In vivo determination of carotenoid resonance excitation profiles of *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Porphyridium purpureum*

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Abstract

Under stress, certain species of algae produce pigments such as astaxanthin, β-carotene, or phycoerythrin for protection against photosynthethic stress. These pigments are of high commercial demand, for example as food additives. Measuring and maintaining stress conditions precisely is necessary for efficient pigment synthesis in algae cultures.

We present resonance excitation maps of three common species of algae, *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Porphyridium purpureum*, exploring the feasibility of resonance Raman spectroscopy for stress monitoring *in vivo*. The resonance excitation profiles extracted from the resonance excitation maps of different stress states for individual Raman lines of carotenoids are a helpful guide to pick optimal excitation wavelengths for maximum resonance enhancement in living algae cultures of these species under laboratory conditions.

Keywords: Excitation profile, Resonance Raman spectroscopy, *Chlorella vulgaris*, *Haematococcus pluvialis*, *Porphyridium purpureum*

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1 Introduction

Pigment production in algae cultures is of high commercial interest especially for food additives and cosmetics[1]. Efficient pigment production requires maintaining a tightly balanced level of stress to force the alga cells to synthesise secondary carotenoids and other types of pigments, such as phycoerythrin. Four different experimental techniques are routinely used to gain insight into the internal stress states of algae: reflectance spectroscopy[2], absorption spectroscopy[3], fluorescence measurement[4], and Raman spectroscopy[5].

Reflectance measurements often rely on sunlight excitation for observing the colour of algae ponds, simplifying application in the field[2][6]. Absorption spectroscopy gives good quantitative results even for small changes of the pigment concentration in the samples[3]. However, very dense cultures may be opaque, requiring dilution before the analysis. Additionally, contaminants like bacteria and soil dust often impair open ponds and may have significant influence on the reflectance and absorption spectra. Chlorophyll fluorescence is a measure for photosynthetic stress in algae and plants in general[7][8]. Visible excitation of chlorophyll gives fluorescence with wavelengths longer than 680 nm. Being easy to detect, chlorophyll fluorescence is well suited as a feedback signal to control stress levels, both in algae cultures[9] and plant crops[10]. However, the typical setup for detecting the fluorescence of chlorophyll is not sensitive for carotenoid fluorescence seen between 505 nm and 530 nm[4].

Raman spectroscopy is a valuable tool for probing carotenoids and other pigments[11], especially resonance Raman spectroscopy is a well established experimental technique for carotenoid detection. Functional aspects and the configuration of carotenoids within the photosystem were also analysed with resonance Raman spectroscopy[12][13][14][15]. Due to the elaborate setup required, few detailed carotenoid resonance excitation profiles related to photosynthesis are available in literature, most notably the work of Ruban *et. al.* done with tunable dye lasers on pure carotenoids and isolated thylakoid membranes[12].

In this work, we present resonance excitation maps of the different stress states of three species of algae, *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Porphyridium purpureum*, and extract resonance profiles therefrom.

Resonance excitation maps are a compilation of Raman spectra measured over a range of excitation wavelengths. They plot the correlation between the intensity of individual Raman lines and the excitation wavelength. Raman lines appear as vertical lines in resonance excitation maps. Non-Raman components such as atomic lines appear as diagonal lines. Resonance excitation profiles are a subset of a resonance excitation map, plotting the intensity of a single Raman line versus the excitation wavelength. Individual Raman lines of the same substance may have different resonance conditions[16]. Solvent lines usually are non-resonant,

therefore appearing with constant intensity over the excitation wavelength range. The carotenoid content of the algae yields the three strong vertical resonant bands in all resonance excitation maps presented in this article.

For green algae (*Chlorophyceae*), including *Chlorella vulgaris* and *Haematococcus pluvialis*, high contents of α- and β-carotene, violaxanthin, neoxanthin, and lutein are to be expected[17]. Additionally, green algae are known to include the violaxanthin cycle, consisting of violaxanthin, zeaxanthin, and small amounts of the intermediate antheraxanthin[18]. In brief, under stress violaxanthin is partially transformed to zeaxanthin within the photosystem[19]. According to Ruban *et. al.*, excitation at 488.0 nm is selective for violaxanthin, whereas excitation above 500 nm (e.g. at 528.7 nm) is selective for zeaxanthin[12]. Therefore, violaxanthin cycle action would present itself as a shift of the resonance maximum towards longer excitation wavelengths under light stress conditions.

