authors also acknowledge support from the Grant Agency of the Ministry of Education, science, research and sport of the Slovak Republic under contract No. VEGA 2/0123/18, Slovak Research and Development Agency under the contract APVV-15-0227, as well as the International Visegrad Fund grant No 21720055. Also, we thank Nives Novosel for help with algal cell cultivation.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btecx.2020.100018.

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