Challenge

Determination of water quality in surface water based on chlorophyll a concentration as indicator for phytoplankton levels in freshwater

Solution

Deploying the SPECORD 50 PLUS UV/Vis spectrophotometer as reliable analysis tool to probe the chlorophyll a concentration based on the absorbance at 665 nm following a simple sample preparation procedure

Probing the *Chlorophyll a* Concentration as an Indicator to Monitor Trophy Variations in Surface Water by Means of UV/Vis Spectrophotometry According to the DIN 38409-60

Introduction

UV/Vis spectrophotometry is a widely used technology in the analysis of water quality. Beyond detection of standard water parameters (e.g., phosphorus, ammonium, and nitrate)[1], it is particularly helpful in the detection of plant pigments such as chlorophylls, phycobilins, and xanthophyls.[2] Herein, Analytik Jena's longstanding experience in UV/Vis spectrophotometry combines suitable spectrophotometer performance and software, as well as the right accessory pallet, reaching from single-sample up to automated solutions. In this respect, the SPECORD 50 PLUS spectrophotometer combines simplicity in operation and outstanding spectroscopic performance, as well as automation capabilities.

Trophy degree (growth of phytoplankton or cyanobacteria) monitored through chlorophyll a spectrophotometric quantification, has been adopted in the DIN 38409-60: "Spectrometric determination of the chlorophyll a concentration in water" as part of the German standard methods for the examination of water, wastewater, and sludge [3]. Chlorophyll a concentration together with further biomass and bioactivity parameters leverage insight to the metabolic performance of phytoplankton (cyanobacteria) in aquatic environments, discussion of these parameters is however beyond the scope of the current application note. In addition to chlorophyll a determination, pheophytin concentration is probed.

Effective monitoring tools in water analysis require relatively simple to negligible sample preparation, fast detection and assessment, and a reasonable degree of precision in terms of a quantitative determination. The simple chlorophyll a determination method allows discrimination between chlorophyll a and further photosynthetic accessory pigments such as phycobilin molecules (e.g., phycocyanobilin), chlorophyll b and β-carotenes (see fig. 1), solely based on its absorbance properties. Like all phytopigments, chlorophyll is sensitive to light, acidic and enzymatic degradation (e.g., through chlorophyllases).

Materials and Methods

Samples and reagents

The surface water samples were collected from a dam within the Saale water system (Thuringia, Germany), filtration, pH modulation, ethanolic extraction, and proper storage conditions were carried out in line with the DIN 38409-60 specifications.

Samples were filtered using a fiber glass filter and under 30–50 kPa vacuum. Depending on trophy degree samples volume varies from 0.10 up to 5.00 L. Directly after filtration ethanolic extraction follows. Hot ethanol (78 ± 2 °C) inhibits overall enzymatic activity, thus chlorophyll degradation through chlorophyllases is then arrested. If required (e.g., higher turbidity by simple visual inspection), samples might need homogenization (e.g., mechanical particle disruption through shear forces). Alternatively, if the sample volume is rather low (< 25 mL) or if the turbidity is very high, additional clarification through filtration or centrifugation is recommended. The turbidity correction is achieved through the absorbance measurement at 750 nm. Chlorophyll is very sensitive towards photochemical and chemical oxidation. Here, sample processing must occur within the first 24 h of collection with negligible light exposure and samples should be stored at 5 ± 3 °C. In the current work, an exemplary set of 8 different samples were tested according to the DIN 38409-60 standard.

