

BLUE-FLUORESCENCE OF NADPH AS AN INDICATOR OF MARINE PRIMARY PRODUCTION

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ABSTRACT

Nicotinamide Adenine Dinucleotide Phosphate (NADPH) is the primary product of photosynthesis and can therefore serve as an indicator of biomass and photosynthetic activity. Pure NADPH which is the reduced form of NADP shows an absorption maximum at 340 nm and a maximum of emission at 460 nm. NADPH concentrations in terrestrial vegetation have already been studied since 1957 in great detail with optical methods. However, its potential as a biomass parameter of oceanic phytoplankton which can be assessed *in situ* and remotely with fluorescence spectroscopy has not yet been investigated.

In this paper, we report on laboratory investigations of the blue-fluorescence spectrum in algal suspensions of *Chlorella* and *Thalassiosira* when excited with UV-A light. It is shown that cell densities of about 10^6 per litre as they are typically found under natural conditions are too low for precise detection of NADPH fluorescence, while concentrated samples with 10^8 - 10^{10} cells per litre exhibit significant blue-fluorescence which can be related to NADPH. Inhibition of photosynthetic activity by addition of DCMU decreases the strength of blue-fluorescence remarkably. Since NADPH is an end product of photosynthesis, changes of PAR illumination levels should directly affect its concentration and hence the intensity of blue-fluorescence. However, no effect of illumination on blue-fluorescence could be observed in our study. Possible reasons of these observations are discussed, and perspectives for practical applications of the method used are proposed.

Keywords: NADPH fluorescence, phytoplankton, photosynthetic activity, primary production

INTRODUCTION

The determination of biomass and primary production of photosynthetically active phytoplankton is done by analysing organic carbon content and fixation. Alternative methods for assessing phytoplankton biomass are based on the concentration of extracted chlorophyll *a* which is measured using spectrophotometry, fluorescence spectroscopy or high performance liquid chromatography (HPLC) (1). Primary production is determined by directly measuring C^{14} bicarbonate fixation or oxygen production (2). As opposed to this, productivity can be estimated only indirectly on the basis of optical seawater properties.

With *in situ* fluorometers, phytoplankton distributions are easily detected measuring the fluorescence emission of chlorophyll. But there is a great variety of physiological processes which influence the chlorophyll fluorescence yield (3), thus making the quantitative interpretation of these signals very difficult. Various fluorescence methods have been developed to investigate the physiological state of phytoplankton such as pulse-amplitude modulated fluorescence excitation (4,5), the pump-and-probe-method (6,7), laser-induced fluorescence saturation spectroscopy (8), and the detection of delayed chlorophyll fluorescence (9,10).

The usefulness of many of these methods to determine phytoplankton biomass has been demonstrated with submersible *in situ* sensors. However, only the pump-and-probe fluorescence lidar can be applied for remote sensing, with instruments installed onboard aircraft, to derive data on the physiological state of phytoplankton. Thus, more methods are highly desirable for investigations of primary production which can be applied *in situ* or with remote sensing.

A parameter for direct measurements of the photosynthetic activity is the fluorescence of NADPH, the reduced form of Nicotinamide Adenine Dinucleotide Phosphate (NADP), representing the primary end product of photosynthesis. Photosynthesis takes place at the inner membrane of chloroplasts forming the thylakoids. The light reaction of photosynthesis starts with the splitting of two water molecules into oxygen, protons and electrons using the energy of a photon absorbed by chlorophyll of photosystem II (PS II). (PS II shows the typical fluorescence emission maximum at 680 nm.) The electrons pass an electron-transport-chain (ETC) via several redox reactions until reaching PS I where NADP is reduced. At the end of the light-driven ETC electrons from water oxidation are used to produce NADPH which is essential for biomass production in the Calvin cycle. The arrangement of redox donors and acceptors involved in ETC according to their redox potential results in the so-called Z-scheme (Figure 1).

