# Violaxanthin cycle kinetics analysed *in vivo* with resonance Raman spectroscopy

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**Abstract**

Measuring the kinetics of the violaxanthin cycle imposes an experimental challenge. Traditionally, carotenoid analysis was done laboriously with high performance liquid chromatography. In this work, we present the first *in vivo* approach to directly measure the kinetics of the violaxanthin cycle, using resonance Raman spectroscopy in combination with baseline correction and principal component analysis. Applying the new approach allows measuring thousands of data points as opposed to the few possible with chemical analysis over the course of a violaxanthin cycle kinetics experiment. *In vivo* analysis of the violaxanthin cycle is necessary to fully understand adaptation kinetics to varying light conditions, the knowledge of which is especially important for assessing the stress tolerance of plants in the wake of the increasing climate change. Three experiments on the green alga *Dunaliella salina* were performed, featuring both the light-to-dark and the dark-to-light transition response of the algae.

**Keywords:** Violaxanthin cycle, Kinetics, Stress adaptation, Resonance Raman, *Dunaliella salina*

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# 1 Introduction

Sunlight, required for photosynthesis, varies considerably in intensity for one location on several time scales. The largest difference is between day and night[1], and on a longer timescale, the seasonal changes[2] reflect themselves in the amount of irradiation per day, both of which are regular and predictable. However, there are also less calculable fluctuations on shorter timescales: Clouds may suddenly change full sunlight to shade[3], and plants in the understory of a forest are subject to even faster changes when the leaves of the trees above are moved by wind, giving what is called sunflecks[4].

Besides light intensity, photosynthesis depends on additional environmental variables such as temperature[5], water availability[6], and carbon dioxide concentration[7], all within specific limits. To maintain a sufficient photosynthetic rate and protect their photosynthetical apparatus from potentially destructive overload conditions, plants developed regulation mechanisms to cope with light intensity fluctuations on various time and intensity scales.

On the seasonal scale, plants slowly adapt their total chlorophyll content, growing different sun and shade leaves[8]. In temperate climate regions, many plants shed their leaves or change colour in winter to survive the difficult conditions of low temperatures and limited water availability in combination with bright sunlight during the day[9]. On the timescale of a day, some plants can adjust the angles of their leaves towards the sun[10]. This is especially obvious during drought conditions, when leaves hang down floppily in order to minimise irradiance per area.

Although some species are capable of fast movements[11], the adaptation to varying light intensity on a shorter timescale is done with chemical regulation mechanisms. On the fastest timescale within seconds, adjusting chlorophyll fluorescence allows plants to quench excess energy by radiation[12][13]. Chlorophyll, which absorbs red and blue light, shows fluorescence in the dark red. The amount of fluorescence depends, among other factors, on the pH level in the thylakoids[14], controlled by photochemical reactions.

Besides chlorophyll, the so called antenna system complexes also contain accessory pigments. These are carotenoids, which can be divided into the yellow oxygen-containing xanthophylls and orange-to-red oxygen-free carotenes. Among other functions, accessory pigments interact with chlorophyll molecules in order to exchange energy. The direction of the energy transfer depends on the individual energy levels of the involved carotenoids. Especially two xanthophylls, violaxanthin and zeaxanthin, are well known to provide a regulatory function called the violaxanthin cycle[15]. Although the exact mechanism is disputed in current literature[16], violaxanthin assists the collection of sunlight, transfering the absorbed energy into chlorophyll and hence to the photochemical reactions.

Zeaxanthin, on the other hand, removes excess energy via heat dissipation. In a reversible enzymatic reaction involving violaxanthin de-epoxidase and zeaxanthin epoxidase[5], plants transform violaxanthin over the intermediate step antheraxanthin into zeaxanthin within a timescale of minutes to a few hours, thus adapting the ratio of collected versus dissipated light energy. The total amount of violaxanthin, antheraxanthin, and zeaxanthin is called the violaxanthin cycle pool size, which gives the total regulation capacity of this mechanism and can be adjusted by synthesising additional violaxanthin[16].

Photoinhibition, involving the temporary self-destruction of the photosystem, is another last-resort protection against extreme light intensities[17]. However, it occurs only under extreme conditions and subsequently needs a long recovery time.

Jointly, all of these mechanisms determine the ability of a plant to grow and survive under widely varying light conditions.