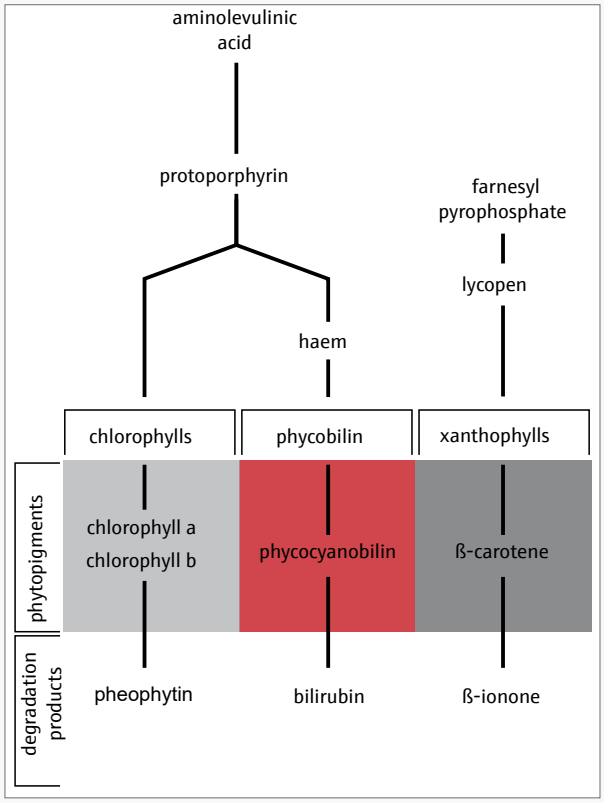

Figure 1: Relevant phytopigments classes, key examples, condensed biosynthesis pathway, and degradation product.

Instrumentation and software settings

The whole measurement series was performed using a SPECORD 50 PLUS spectrophotometer equipped with a standard cuvette holder and a 50 mm glass cuvette. The optical properties of the SPECORD 50 PLUS offer by far higher performance then the DIN 38209-60 specified requirements in terms of spectrophotometer's spectral bandwidth (1.4 nm), photometric accuracy (±0.003 A), and wavelength accuracy (≤ ±0.5 nm). Here, the DIN 38209-60 requirements are ≤ 2 nm, ≤ ±0.005 A at 1 A and ≤ ± 1 nm, respectively. The spectrophotometer was operated using the ASpect UV software (version 1.5.0) in the photometry module. Here a simple method for recording the absorbance values at 665 nm and 750 nm was defined (see table 1), blanks and samples were incorporated in a predefined sample table.

Table 1: Details of the measured absorbance values as specified for ASpect UV software settings.

	A_{665}	A_{750}
Measurement mode	Absorbance	Absorbance
Wavelength [nm]	665	750
Integration time [s]	0.1	0.1

Measurements

Unknown samples require measurement of the spectra within 500 and 800 nm, this allows a feasibility study in terms of the overall concentration and ratio of the probed phytopigments (e.g., phycobilin molecules, and corresponding degradation products). For the chlorophyll a, absorbance measurements at the given wavelengths (see table 1) of reference (distilled water), blank and samples were performed in sequence. Ethanol (90%) was used as blank measurement. Its contribution was automatically subtracted in the ASpect UV software for every subsequent sample absorbance measurement. To determine and subtract pheophytin contribution, absorbance was measured prior (A_v for *Absorbanz vor* in German) and after (A_n for *Absorbanz nach* in German) acidification (see table 2, results and disussion). Details of this step are given in the photometric procedure of the DIN 38409-60. Chlorophyll a concentration (ρ_c), pheophytin contribution (ρ_p), chlorophyll a mass, or chlorophyll a before acidic treatment (ρ_g) as well as an acid-ratio R are calculated on the basis of the recorded absorbance values (see results and discussions).

For optimal evaluation, it is recommended to work within absorbance values between 0.02 and 1.00 A, thus the selection of the sample volume, extract volumes, and cuvette pathlength is paramount for this purpose. For example, in the current case 2.00 L sample volume and 25 mL extract require a 50 mm cuvette, thus the low chlorophyll concentration can be properly measured through the deployment of a cuvette with longer path length.
As specified by the DIN 38409-60, the limit of determination must be actively calculated for each laboratory individually on an annual basis. Chlorophyll a is not available as stable standard or reference material, therefore the DIN 32645[4] for blank method should be employed. Here an analyte-free sample composed by a matrix which matches the analyzed target samples (e.g., surface water filtrate of prepared samples, strongly diluted sample in a drinking matrix or plain drinking water) is then used for a 10-fold determination. Further details regarding the procedure and the succeeding data evaluation are given in the DIN 38409-60, the key details are discussed below.