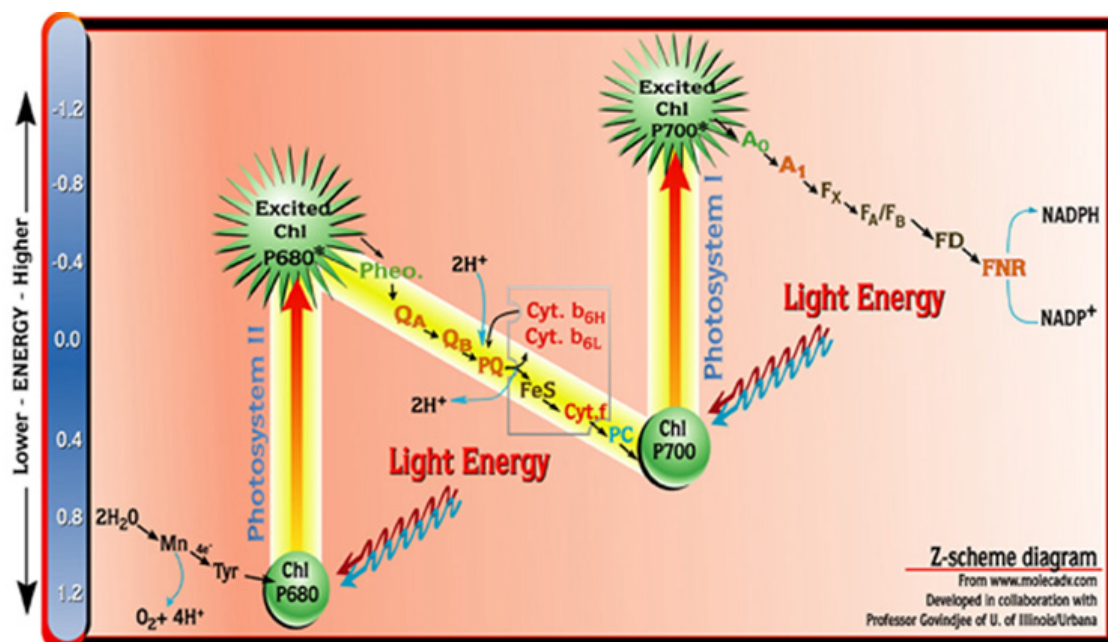


Figure 1: Z-Scheme diagram of photosynthesis. Abbreviations: Mn for a manganese complex containing 4 Mn atoms; Tyr: tyrosine; P680: reaction centre chlorophyll in PS II; Pheo: pheophytin molecule; QA and QB: plastoquinone molecules; FeS: iron sulphur protein; Cyt b and Cyt f: Cytochrome molecules; PC: copper protein plastocyanin; P700: reaction centre chlorophyll in PS I; A₀: chlorophyll molecule; A₁: phyloquinone molecule; F_x, F_A, F_B: iron sulfur centres; FD: ferredoxin; FNR: Ferredoxin-NADP-oxido-Reductase.

Adopted with permission from: <http://www.life.uiuc.edu/govindjee/ZSchemeG.html>,

see also <http://www.life.uiuc.edu/govindjee/textzsch.htm>

NADPH absorbs light in the UV-A spectral range, resulting in fluorescence at blue wavelengths. First investigations were already performed in 1950-60, with phytoplankton and plant leaves, or separated chloroplasts from plant leaves (11,12,13,14), and a correlation of actinic illumination and intensity of blue-fluorescence of NADPH has been observed. Later on, NADPH fluorescence excited in higher plant leaves and their separated chloroplasts were studied with similar results (15,16). Phytoplankton, however, was further studied in a few cases only (17).

In this paper, we report on experiments (acceleration of electron transport chain by PAR illumination and brake of electron transport chain with a herbicide) on blue-fluorescence of several phytoplankton species with the aim to use NADPH fluorescence as a direct indicator of living algal biomass, which should be more precise than chlorophyll fluorescence.

METHODS

Absorption properties of NADPH (Sigma, N 6505, tetrasodium salt) were measured with a Perkin Elmer Lambda 18 spectrophotometer equipped with quartz cuvettes having a path length of 1 cm.