Very few measurements were conducted for the violaxanthin cycle kinetics so far[1][5][16][18][19]. Experimental access is difficult and relied on laborious chemical sample analysis with high performance liquid chromatography[20], requiring destructive sample preparation. *In vivo* analysis of the violaxanthin cycle is necessary to fully understand adaptation kinetics for varying light conditions, the knowledge of which is especially important for assessing the stress tolerance of plants in the wake of the increasing climate change.

Carotenoid detection with Raman spectroscopy is a well established experimental technique. Many studies on functional aspects and the configuration of carotenoids within the photosystem[21][22][23][24] were done with resonance Raman spectroscopy. First hints on the observability of the violaxanthin cycle in Raman spectra were published accompanying research on photoinhibiton[17].

As all higher plants and green alga share the same photosynthetic configuration[5][25], the green alga *Dunaliella salina* was chosen as a model system. As a unicellular microalga, many independent individuals are easily maintained and by quickly stirring the culture, stress due to prolonged exposure to the measurement beam is avoided. As this species is used commercially for the production of beta carotene, it is readily available from algae culture collections. In addition, *Dunaliella salina* is well known for its extreme salt tolerance, which prevents infections often impairing freshwater algae cultures[26].

In this work, we present the first *in vivo* analysis of the violaxanthin cycle kinetics by means of resonance Raman spectroscopy on *Dunaliella salina* in combination with baseline correction and principal component analysis.

# 2 Experimental aspects

## 2.1 Culture conditions and biological setup

The medium was prepared according to the recipe for Dunaliella medium[27] ("Dun" recipe, Culture Collection of Algae at the University of Göttingen, Germany) replacing the 30 ml soil extract with 5 ml inorganic salt micronutrient solution as described for common seawater medium ("SWES" recipe) and 25 ml distilled water to avoid introducing unwanted features in the Raman spectra by usually unknown soil components. The green alga *Dunaliella salina* (SAG strain 184.80) was cultivated successfully at a temperature of $22°C$ in this modified medium for half a year. The algae were grown in an airlift agitation reactor specifically made from borosilicate glass. The cultures were kept in the low light regime by 2.5 $\mu\text{mol} * \text{s}^{-1} * \text{m}^{-2}$ photons of photosynthetically active radiation, provided by red and blue LED strips (628 nm and 467 nm, Barthelme, Nürnberg, Germany). Aeration was provided by compressed air through a gas wash flask filled with distilled water, minimising evaporation of the culture medium, and a sterile filter with 200 nm pore size.

For each experiment, a volume of 200 ml culture solution in the exponential growth phase was transferred into a 250 ml Erlenmeyer flask. At low cell density, all cells in the culture flask are equally illuminated, experiencing the same level of photo stress. However, the achievable signal to noise ratio of recorded Raman spectra is also low. At high cell density, attenuation of the stress illumination in the culture itself prevents stress of the individual cells. Therefore, a medium cell density in the order of $1.5 * 10^6$ cells/ml as confirmed with a Thoma cell counting chamber was used in the experiments.

## 2.2 Physical setup

A 250 ml Erlenmayer flask containing 200 ml of algae culture was placed on a magnetic stirrer and next to a high-power LED illumination source. Phlatlight LEDs (Luminous Devices, Woburn, USA, CBT-90-RX-L15-BN101 for red illumination at 611 - 631 nm and CBT-90-B-L11-J101 for blue illumination at 450 - 470 nm) mounted on a heatsink to avoid heating the sample flask were used. The illumination output was adjusted to $3,000$ $\mu\text{mol} * \text{s}^{-1} * \text{m}^{-2}$ photons of photosynthetic active radiation as measured with a Li-190SA (Li-Cor, Lincoln, USA) quantum sensor at the outside surface of the flask. The temperature was kept constant at $22°C$. Illumination schedules were determined empirically for each experiment. Details are given in the experimental sections.

As carotenoids, both violaxanthin and zeaxanthin exhibit resonance effects. These can be exploited by choosing appropriate Raman excitation wavelengths