Spectrophotometric data analysis

For the elucidation of the key chlorophyll indicator values the following equations and variables were employed.

(1) Determination of chlorophyll a concentration upon acidification

$$\rho_c = \left((A_{665v} - A_{750v}) - (A_{665n} - A_{750n})\right) \cdot \frac{R}{R-1} \cdot \frac{V_E}{V_P \cdot d \cdot \alpha}$$

(2) Mass concentration of phaeopigments

$$\rho_p = \frac{R}{R-1} \cdot \left(R \cdot (A_{665n} - A_{750n}) - (A_{665v} - A_{750v})\right) \cdot \frac{V_E}{V_P \cdot d \cdot \alpha}$$

(3) Mass concentration of chlorophyll a before acidic correction

$$\rho_g = (A_{665v} - A_{750v}) \cdot \frac{V_E}{V_P \cdot d \cdot \alpha}$$

(4) Acid-ratio R to validate DIN 38409-60 fulfillment. For this purpose, the given range should be within 1.0 and 1.7.

$$R = \frac{(A_{665v} - A_{750v})}{(A_{665n} - A_{750n})}$$

With the following variables:

ρ_C	Chlorophyll a mass concentration in microgram per liter [µg/L]
A_{665v}	Extract absorbance [A] at 665 nm prior acidification
A_{750v}	Extract absorbance [A] at 750 nm prior acidification (turbidity correction)
A_{665n}	Extract absorbance [A] at 665 nm after acidification
A_{750n}	Extract absorbance [A] at 750 nm after acidification (turbidity correction)
R	A_{665v} /A_{665n} ratio for pure chlorophyll a, in this case R = 1.7
V_E	Extract volume in milliliter [mL]
V_P	Filtered water sample volume in liter [L]
d	Cuvette path length in centimeter [cm]
α	Specific absorbance coefficient for chlorophyll a, in this case 90% ethanolic solution $\alpha = 82 \cdot 10^{-3}$ [mL · cm^{-1} · µg^{-1}]

Measured and calculated values are summarized in table 2 (see results and discussion).

Results and Discussion

To understand the use of this spectrophotometric technique, it is pertinent to highlight some spectrophotometric absorbance and therefore structural details related to chlorophyll a and b.[5,6] These molecules are structurally similar but chlorophyll a incorporates a CH_3 group (methyl) directly at the aromatic ring, whereas in chlorophyll b the methyl side chain is substituted by an CHO (acyl) group (see fig. 2A). Overall, the chlorophyll (a or b) aromatic ring has a primary absorbance maximum in the region between 600 and 700 nm. Due to the given structural differences, chlorophyll a and chlorophyll b clearly differ in their characteristic absorbance properties (see fig. 2B). According to the DIN 38409-60 standard, the absorbance maximum of chlorophyll a in ethanol corresponds with the absorbance at 665 nm (A_{665}), whereas chlorophyll b has a broader and less prominent maximum between 610 and 630 nm with a peak at approx. 620 nm.[7] The absorbance of chlorophyll a also overlaps with further phytopigments (e.g., phycocyanobilin), especially below 650 nm. This spectral perturbation is weak, and it is quantitatively suppressed, as described in the DIN 38409-60 standard, through acidification. This refers to the absorbance measurement at 665 nm prior (A_{665v}) and after (A_{665n}) acidification. To compensate potential background contribution from other phytopigments, the effective absorbance is then estimated from the difference of the absorbance A_{665} and A_{750}. The latter one corresponds to the sample absorbance at 750 nm which should be relatively low. Here, the red absorbing phycobilin pigments have the most prominent contribution, but since they are less soluble in ethanol than chlorophyll a, this corresponds to a minor perturbation. The corrected value is then subsequently employed for quantitative determination (see acidification procedure).