Chlorella vulgaris is a very common green alga, ubiquitous in freshwater habitats around the world. *Chlorella vulgaris* grows steadily under a wide variety of conditions, but does not form secondary carotenoids quickly[20]. Its main commercial application is lipid production[21] for biofuel[22]. The storage location[23] and pathways for lipid[24] and carotenoid[25] synthesis differ, therefore it is unlikely that lipid production will influence carotenoid measurements. Additionally, lipid Raman spectra[26] differ from carotenoid Raman spectra and can be easily spotted, if present.

Haematococcus pluvialis, also a green alga, is well known for its cells to synthesise large amounts of astaxanthin under stress to form an opaque protective shield in their cell walls[5], a condition known as the red cyst state. Boussiba *et. al.* found by HPLC analysis that astaxanthin accounts for more than 99 % of the total carotenoid content in stressed *Haematococcus pluvialis* cysts[27]. Given the very high concentration and localisation of astaxanthin in the cell wall, the resonance excitation map of the red cysts therefore is expected to closely mirror the resonance behaviour of astaxanthin embedded in lipid membranes. Along with increased astaxanthin content, the cells also undergo a morphological change from actively swimming green cells to immobile, circular red cells[28] in order to survive subsequent desiccation and airborne distribution by wind[29]. Although *Haematococcus pluvialis* also contains the violaxanthin cycle, its action would be hardly visible in the resonance excitation maps due to the distinctive change in carotenoid composition accompanying stress adaptation with astaxanthin.

As a comparison to green algae containing many different carotenoids, the red alga *Porphyridium purpureum* containing only the two similar carotenoids β-carotene and zeaxanthin was selected[17]. In addition, *Porphyridium purpureum* under stress synthesises the non-carotenoid pigment phycoerythrin[30], resulting in two different states without the appearance of additional carotenoids.

2 Experimental aspects

2.1 Culture conditions and biological setup

The medium for all algae cultures was prepared according to the recipe for ES medium[31] ("Basal Medium ES" recipe, Culture Collection of Algae at the University of Göttingen, Germany) replacing the 30 ml soil extract with distilled water to avoid introducing unwanted features in the Raman spectra by unknown soil components. The algae *Chlorella vulgaris* (SAG strain 211-11b), *Haematococcus pluvialis* (SAG strain 34-1a), and *Porphyridium purpureum* (SAG strain 1380-1a) were cultivated successfully at a temperature of $22^{\circ}C$ in this modified medium for one year. Culture conditions were the same as in[19]. Briefly, the algae were grown in an airlift agitation reactor specifically made from borosilicate glass. The cultures were subjected to photosynthetically active light with an intensity of 2.5 $\mu\mathrm{mol} * \mathrm{s}^{-1} * \mathrm{m}^{-2}$, provided by red and blue LED strips (628 nm and 467 nm, Josef Barthelme GmbH & Co. KG, Nürnberg, Germany). Aeration was provided by compressed air through a gas wash flask filled with distilled water to minimise evaporation of the culture medium, and a sterile filter with 200 nm pore size. The unstressed cultures were maintained in exponential growth state by dilution with fresh medium every two weeks.

For preparing stressed sample cultures of *Haematococcus pluvialis* and *Porphyridium purpureum*, the cultures were left in the reactor without replacing depleted medium, causing nutrient deficiency over time[27][30]. Different stress levels were obtained by observing the colour of the cultures over time, as classified for *Haematococcus pluvialis* in[28]. Slightly stressed states were measured when the first cells in the cultures changed colour, approximately three weeks after the final dilution with fresh medium. Highly stressed states were measured after several weeks without further changes of the culture colouring. Fresh samples of 2 ml of shaken culture were taken for every individual spectrum.