close to the 0-0 transitions. We employed an excitation wavelength close to the 0-0 transition of violaxanthin and within the Soret band of chlorophyll b, but far from the 0-0 transition of zeaxanthin[21]. An Excelsior single mode laser (Spectra physics, Santa Clara, USA) with 50 mW continuous wave output at 473 nm was used for excitation. Resonance Raman measurements were performed with a custom-built sea-water resistant fibre bundle (CeramOptec, 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 arranged into a line in the ferrule on the spectrometer side. Raw spectra were taken with a SR500 spectrograph (Andor, Belfast, Northern Ireland), equipped with a 473 nm RazorEdge long-pass filter (Semrock, Rochester, USA), a 20 µm slit, either with a 1200 lines/mm or 2400 lines/mm grating blazed for 500 nm and an Andor Newton DU940P camera. On average, the setup maps 0.8 $cm^{-1}$ on every pixel with the 1200 lines/mm grating and 0.3 $cm^{-1}$ with the 2400 lines/mm grating. Spectra were taken with 20 x 1 s accumulation time and cosmic ray removal by means of comparing individual accumulated spectra and interpolating across detected spikes. While the accumulation time for each spectrum was exactly 20 s, the data transition from the camera chip to storage required 0.885 s, giving a total acquisition time for each spectrum of 20.885 s. A typical raw spectrum is shown in Fig. 1.

In order to avoid triggering stress reactions solely with the beam necessary for capturing spectra, the fibre bundle was positioned freely in the stirred culture solution so that individual algae cells were in the beam only for very brief periods of time, maintaining a steady glow of chlorophyll fluorescence. In combination with the small irradiated probe volume, the culture could be maintained without visible stress reactions in this setup for many days.

Wavelength calibration was based on the spectral lines of a neon glow lamp. For testing long-term stability, eight neon glow lamps were embedded in a tile of acrylic glass and placed between the flask and the magnetic stirrer plate so that calibration lines were available in the otherwise unchanged experimental setup.

Baseline correction was done as described in Koch *et. al.*[28] 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. Line positions were determined by fitting Lorentzian line shapes with a least squares algorithm. Principal component analysis (PCA) was done with "MCR-ALS command line", obtained from http://www.mcrals.info/ and run on GNU Octave[29]. The concentrations of components are derived with PCA from Raman intensities as in Fig. 1.

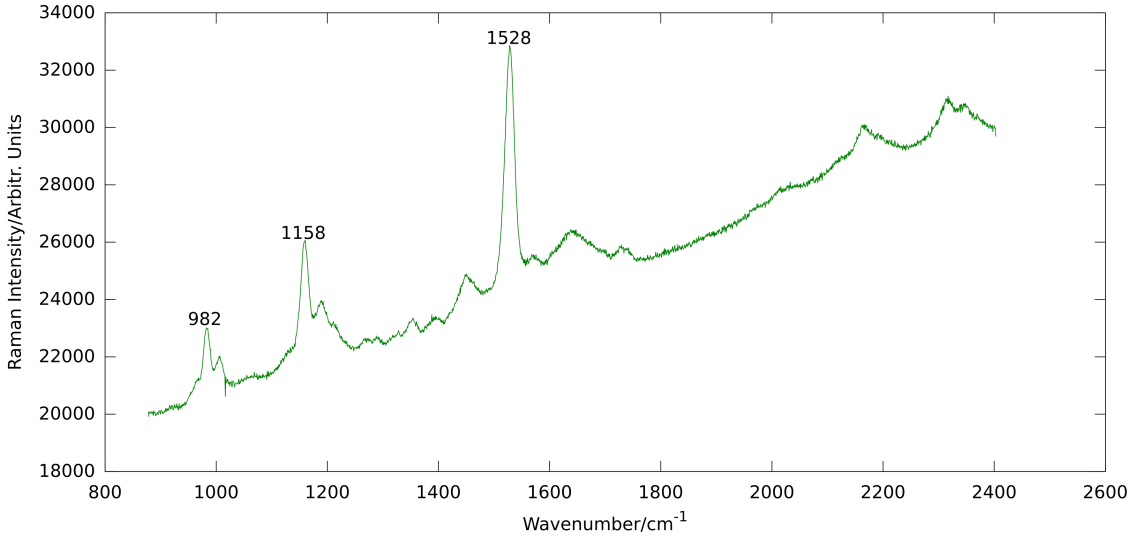


Figure 1: Typical raw resonance Raman spectrum of *Dunaliella salina* culture solution adapted to darkness with 473 nm excitation. The strongest signals visible are the $\nu_1$ (1528 cm$^{-1}$), $\nu_2$ (1158 cm$^{-1}$) and $\nu_3$ (982 cm$^{-1}$) bands of the carotenoids found in the algae. Chlorophyll bands are visible between 1600 cm$^{-1}$ and 1800 cm$^{-1}$. The fluorescent background visible here was removed subsequently by baseline correction.