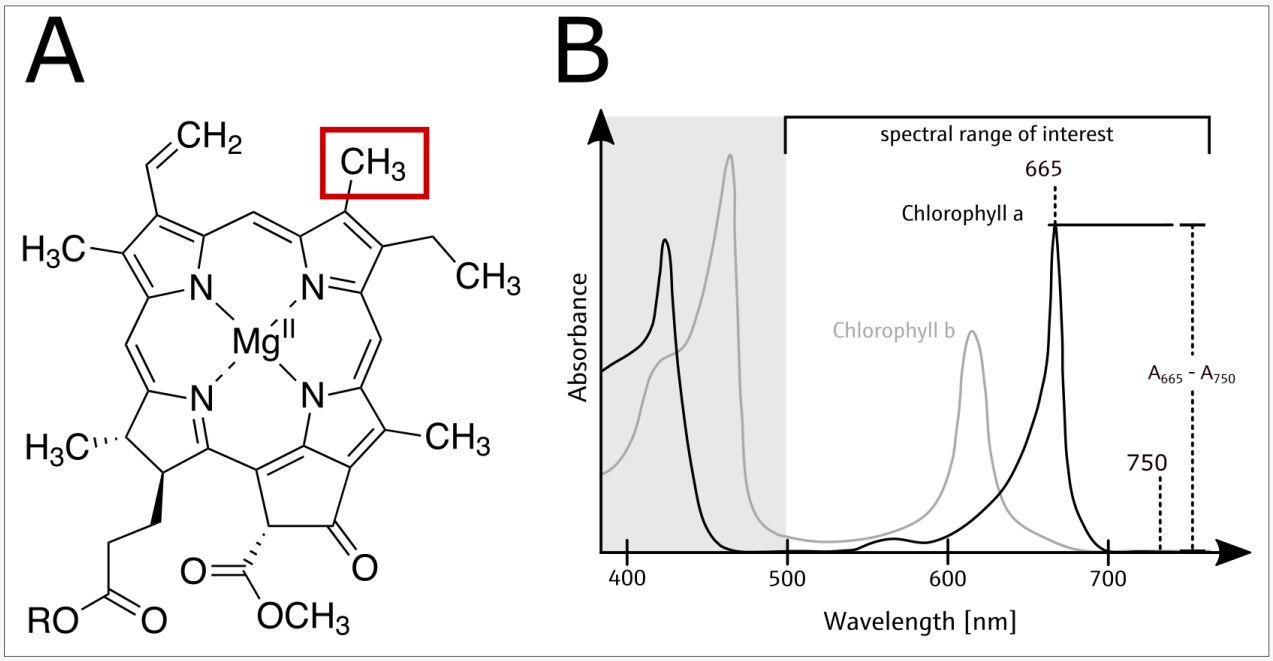

Figure 2: (A) Chemical structure of chlorophyll a, with highlighted methyl group (CH_3, red box). Chlorophyll b incorporates an acyl (CHO) group instead.
(B) Graphical representation of the absorbance spectra of chlorophyll a (black) and chlorophyll b (grey), key spectroscopic features to determine chlorophyll a concentration (A_{665}), background absorbance (A_{750}) and the background-corrected absorbance ($A_{665} - A_{750}$) are indicated. The relevant region, acc. to the DIN 38409-60 is indicated (spectral range of interest: 500 to 800 nm).

Absorbance measurement of freshwater samples

All recorded values are summarized in table 2. Absorbance values at 665 nm prior (A_{665v}) and after (A_{665n}) acidification treatment scatter between approx. 0.350 and 0.040 A. Upon acidification, absorbance decreases substantially, the reduction oscillates between 12 and 32%. Thus, a higher A_{665n} value indicates a higher chlorophyll a concentration and thus a strong pheophytin contribution. The later one strongly depends on the absorbance A_{750}. As expected, these values (both A_{750v} and A_{750n}) do not correlate with the overall absorbance at 665 nm[7]. Here, the value oscillates between 0.005 and 0.095 A. The A_{750} value also provides an indicator for the lower detection limit, since chlorophyll a and further pigments absorbance is not expected. As anticipated, the corrected chlorophyll a ρ_C concentration is considerably lower than the chlorophyll concentration ρ_g prior correction. In addition, the ρ_p (derived from the absorbance measurements) is certainly higher since it comprehends a larger amount and diversity of phyto-molecules. The determined acid-ratio R shows that the measured values are within the limits given by the DIN 38409-60 standard.