Emission spectra were taken with a Shimadzu RF-1501 spectrofluorometer, with a 10 nm spectral bandwidth of the excitation and emission monochromators. For suppression of straylight and higher orders of diffraction, a UG11 colour glass filter (Schott) was used in the excitation channel and a KV418 edge filter in the emission channel. Standard 1 cm x 1 cm quartz cuvettes were used in all measurements. The spectra were corrected for the wavelength-dependent sensitivity of the instrument. The spectral correction was determined measuring the emission spectra of a 10^{-7} M Quinine sulfate solution and the emission of a calibrated tungsten lamp, and comparing each with their known standard spectrum. Water Raman scattering was eliminated from the emission spectra by subtracting the emission spectrum of purified water measured with identical instrumental settings. Spectra of purified water were also used to normalize the sample fluorescence to the wavelength-integrated water Raman scatter band, yielding fluorescence data in Raman units (18).

Algal suspensions of *Chlorella vulgaris* were kept in a *Basal* culture medium without soil extract (19), and *Thalassiosira rotula* in an *f/2* culture medium diluted with seawater (20) at room temperature. Two LEDs with emission at 680 nm and with 35 nm bandwidth were used for PAR illumination of the algal samples.

The herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, Sigma, D 2425) was used to inhibit photosynthesis. A volume of 100 μ l of a 1 mM DCMU solution in 40 mM phosphate buffer (KH_2PO_4 , pH=6.5) was added to 1.5 ml algal suspension. The DCMU solution itself does not absorb excitation radiation at 360 nm or fluorescence emission centred at 460 nm, and it does not fluoresce in the spectral region of NADPH-fluorescence.

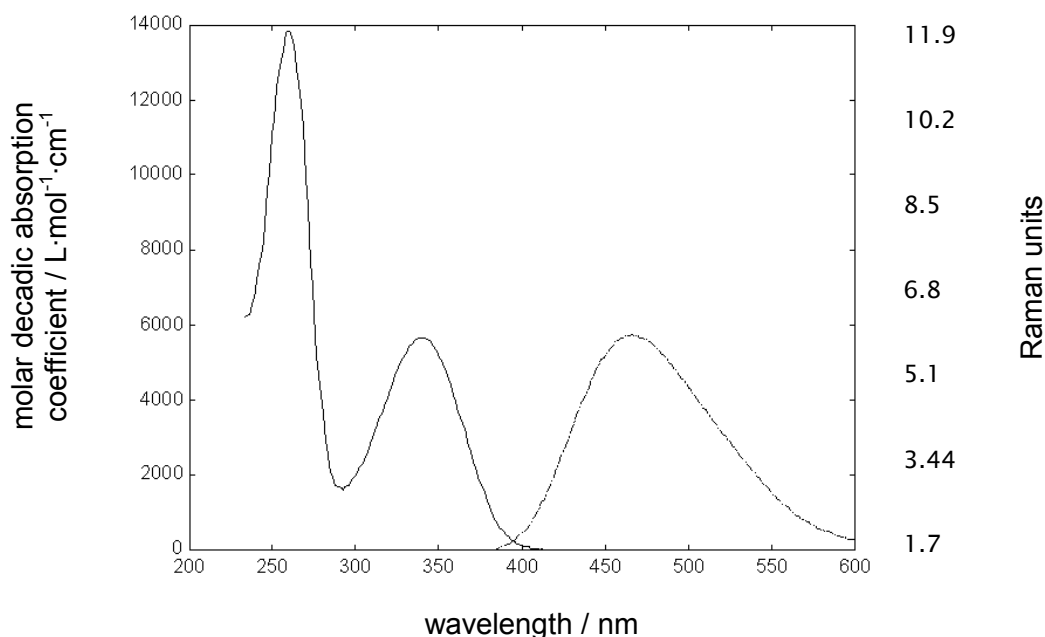


Figure 2: Absorption and emission (dashed line, excitation at 360 nm) spectra of an aqueous NADPH solution (40 μ M). Due to subtraction of the spectrum of pure water, the water Raman scattering peak is not visible.

RESULTS

The absorption maximum at 260 nm is caused by the Adenine group of the NADPH molecule, that at 339 nm by the Nicotinamide group (Figure 2). The molar decade absorption coefficient of NADPH at 340 nm is $5660 \text{ L}\cdot\text{mol}^{-1}\cdot\text{l}^{-1}$. These results agree with those given in the literature.