# 3 Results and Discussion

## 3.1 Violaxanthin cycle kinetic features as determined by principal component analysis (PCA)

In an initial experiment, 200 ml of *Dunaliella salina* culture solution were subjected to an illumination scheme repeating 42 minutes of stress, induced by blue illumination, followed by 459 minutes of subsequent darkness for relaxation. The spectral features as shown in Figs. 2 and 3 are the result of eight repetitions in total.

In accordance with spectral deconvolution results from Ruban *et. al.*[21], four components were used. As expected, two components of the PCA results maintain a nearly constant level during the violaxanthin cycle reactions (see Fig. 4). These two PCA components represent a mixture of chlorophylls (most probably chlorophyll b due to excitation within its Soret band) and different carotenoids embodied in the algae which are not directly involved in the violaxanthin cycle reactions. Due to characteristics of the PCA, components that do not change independently over the course of the experiment cannot be resolved. Therefore, an

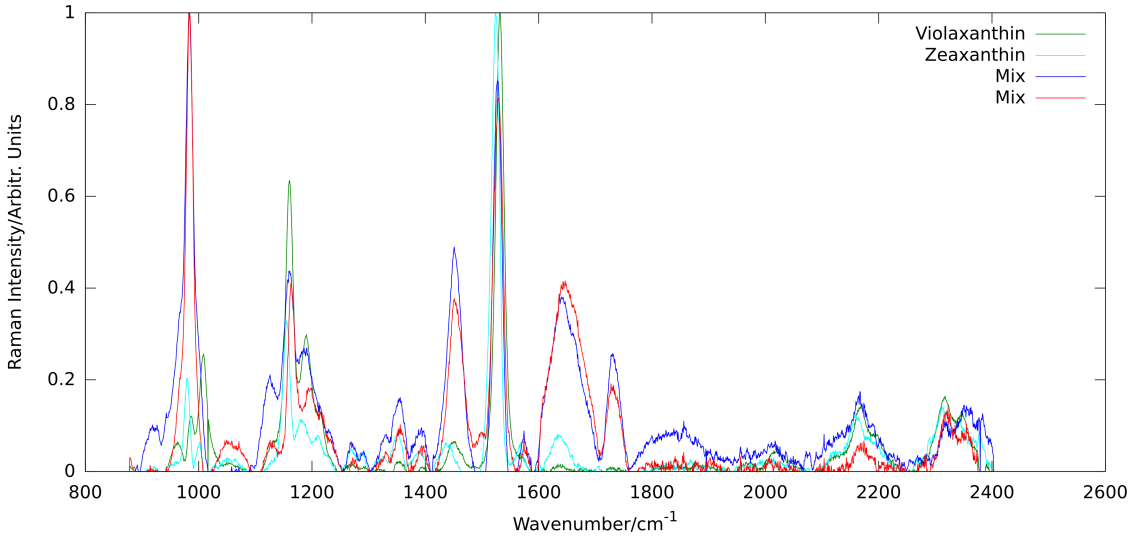


Figure 2: Spectra of violaxanthin (green trace), zeaxanthin (cyan) and two additional chlorophyll and carotenoid mixture components (red and blue) as determined by PCA of eight illumination repetitions on *Dunaliella salina*.