Light stress was used to screen *Chlorella vulgaris* for violaxanthin cycle action by comparing light stressed and dark adapted samples of the otherwise unstressed culture in exponential growth state. For dark adaptation of *Chlorella vulgaris*, a flask with unstressed culture was kept in darkness overnight prior to the measurement. Algae cells on the bottom of a cuvette do not move and are subjected to the laser beam, therefore short-term light adaptations of the violaxanthin cycle due to the excitation beam are to be expected. To ensure consistent dark adaptation for every spectrum in the resonance excitation map, fresh samples of 2 ml of shaken, dark-adapted culture were taken for every individual spectrum. For light stress adaptation of *Chlorella vulgaris*, a small culture sample of 2 ml was subjected to intense light of 1950 $\mu\mathrm{mol} * \mathrm{s}^{-1} * \mathrm{m}^{-2}$ photons of photosynthetically active radiation for one day prior to the measurement, with the culture returned to the stress

light between the recording of individual spectra to maintain the stressed state. The intensity for stress light was selected according to values given in[32]. Due to the small total sample size, the light stress experiment was repeated twice. According to Koch *et. al.*, the violaxanthin cycle of the green alga *Dunaliella salina* reacts to light stress within 3.2 minutes[19]. For *Chlorella pyrenoidosa* an equilibrium for the dark-to-light adaptation of the violaxanthin cycle was measured after 30 minutes with thin-layer chromatography[33], so it is safe to assume that *Chlorella vulgaris* will reach equilibrium adaptation after one day of darkness or intense light, respectively.

For sufficiently strong Raman signals without resonance effects, high cell densities are necessary, which can be achieved reproducibly in a layer of cells sunk to the bottom of a fused silica cuvette (117.104-QS, Hellma Analytics GmbH & Co. KG, Müllheim, Germany). The sunken layer of cells was subsequently measured through the bottom of the cuvette using a custom-built fibre bundle (detailed in 2.2). The temperature was kept constant at $22°C$ for the measurements.

2.2 Physical setup

Resonance Raman measurements were performed with a custom-built fibre bundle (CeramOptec GmbH, Bonn, Germany) with a 800 µm fused-silica fibre for excitation, surrounded by a rotation symmetric configuration of eighteen fused-silica fibres with 200 µm cores for detection, arranged into a line in the ferrule on the spectrometer side. For excitation, a widely tunable optical parametric oscillator (PG122/UV, EKSPLA uab, Vilnius, Lithuania) was used, giving 4 ns pulses at 10 Hz. Raw spectra were taken with a SR500 spectrograph (Andor Technology Ltd, Belfast, Northern Ireland) equipped with a set of long-pass filters (446 nm, 458 nm, 473 nm, 488 nm, 496 nm, 514 nm, and 532 nm; Semrock Inc., Rochester, USA), a 100 µm slit, a 1200 lines/mm grating blazed for 500 nm, and an Andor Newton DU940P camera. The resolution of the spectrometer in this configuration is 0.8 nm, as determined with a neon glow lamp, giving 27 cm^{-1} to 39 cm^{-1} resolution depending on excitation wavelength.

Spectra were taken with an excitation energy of 200 mJ per spectrum. To ensure the same energy was irradiated for each of the 46 excitation wavelengths, a custom-built pulse energy detector was employed. Before entering the bundle, the pulses from the optical parametric oscillator were fed through a 800 µm fused-silica fibre, decladded on 8 mm and bent at the decladded section. A photodiode (S1336-18BQ, Hamamatsu Photonics K.K., Hamamatsu, Japan) was placed on the outer side of the bend to detect the emergent light. Calibration was done in comparison to a standard laser power detector (ED-100AUV V5, Gentec Electro-Optics Inc., Quebec, Canada). Aquisition time per individual spectrum varied with the pulse energies available from the optical parametric oscillator but were

below 60 s. Cosmic rays were removed by means of comparing individual accumulated spectra and interpolating across detected spikes as described in[19].

Spectrometer wavelength offsets were calibrated using the Raman lines of ethanol measured with the same setup. Baseline correction was done as described in Koch *et. al.*[34] with the default configuration stated therein. In brief, the morphological baseline correction separates Raman signals from the contribution of fluorescence by means of their different spectral feature widths. Resonance excitation profiles were determined by fitting Lorentzian line shapes with a least squares algorithm.

3 Results and Discussion

Baseline corrected Raman spectra of all three algae species are shown in Fig. 1, with the carotenoid bands being the most prominent features in every spectrum.