The fluorescence emission of algal samples (Figs. 3 and 4) of $12 \cdot 10^9$ cells L^{-1} (*Chlorella vul.*) and $2 \cdot 10^7$ cells L^{-1} (*Thalassiosira rot.*) with 360 nm excitation showed a maximum at 457 nm and 450 nm, respectively. However, PAR-illumination did not change the intensity of fluorescence at this wavelength (data not shown). For this experiment a dark adapted (30 min) algal suspension was permanently illuminated with light at 680 nm and the intensity of 460 nm blue-fluorescence which is assumed to be related to the NADPH concentration was detected over the next 30 min.

Treatment with the herbicide DCMU, interrupting the ETC between PS I und PS II, caused both an increase of chlorophyll *a* fluorescence at 680 nm and a decrease of the 460 nm blue-fluorescence of about 17 to 27 % (Figs. 3 and 4). The different peak ratios of 460 nm to 680 nm in Figs. 3 and 4 result from a stronger re-absorption of blue-fluorescence in the more concentrated cell suspension caused by photosynthetic pigments (chlorophylls and carotinoids) (21).

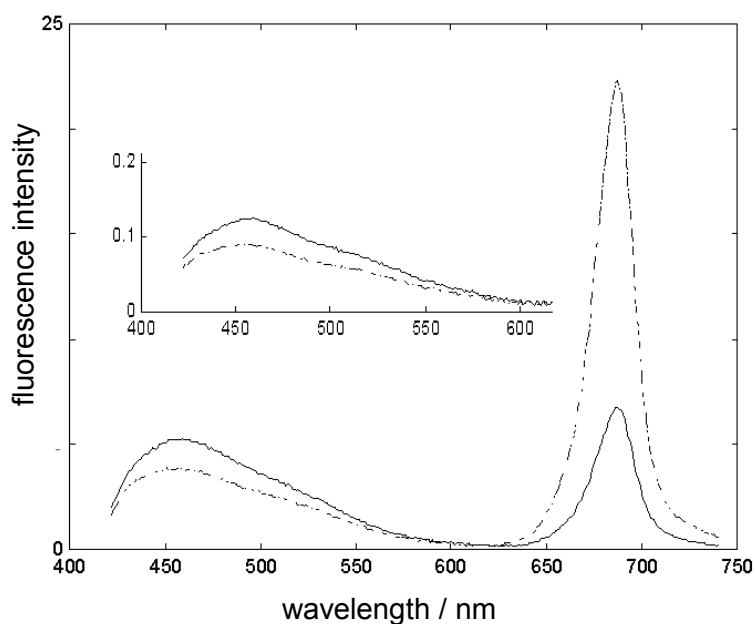


Figure 3: Emission spectra of algal suspension (solid line) and changes after treatment with DCMU (dashed line), the small diagram shows the corrected spectra (ordinate values in Raman units $\times 0.16$), *Chlorella vulgaris*, $12 \cdot 10^9$ cells L^{-1} , $\lambda_{ex} = 360$ nm.