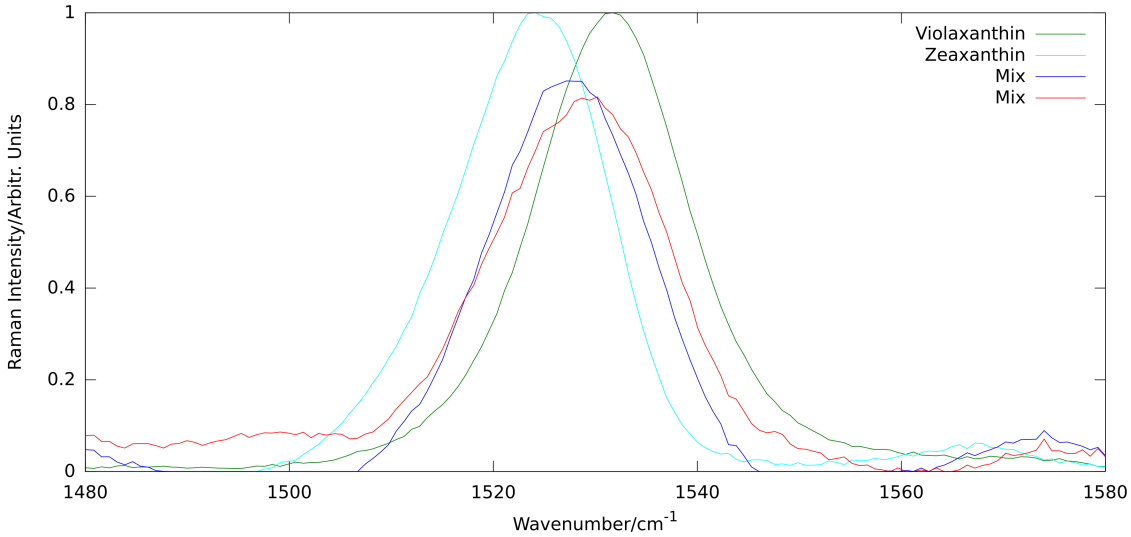


Figure 3: The Raman lines centered at 1525 $cm^{-1}$. Note the clearly visible difference in peak positions for violaxanthin (green trace) and zeaxanthin (cyan).

identification of the carotenoids involved in both of the mix components is not possible. Nevertheless, the line positions suggest that lutein may be involved. As

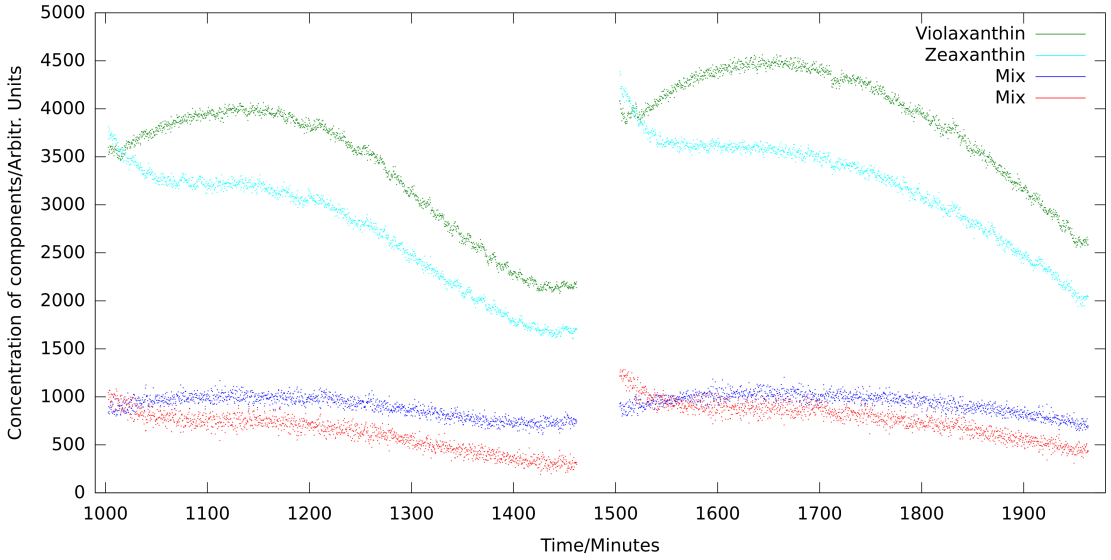


Figure 4: Changes in the concentrations of violaxanthin (green trace), zeaxanthin (cyan) and two additional chlorophyll and carotenoid mixture components (red and blue) over two illumination repetitions as determined by PCA from the intensities of the individual component signals. Spectra were evaluated only over the dark period, resulting in the gap during illumination. For both repetitions, after the end of illumination, a decrease of the zeaxanthin concentration together with an increase of violaxanthin concentration can be observed (light-to-dark adaptation), subsequently followed by a more distinct concentration decrease of both zeaxanthin and violaxanthin (pool size adjustment).

PCA determines the Raman spectra of individual components, a comparison with isolated carotenoids seems appropriate, although due to solvent effects, isolated carotenoids in solvents may exhibit a line shift compared to carotenoids embedded in biological material[30]. However, the solvent pyridine is known to reproduce line positions of carotenoids embedded in biological materials quite well[24]. The component line positions of the $\nu_1$ band for zeaxanthin (1524 $cm^{-1}$) and violaxanthin (1531 $cm^{-1}$) compare well to the values found by Ruban *et. al.*[21] in pyridine solution at 473 nm excitation for isolated zeaxanthin (1522 $cm^{-1}$) and for isolated violaxanthin (1529 $cm^{-1}$).