Since *Haematococcus pluvialis* and *Chlorella vulgaris* are both green algae, the resonance excitation maps of the unstressed state of *Haematococcus pluvialis* and the unstressed, dark-adapted state of *Chlorella vulgaris*, both without secondary carotenoids present, are comparable and result in similar resonance excitation profiles (Figs. 2b and 3b). In comparison, the red alga *Porphyridium purpureum* (Fig. 4b) holds only the two carotenoids β-carotene and zeaxanthin and has its unstressed maximum resonance at the longer wavelength of 505 nm instead of 495 nm (*Chlorella vulgaris*) or 500 nm (*Haematococcus pluvialis*).

Variations of the carotenoid line positions with excitation wavelenghts are clearly visible in all resonance excitation maps presented in this work. It is known that the line positions of the ν_1 Raman band vary across different carotenoids[35]. Therefore, with tuning the excitation wavelength, partially selective excitation of different carotenoid populations within the algae cells is likely. However, a close examination of the resonance excitation maps reveals that the ν_1, ν_2, and ν_3 Raman bands shift in unison, an artifact caused by an unreliable mechanism in the spectrometer that resulted in varying offsets whenever the grating was moved.

3.1 Chlorella vulgaris

Chlorella vulgaris was examined in two conditions: in an unstressed state adapted to darkness, and stressed by intense light.

The resonance excitation profiles for dark-adapted (Fig. 2a) and light-stressed (Fig. 2c) samples are similar (Figs. 2b and 2d, respectively), showing a resonance maximum for an excitation wavelength of 500 nm. All three resonance excitation profiles for the ν_1 (1525 cm^{-1}), ν_2 (1150 cm^{-1}) and ν_3 (1005 cm^{-1}) Raman bands of the carotenoid content of the algae culture show a similar shape.

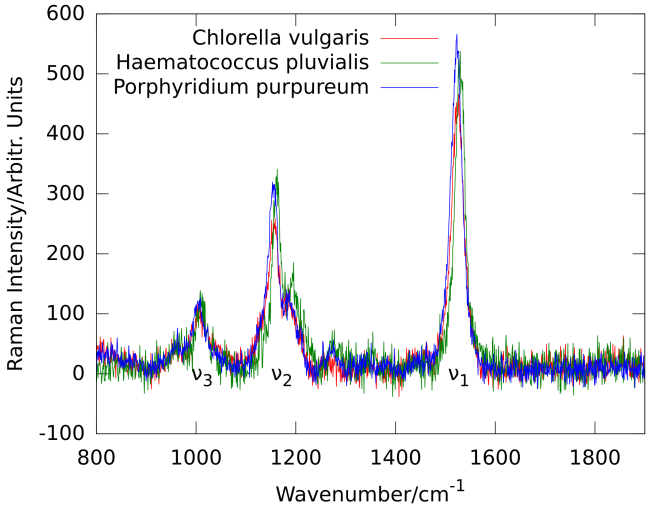


Figure 1: Baseline corrected Raman spectra of *Chlorella vulgaris* (unstressed, dark-adapted), *Haematococcus pluvialis* (unstressed), and *Porphyridium purpureum* (unstressed) recorded with 480 nm excitation.