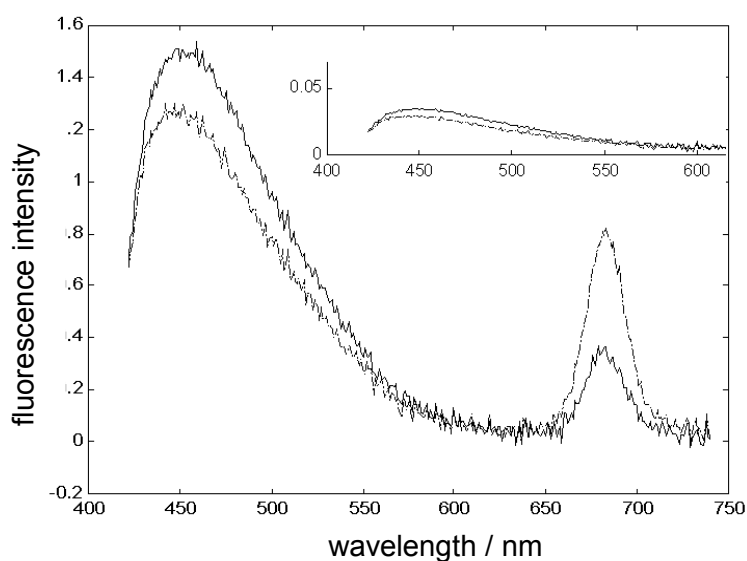


Figure 4: Emission spectra of algal suspension (solid line) and changes after treatment with DCMU (dashed line), the small diagram shows the corrected spectra (ordinate values in Raman units $\times 0.16$), *Thalassiosira rotula*, $2 \cdot 10^7$ cells L^{-1} , $\lambda_{ex} = 360$ nm.

Time-scans of blue-fluorescence performed with algal suspensions adapted to darkness (30 min) did not show any effect on the blue fluorescence at continuous illumination with radiation of 360 nm, although the 680 nm chlorophyll *a* fluorescence emission showed the typical behaviour which is denoted as Kautsky effect (Figure 5).

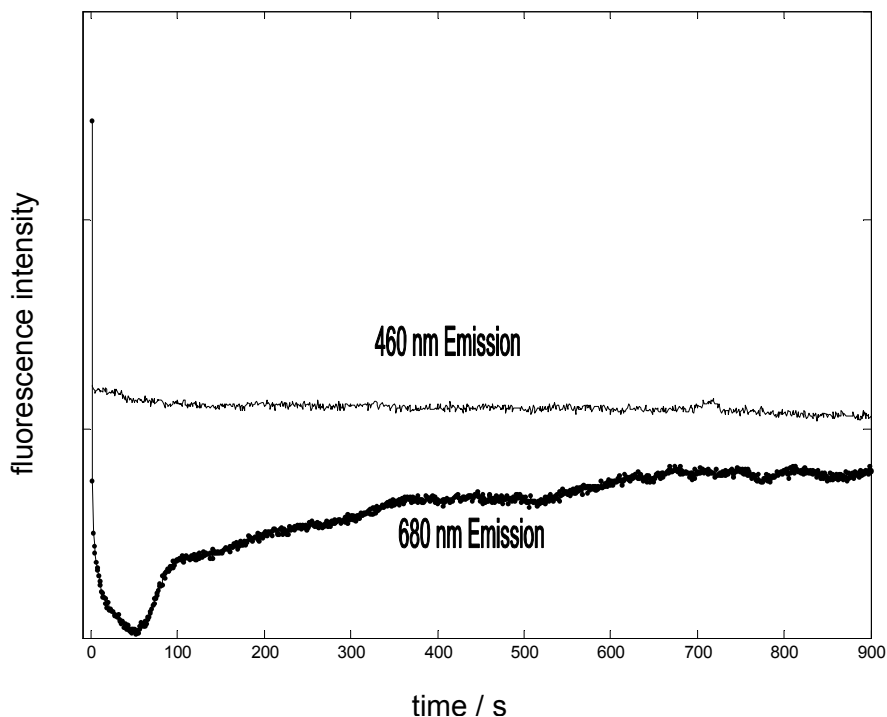


Figure 5: Fluorescence-time-scan of a dark adapted suspension of *Chlorella vulgaris*. Illumination at 360 nm wavelength starts at $t = 0$ (arbitrary intensity ratio of both spectra) .

DISCUSSION

The results shown above support the hypothesis that cellular NADPH contributes to fluorescence at around 460 nm wavelength. Fluorescence changes effected by treatment with DCMU reveal the connection of this blue-fluorescence to photosynthesis. We have expected similar changes in blue fluorescence to occur with different levels of photosynthetically active radiation. However, this could not be verified in our experiments. Our results do not confirm that NADPH is the sole source of the blue-fluorescence.