Raw Raman spectra were captured in the presence of stress illumination, but were not suitable for analysis as the blue stress illumination completely overwhelmed the weak Raman signals in the recorded spectra. Therefore, only the light-to-dark response was observed in the first two experiments.

As can be seen in Fig. 4, immediately following the end of the illumination, the light-to-dark adaptation occurs as a decrease of the zeaxanthin content in

conjunction with an increase of violaxanthin. This is similar to the kinetics seen in pea plants as reported by Jahns[18]. The light-to-dark adaptation is studied in greater detail in the following two experiments. On a larger time scale, a subsequent decrease of both violaxanthin and zeaxanthin can be seen, corresponding to a reduction of the violaxanthin cycle regulation capacity in extended periods of darkness. Pool size adjustments of the carotenoids involved in the violaxanthin cycle are known, but on a larger time scale of a few days, as reported by Nichelmann *et. al.*[16].

## 3.2 Light-to-dark transition kinetics

In order to quantify the response time of the violaxanthin cycle, the light-to-dark transitions were queried in detail by using 21 minutes of stress, induced by blue illumination, followed by 230 minutes of subsequent darkness (see Figs. 5 and 6).

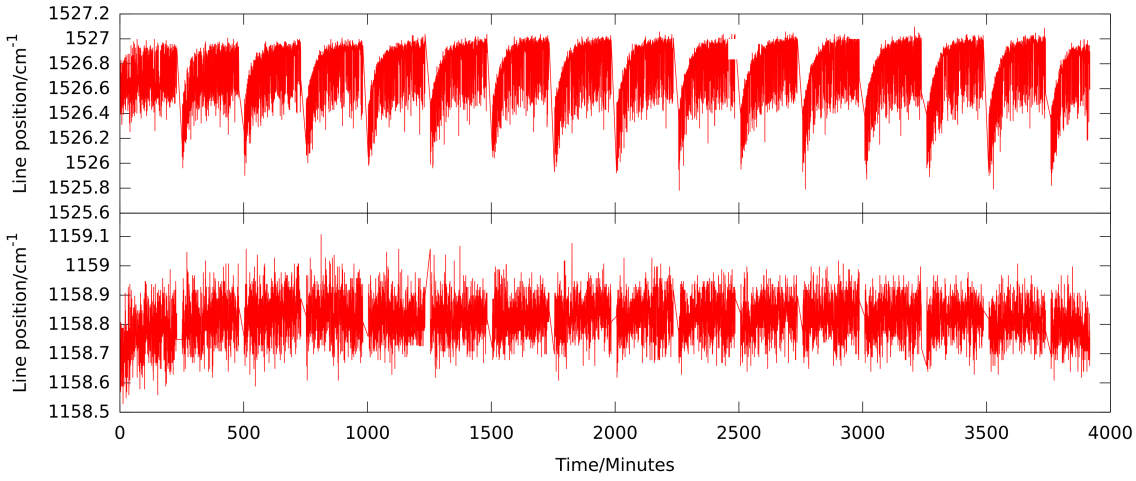


Figure 5: Line positions as determined by Lorentzian fitting to the $\nu_1$ (upper panel) and $\nu_2$ (lower panel) bands of 10,354 raw Raman spectra during 16 repetitions. Those bands represent a mix of all carotenoids available in the algae cells. While the position of $\nu_2$ at 1158.8 $cm^{-1}$ remains nearly constant, the $\nu_1$ band exhibits a periodic shift of 1 $cm^{-1}$ between 1526 $cm^{-1}$ and 1527 $cm^{-1}$ in response to the repetition of illumination and subsequent darkness.

To compare the measurements to known characteristics of the violaxanthin cycle prior to more sophisticated analysis, the line positions of the $\nu_1$ and $\nu_2$ band of the involved carotenoid mix were identified with Lorentizan fitting. Within