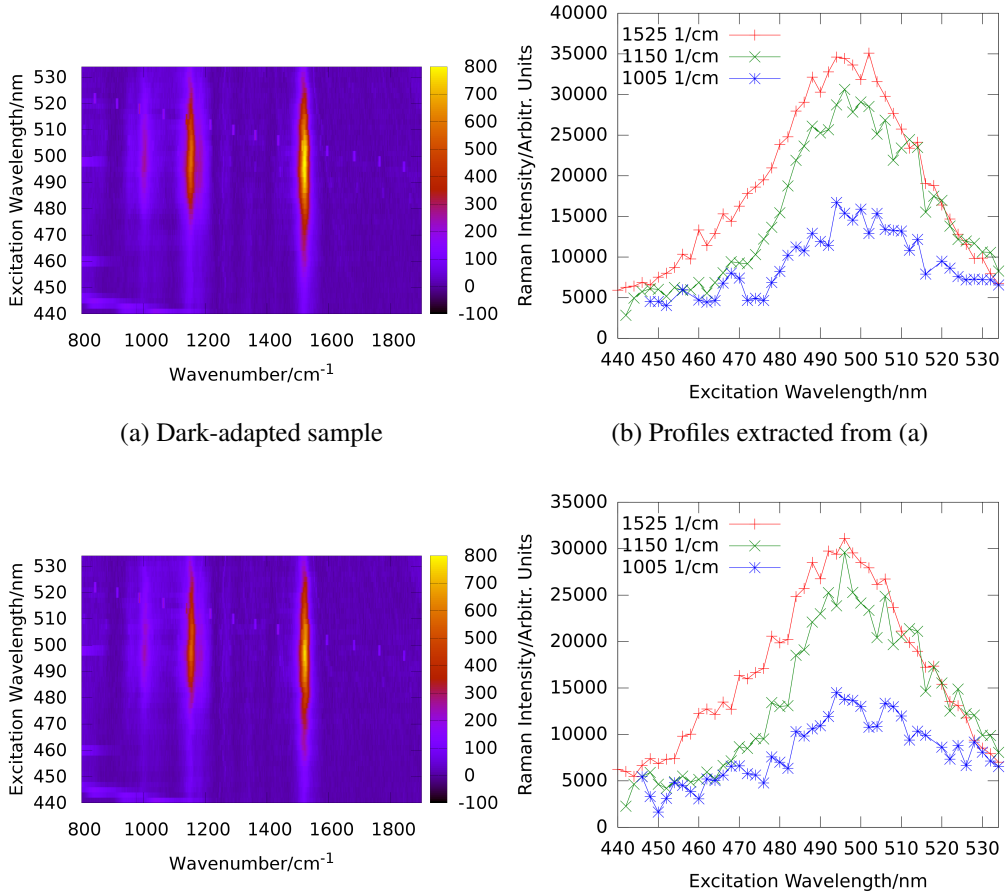
Within one day, *Chlorella vulgaris* cannot synthesise secondary carotenoids[20], but violaxanthin cycle action can be expected. However, no signs of violaxanthin cycle action were observed in the comparison of the resonance excitation profiles (Figs. 2b and 2d) either. This may be due to the fact that the violaxanthin cycle pool size is small in comparison to the overall carotenoid content in *Chlorella vulgaris* as determined with HPLC analysis in[20].

3.2 Haematococcus pluvialis

Haematococcus pluvialis was examined under three conditions: in unstressed green, slightly stressed red tinged, and highly stressed red state.

The unstressed green state of *Haematococcus pluvialis* (Fig. 3a) shows a resonance maximum at an excitation wavelength of 500 nm, with the resonance excitation profiles of the ν_1 (1525 cm^{-1}) and ν_2 (1150 cm^{-1}) Raman bands of the carotenoid content of the algae culture showing a similar shape (Fig. 3b).

However, *Haematococcus pluvialis* is known to produce large amounts of the secondary carotenoid astaxanthin[5] when stressed with nitrogen deficiency[27], increasing its carotenoid resonance (Fig. 3c) in the range from 510 nm and above without changing the shape of the resonance excitation profiles towards shorter excitation wavelengths (Fig. 3d). For red cyst cells having large amounts of astaxanthin in the cell wall shielding the carotenoids within the cell (Fig. 3e), a



(a) Dark-adapted sample

(b) Profiles extracted from (a)

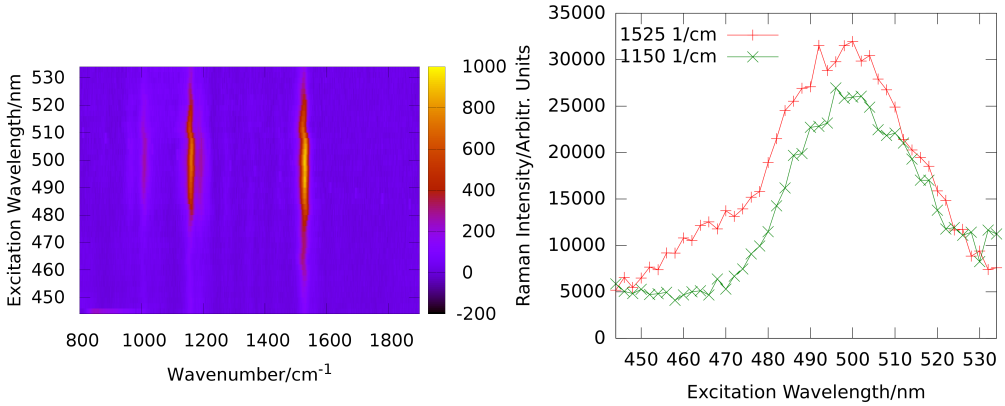
(c) Light-stressed sample

(d) Profiles extracted from (c)

Figure 2: Resonance excitation maps (a, c) and resonance excitation profiles (b, d) of the 1525 cm^{-1}, 1150 cm^{-1} and 1005 cm^{-1} line of two different states of *Chlorella vulgaris*.