Duysens *et al.* (11,12,13) studied the fluorescence of pyridine nucleotide in photosynthetically active cells. Their measurements with purple bacteria and cyanobacteria showed a remarkable increase in NADPH fluorescence at around 450 nm when excited with radiation of 366 nm and illuminated with photosynthetically active radiation. In addition, Amesz *et al.* (14) examined the fresh water green alga *Chlorella vulgaris* in a similar way and observed an increase in fluorescence of 20 to 30 % upon illumination with PAR. A decrease by about the same order is observed in our studies when using DCMU.

Measurements with extracted chloroplasts of plant leaves revealed increasing NADPH fluorescence and a fast response upon PAR illumination changes. Heber *et al.* (22) determined the concentration of reduced and oxidized pyridine nucleotides in chloroplasts via enzymatic cycling. The authors obtained a rapid reduction of pyridine nucleotides upon illumination: after 15 to 30 s of illumination, NADPH concentration reached a maximum which then decreased rapidly. However, such a fast decrease in fluorescence was not found in the following fluorimetric investigations on separated chloroplasts.

Brogliola (16) investigated the effect of PAR on laser-induced blue-fluorescence of spinach chloroplasts. She ascribed the PAR-induced increase of blue-fluorescence excited at 340 nm and de-

tected at 460 nm to photosynthetically produced NADPH. Measurements of Cerovic *et al.* (15) on plant leaves gave very similar results. The authors conclude that with an excitation shorter than 350 nm a light-induced variation of blue-fluorescence in intact isolated chloroplasts only reflects changes in the NADPH concentration/redox state. The detected increase of blue-fluorescence was only a few percent and they resume that the blue fluorescence is not a suitable indicator of photosynthetic activity of intact leaves.

In our studies on living phytoplankton cells this effect of PAR-illumination could not be detected, probably because of the metabolic exchange between chloroplasts and other cell compartments. This exchange can rapidly reduce the high concentration of NADPH after PAR illumination and hence maintain a rather constant steady-state. This effect probably does not appear on separated chloroplasts.

Furthermore, our results show that concentration of NADPH in *in vivo* samples seems to be too small for fluorimetric measurements. About 2 to 3 orders of magnitude higher cell densities were necessary to excite blue-green-fluorescence up to a level which would be required in practical applications using standard laboratory or *in situ* fluorimeters.

Duysens *et al.* (12) already tried to measure the change in fluorescence intensity at 460 nm when illuminating with PAR, but their attempt failed. Under quite the same conditions Amesz *et al.* (14) were successful in measuring a change in NADPH fluorescence on samples with about 100 times higher cell densities of about 10^{11} cells L⁻¹ which, however, are unrealistic for *in situ* conditions.

In our study cell numbers of about 10^8 - 10^{10} cells L⁻¹ were used, and PAR-induced fluorescence changes were not apparent. But even at higher cell densities the impact of re-absorption of fluorescent light due to photosynthetic pigments is not negligible.

CONCLUSIONS

Phytoplankton cell densities in productive oceanic regions are typically in the order of 10^4 - 10^6 cells L⁻¹. Based on the findings presented here and in agreement with the results reported elsewhere in the literature, we conclude that these cell densities would be much too small for biomass/photosynthetic activity measurements with remote sensing using fluorescence lidar. In natural waters, these measurements are further hampered by gelbstoff which due to its broad fluorescence emission significantly contributes to the spectra of the blue-fluorescence from NADPH. The same holds with measurements using laboratory instruments or *in situ* probes. But also pump-and-probe based methods, which can be reliably used to measure the physiological state of marine phytoplankton, do not lead to meaningful results since there is apparently no specific effect of PAR on the blue-fluorescence of NADPH.

However, these restrictions may be overcome in different ways: 1) Flow-cytometric investigations of individual cells (7), thus avoiding the gelbstoff background fluorescence, with high light-gathering efficiency which may lead to reliable signals from NADPH. 2) Phytoplankton cells can be concentrated with tangential filtration, leading to cell densities from which the feasibility of direct measurements of NADPH fluorescence is expected. 3) Fluorescence lifetime measurements are feasible using pulsed fluorescence excitation. The 9 ns fluorescence lifetime of NADPH (23) is much shorter than that of gelbstoff fluorescence which, in addition to the different spectral signatures of their emission bands, allows these fluorophores to be discriminated using time-resolved spectroscopy.

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