Table 2: Sample volume (V_P), sample extract volume (V_E), absorbance prior (A_{665v} and A_{750v}) and absorbance after acidification (A_{665n} and A_{750n}), chlorophyll a concentration (ρ_C), mass concentration of phaeopigments (ρ_p), mass concentration of chlorophyll a before acidic correction (ρ_g) and acid ratio R for each sample.

Sample	V_P [L]	V_E [mL]	A_{665v}	A_{665n}	A_{750v}	A_{750n}	ρ_C [µg/L]	ρ_p [µg/L]	ρ_g [µg/L]	acid-ratio R
1	2	25	0.0922	0.0807	0.0067	0.0076	0.92	2.87	2.61	1.17
2	2	25	0.1008	0.0939	0.0088	0.0092	0.54	3.85	2.80	1.09
3	2	25	0.2697	0.2363	0.0085	0.0088	2.50	9.30	7.96	1.15
4	2	25	0.2333	0.2033	0.0062	0.0065	2.24	7.96	6.92	1.15
5	2	25	0.3033	0.2068	0.0064	0.0064	7.15	3.24	9.05	1.48
6	2	25	0.3241	0.2550	0.0063	0.0059	5.09	7.82	9.69	1.28
7	2	25	0.0735	0.0576	0.0053	0.0055	1.19	1.51	2.08	1.31
8	2	25	0.0611	0.0488	0.0048	0.0050	0.93	1.34	1.72	1.29

Conclusion

The herein described spectrophotometric method leverages key insights into the development of phytoplankton in surface water. The water quality parameter chlorophyll a is a labile compound, which offers a narrow window for a reliable quantification. However, the method described in the DIN 38409-60 provides rapid and accurate determination with relatively simple sample preparation procedure. Furthermore, the easy spectrophotometric determination leverages access to a variety of components (e.g., other chlorophyll and carotenoids, phycobilin molecules). In this respect, the SPECORD 50 PLUS (see fig. 3) provides powerful spectroscopic performance, suitable accessories to fulfill the standard requirements in terms of cuvette pathlengths (50 mm), and a cuvette changer for higher throughput (6-fold cuvette changer, both for 10 and 50 mm pathlength). Finally, the ASpect UV software allows the predefinition of methods, blanks, and sample tables. Higher sample throughput (up to 116 samples) in the chlorophyll a determination can be also achieved using the APG autosampler with the sipper system.

Figure 3: SPECORD 50 PLUS

References

[1] Analytik Jena Application Note: Spectrophotometric Determination of Standard Parameters in Wastewater According to Standard Methods

[2] Analytik Jena Application Note: Palm Oil – UV/Vis Spectrophotometric Quality Assessment Along the Value Chain Using the Deterioration of Bleachability Index (DOBI) and Carotene Content According to ISO 17932:2011

[3] DIN 38409-60: German standard methods for the examination of water, waste water and sludge - Parameters characterizing effects and substances (group H) - Part 60: Spectrometric determination of the chlorophyll-a concentration in water (H 60)

[4] DIN 32645: Chemical analysis - Decision limit, detection limit and determination limit under repeatability conditions - Terms, methods, evaluation

[5] Sanchini, A., and Grosjean, M.; Quantification of chlorophyll a, chlorophyll b and pheopigments a in lake sediments through deconvolution of bulk UV–VIS absorption spectra; J. of Paleolimn. 2020, 64, pages 243-256

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