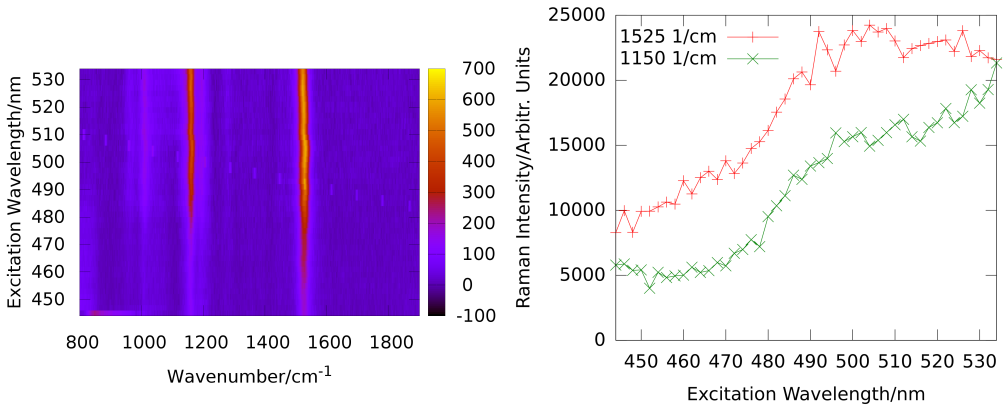
resonance excitation profile with a resonance maximum beyond the upper boundary of the excitation wavelengths accessible in our experiment was found (Fig. 3f). Despite different solvent environments, this is similar to the observation by Salares *et. al.*[36] who found a resonance maximum for excitation wavelengths close to 520 nm with pure astaxanthin dissolved in chloroform at room teperature.

Comparing Figs. 3b, 3d, and 3f shows that the resonance maximum of the carotenoid Raman lines of *Haemaotococcus pluvialis* shifts towards longer wavelengths with increasing astaxanthin content. Therefore, monitoring of pigment production in *Haematococcs pluvialis* using resonance excitation profiles of carotenoid Raman lines of the culture is possible.



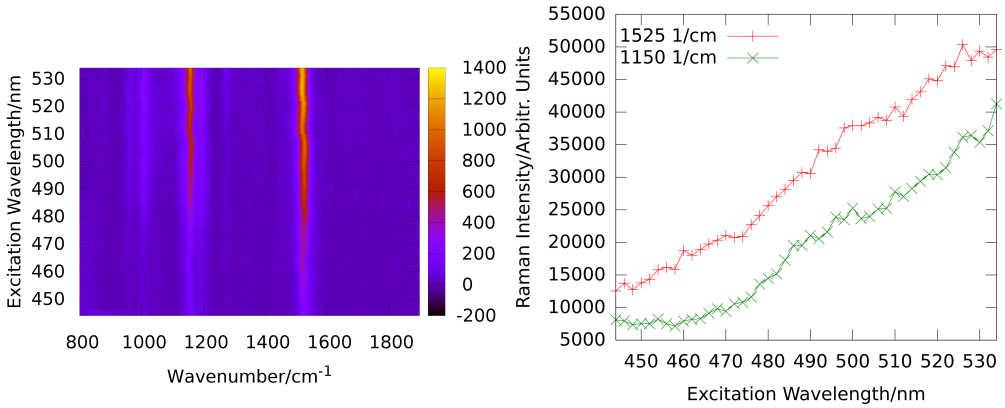
(a) Unstressed green sample

(b) Profiles extracted from (a)



(c) Slightly stressed red tinged sample

(d) Profiles extracted from (c)



(e) Highly stressed red sample

(f) Profiles extracted from (e)

Figure 3: Resonance excitation map of (a) an unstressed green, (c) a slightly stressed red tinged, and (e) a highly stressed red *Haematococcus pluvialis* culture, along with the corresponding resonance excitation profiles (b, d, f).