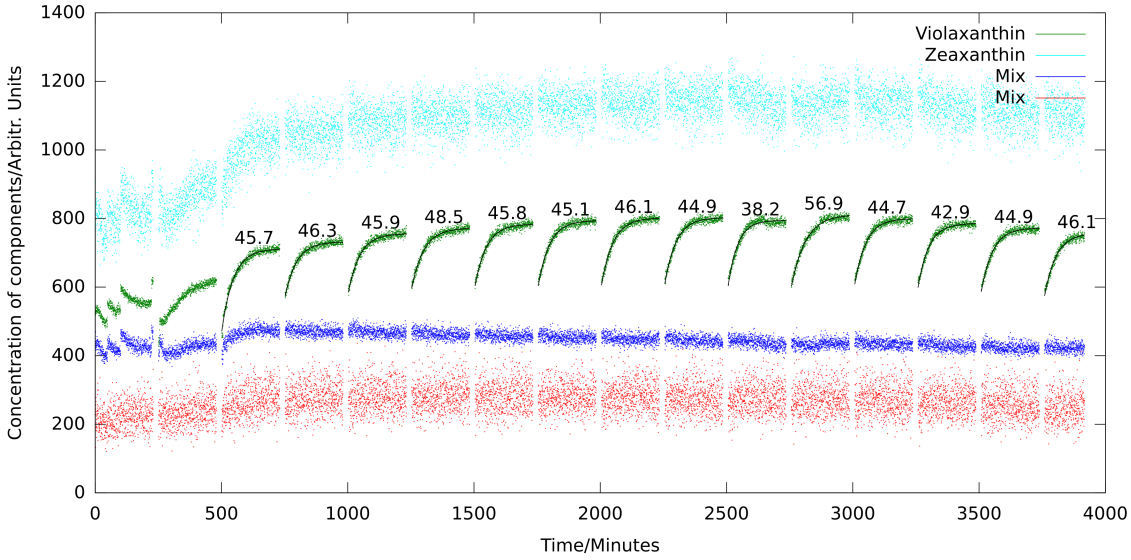


Figure 6: Light-to-dark cycle kinetics determined by fitting individual exponentials to 14 repetitions of the violaxanthin trace (green) after PCA of the same data as in Fig. 5. Embedded numbers give the time constant in minutes for each fitted light-to-dark response with a mean of 46 minutes. Complementary kinetics are barely visible in the zeaxanthin trace (cyan). The low signal to noise ratio of this component is due to off-resonance excitation and could be improved by employing a second excitation wavelength closer to the 0-0 transition of zeaxanthin. Non-periodic transient responses due to the transfer from the airlift reactor with continuous low light to the stirred Erlenmayer flask with periodic stress illumination and artefacts due to experimental adjustments are visible in the first two repetitions not used for time constant determination. Two additional traces (red and blue) represent chlorophylls and other algal carotenoids not directly involved in the violaxanthin cycle.

the quality of the Lorentzian fit (see Fig. 5), a nearly constant $\nu_2$ band position was confirmed in accordance with the findings of Ruban *et. al.*[21]. Periodic shifts of the $\nu_1$ band position between 1526 $cm^{-1}$ and 1527 $cm^{-1}$ are within positions given in[21] for isolated zeaxanthin (1522 $cm^{-1}$) and isolated violaxanthin (1529 $cm^{-1}$) in pyridine. A downshift of the $\nu_1$ line position of 3 $cm^{-1}$ is to be expected for samples enriched with zeaxanthin[21]. In comparison, a downshift of 1 $cm^{-1}$ was observed here for the $\nu_1$ band of the involved carotenoid mix, as can be seen in Fig. 5. Also, compared to Ruban *et. al.*[21], the smaller shift observed is probably due to incomplete conversion into zeaxanthin, as measurements there were done on prepared thylakoid membranes as opposed to whole algae cells in this work.

The time constant of the light-to-dark response of the violaxanthin cycle was determined by subjecting this dataset to PCA, which results in less noise. Exponential fitting to the violaxanthin trace as in Fig. 6 returns a mean of 46 minutes for the light-to-dark transition time constant.

## 3.3 Dark-to-light transition kinetics

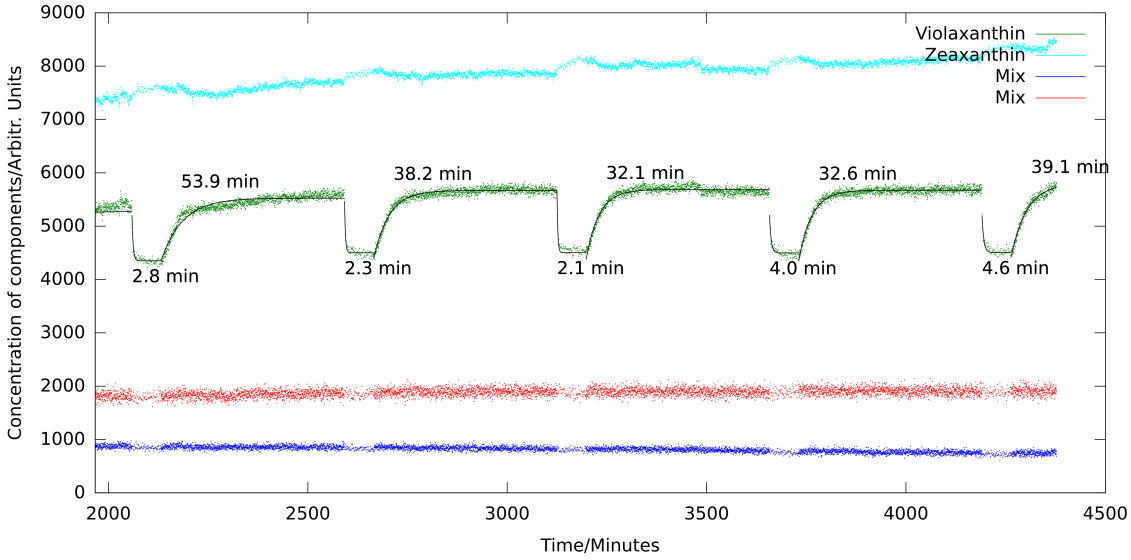


Figure 7: Kinetics of the light-to-dark transition response (upper level of the violaxanthin trace, 39 minutes mean) and dark-to-light response (lower level of the violaxanthin trace, 3.2 minutes mean) of the violaxanthin cycle as determined by exponential fitting to the violaxanthin trace (green) of the PCA result. Complementary kinetics are visible in the zeaxanthin trace (cyan), albeit with a lower signal to noise ratio than in the violaxanthin trace, as the employed excitation wavelength matches the 0-0 transition of violaxanthin more closely than the 0-0 transition of zeaxanthin. Two additional, nearly constant traces (red and blue) represent chlorophylls and carotenoids not directly involved in the violaxanthin cycle reactions.

The dark-to-light transitions were queried in detail by using 73 minutes of stress, induced by red illumination, followed by 459 minutes of subsequent darkness (see Fig.7). The stress illumination was periodically interrupted for 21 seconds to measure one undistorted Raman spectrum subsequently followed by 42 seconds of illumination. Given a light-to-dark response time of 46 minutes, the effects of the interruptions on the violaxanthin cycle were considered negligible. In short, this resulted in a total illumination time of 49 minutes per repetition.

The capture of the data started after three full repetitions of the stress illumination allowing for the algae to adapt to the change in culture conditions.

A time constant for the dark-to-light response of 3.2 minutes and for the light-to-dark response of 39 minutes was found. This compares well with the observations made with HPLC on pea plants in Jahns[18] and on star duckweed in Latowski *et. al.*[5]. In comparison with the second experiment which gave a time constant of 46 minutes, a slightly shorter light-to-dark time constant was found due to different illumination conditions. This is consistent with a similar observation by Jahns and Miehe in[17] regarding the light-to-dark kinetics being partially dependent on the illumination intensity.

# 4 Conclusion

In this article, we presented the first *in vivo* violaxanthin cycle kinetics measurements of *Dunaliella salina*. Time constants were determined for the light-to-dark transition between 39 and 46 minutes depending on experimental conditions. For the dark-to-light transition, a time constant of 3.2 minutes was found.

Especially the line positions for the $\nu_1$ band of violaxanthin and zeaxanthin obtained through principal component analysis were found to be within 2 $cm^{-1}$ in accordance with published values for isolated violaxanthin and zeaxanthin in pyridine at the same excitation wavelength[21], while the kinetics themselves were found to be slightly faster than found for species of higher plants analysed with chemical HPLC[5][18].

This work presents the experimental foundation for *in vivo* measurements of violaxanthin cycle kinetics, allowing studies with many more data points than possible with HPLC, as well as forgoing destructive sampling.

More research is necessary to determine the environmental factors influencing the stress adaptation and the relaxation timing. Especially stress illumination schedule and wavelength should be explored. Data shown here indicates that the first repetitions exhibit a transient response different from the periodic steady state reached when the sample is adapted to the new culture conditions. Changing the illumination schedule during an experiment might give rise to additional transient behaviours. Using a second excitation wavelength closer to the 0-0 transition of zeaxanthin such as the 514.5 nm line of an Argon ion laser will enhance the signal-to-noise ratio of the zeaxanthin traces to more clearly demonstrate the interconversion of violaxanthin and zeaxanthin. Further experiments may also benefit from an additional optical filter between the LED and culture flask to allow simultaneous stress illumination and Raman measurements.

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