3.3 Porphyridium purpureum

Porphyridium purpureum was examined under three conditions: in unstressed green, slightly stressed brown, and highly stressed red state.

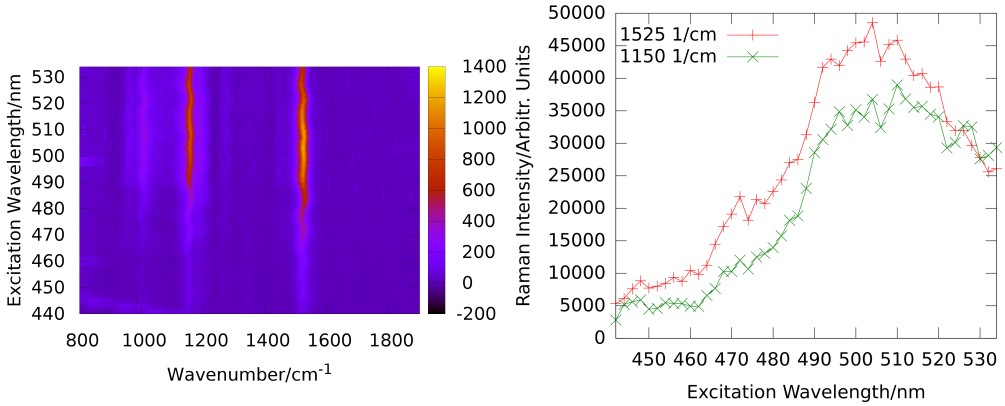
The unstressed green sample (Fig. 4a) shows a resonance maximum close to 505 nm (Fig. 4b), slightly broader than the resonance maximum of the green samples of *Chlorella vulgaris* (Fig. 2b) and *Haematococcus pluvialis* (Fig. 3b).

Porphyridium purpureum is known to synthesise large amounts of the protein based red pigment phycoerythrin when stressed[30]. Together with unstressed green cells, this results in a slightly brown colour of a partially stressed sample (Fig. 4c). Phycoerythrin is not a carotenoid, but nonetheless a large variation in the resonance excitation profiles of the carotenoid bands was found with a resonance maximum shifted beyond 530 nm (Fig. 4d).

Additional stress results in a completely red sample without green cells present and a large amount of phycoerythrin synthesised (Fig. 4e). The resonance excitation map does not change upon transition from the brown to the fully red state. The resonance excitation profiles (Figs. 4d and 4f) are also comparable, indicating either that the maximum stress with regard to carotenoid resonance is already reached in the brown sample or that the phycoerythrin concentration does not correlate directly with the resonance excitation profiles of the carotenoid bands.

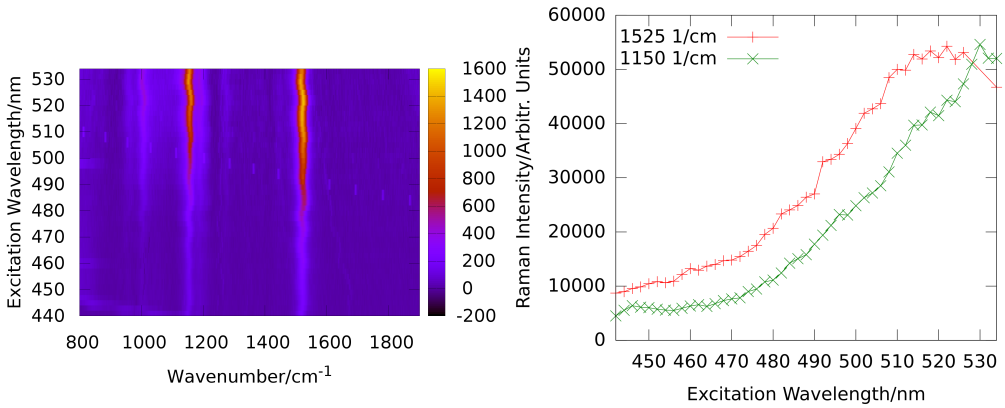
Absorption and Raman spectra of β-carotene and zeaxanthin are very similar[37][38]. Since resonance excitation profiles are linked to absorption spectra[16][36], the resonance excitation profiles for β-carotene and zeaxanthin are expected to be similar. Therefore, the shift of the resonance maximum cannot be explained solely by a transformation of β-carotene into zeaxanthin and vice versa. The Raman bands of phycobiliproteins like phycoerythrin differ from those seen in carotenoids[39], but are comparatively weak and were not observed in the data presented here.

Porphyridium purpureum uses phycoerythrin located in the thylakoid membranes[40] for photosynthesis in green light[41]. While phycoerythrin is a strong fluorophore, the shape of its absorption spectra given in[42] cannot explain the observed redshift of the resonance maximum. However, both β-carotene and zeaxanthin are also located in the thylakoid membranes[43]. Therefore, a change in the environment of the carotenoids contained in *Porphyridium purpureum* due to internal pathways for phycoerythrin synthesis under stress may be possible. This would also lead to the observed changes in the resonance excitation profiles of the unstressed green compared to the slightly stressed brown or fully stressed red state. This hypothesis needs to be checked by additional experiments and is beyond the scope of this work.



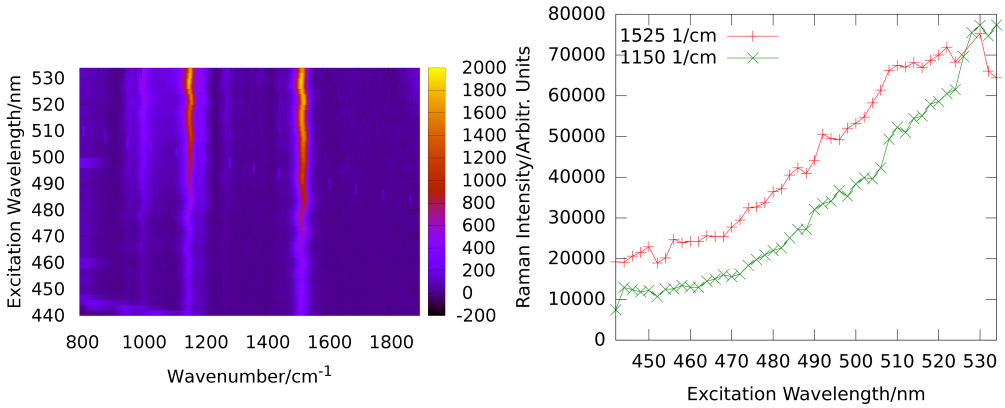
(a) Unstressed green sample

(b) Profiles extracted from (a)



(c) Slightly stressed brown sample

(d) Profiles extracted from (c)



(e) Highly stressed red sample

(f) Profiles extracted from (e)

Figure 4: Resonance excitation map of (a) an unstressed green, (c) a slightly stressed brown, and (e) a highly stressed red *Porphyridium purpureum* culture, along with the corresponding resonance excitation profiles (b, d, f).

4 Conclusion

To determine the resonance conditions of the different stress states of three different species of algae, we successfully measured resonance excitation maps of different states of *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Porphyridium purpureum* and extracted resonance excitation profiles therefrom.

No stress reaction was observed in the resonance excitation maps of *Chlorella vulgaris* in response to intense light.

Synthesis of the secondary carotenoid astaxanthin in response to nutrient deficiency was clearly observed in the resonance excitation maps of *Haematococcus pluvialis* with increasing concentrations of astaxanthin causing a subsequent redshift of the resonance maximum of the carotenoid bands.

Distinctive changes of the resonance excitation profiles of carotenoids were also observed in *Porphyridium purpureum* along with initial phycoerythrin synthesis. Additional stress increasing phycoerythrin production even more was not accompanied with further resonance changes in the carotenoid lines of *Porphyridium purpureum*.

While the redshift in the resonance wavelengths of *Haematococcus pluvialis* is explained by the well-known synthesis of the secondary carotenoid astaxanthin, more physiological research is necessary regarding the cause of the observed redshift of the carotenoid resonance seen in *Porphyridium purpureum* during phycoerythrin synthesis.

Further experiments with improved absolute accuracy of line positions will allow to exploit variations of the carotenoid line positions with excitation wavelengths, giving rise to partially selective excitation of different carotenoid populations within the algae cells.

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