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BSI Standards Publication

# Water quality — Guidance on the use of in vivo absorption techniques for the estimation of chlorophyll-a concentration in marine and fresh water samples

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# EN 16161

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English Version

# Water quality - Guidance on the use of in vivo absorption techniques for the estimation of chlorophyll-a concentration in marine and fresh water samples

Qualité de l'eau - Lignes directrices sur l'utilisation des techniques d'absorption in vivo pour l'estimation de la concentration de chlorophylle-a dans les eaux douces et eaux marines

Wasserbeschaffenheit - Anleitung für die Anwendung der in-vivo-Absorption zur Abschätzung der Chlorophyll a-Konzentration in Meer- und Süßwasser

This European Standard was approved by CEN on 17 May 2012.

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# Foreword

This document (EN 16161:2012) has been prepared by Technical Committee CEN/TC 230 “Water analysis”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by January 2013, and conflicting national standards shall be withdrawn at the latest by January 2013.

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# Introduction

Surveys of chlorophyll and nutrient concentration are fundamental descriptors of primary productivity and eutrophic threat in coastal and inland waters.

Chlorophyll-a concentration can be determined by sampling and laboratory analysis using the techniques described in ISO 10260. Achieving consistent results with this technique requires careful attention during the various steps of the process commonly used, such as during sampling, transport, filtering, freezing, storage and extraction and subsequent pigment estimation.

The *in vivo* technique described here can be applied to surveys where a rapid non-destructive and repeatable measurement capability is required. It can be used either in the field or laboratory. No chemicals are required. Utilised in association with other methods of chlorophyll-a determination such as ISO 10260, HPLC pigment analysis and chlorophyll fluorescence measurements techniques, it can help identify sources of inconsistency or be used as an alternative technique in its own right. As chlorophyll-a estimates can be achieved in times as short as one minute, the technique can enhance surveying capability considerably.

This standard describes procedures to implement and verify performance.

# 1 Scope

This European Standard provides guidance in the use of *in vivo* absorption techniques to quantify chlorophyll-a concentration in marine and fresh waters.

This European Standard is comprised of the following:

— definition of the equipment requirement;

— *a priori* data and mathematical tools;

— recommendations for verification of measurement system performance and consideration of factors that can influence measurements;

— listing of the procedures to be implemented.

# 2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ENV 13005, *Guide to the expression of uncertainty in measurement*

# 3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

**3.1**
**absorption coefficient**
***a***
natural logarithm of the ratio between the light intensity entering and corresponding intensity emerging directly through a sample of water divided by the sample path length (in metres) in cases where the scattering of light is negligible

Note 1 to entry: The unit is $m^{-1}$.

Note 2 to entry: A spectrophotometer often gives the $Log_{10}$ of the ratio in place of the natural logarithm.

**3.2**
**extinction**
***c***
sum of losses of directly transmitted light by absorption and scattering

Note 1 to entry: The unit is $m^{-1}$.

Note 2 to entry: The extinction $c$ is related to absorption $a$ and scattering $b$, by $c = a + b$.

**3.3**
**extractive photometric**
**EP**
method of chlorophyll concentration estimation involving extraction and absorption measurement

**3.4**
***in vivo* photometric**
**IVP**
method of assessing chlorophyll-a concentration through the use of *in vivo* spectral photometry

**3.5**
**package effect**
flattening of a spectral absorption feature arising from excessive absorbing molecule concentration within cells

**3.6**
**resolution**
width at half height of the instrument response function

**3.7**
**scattering coefficient**
***b***
natural logarithm of the ratio between the light intensity entering and corresponding intensity emerging directly through a sample of water divided by the sample path length (in metres) in cases where the absorption of light is negligible

Note 1 to entry: The unit is $m^{-1}$.

**3.8**
**spectrum**
set of data of a sample taken over a defined wavelength range and by a defined resolution

**3.9**
**wavelength range**
range from minimum to maximum wavelength over which a spectrum is described

## 4 Principle

The *in vivo* photometric absorption technique (IVP) is based on:

a) the additive nature of absorption of individual constituents within a suspension;

b) the use of a priori knowledge about the absorption features of chlorophyll-a in the wavelength area of approximately 675 nm;

c) the absence of other components interfering with spectral features of chlorophyll-a in this region;

d) the use of a measurement cell (a cuvette or other sample receptacle) of sufficient length and spectrophotometer of sufficient performance to enable the absorption feature of chlorophyll-a to be identified at the concentration levels required;

e) the availability of a suitable algorithm to identify and quantify the distinctive chlorophyll-a absorption feature within a spectral absorption measurement.

## 5 Apparatus

**5.1 Spectrophotometer**, or equivalent, with the capability to determine the absorption spectrum of an *in situ* sample of water.

The spectral measuring instrument shall be capable of measuring the absorption of the sample in a range between 600 nm and 750 nm with a resolution better than 10 nm. The data capture is carried out in measuring intervals of 5 nm or smaller.

NOTE For example, a spectrophotometer with 2 nm resolution requires the ability to sense changes in absorption around 675 nm of 0,020 3 $m^{-1}$ in order to sense 1 µg/l of chlorophyll-a.

**5.2 Datum**, consisting of the unit response spectrum for chlorophyll-a absorption $a_s(\lambda)$ in the region around 675 nm for the spectrophotometer in use.

See Annex B for suggested methods of determining this.

**5.3 Mathematical routine**, capable of determining the quantity $C$ of unit chlorophyll-a spectra present in the sample spectrum.

# 6 Procedure

## 6.1 Calibration

The reference spectrum should be recorded with a sample of water which is free of constituents with absorption effects likely to mimic or interfere with chlorophyll-a absorption spectral features in the 650 nm to 710 nm region. This measurement should be stored as a system reference spectral signal of the water $I_w(\lambda)$. The temperature of the reference water sample should not differ more than around 10 °C from that of the samples to avoid affecting chlorophyll estimation results at the 1 µg/l level.

If the same measurement cell is used for successive samples, the measuring chamber should be flushed to remove influences of previous measurements before the next measurement (see 6.3). This is particularly necessary in cases where a flow-through measurement cell is utilised. Persistent positive residual chlorophyll-a measurements on clean water samples indicate window fouling by chlorophyllous material. Either the measurement cell should be cleaned or the effects of the persistent fouling should be zeroed out by taking a new clean water reference spectrum.

Water free of chlorophyll-a can be generated by filtering drinking water through a pore size of 0,1 µm. For the measurement of samples with high content of dissolved salts or colour (especially high content of humic substances), a representative water sample can be filtered for use as reference.

When a measurement cell is emptied there is often a residue. In practice, the number of flushes recommended should be sufficient to bring verification of zero measurements to within two standard deviations of zero. If a significant positive residue persists, this might indicate window fouling and either a new reference clean water sample should be taken or the measurement cell should be cleaned.

EXAMPLE A sample of clean water free of chlorophyll inserted after a measurement of a sample with 100 µg/l of chlorophyll-a may record a residual chlorophyll-a concentration of 2 µg/l, or a 2 % residual. Two flushes should reduce this to 2 % of 2 % or 0,04 % and three flushes to 0,008 %.

The effect of temperature difference on the system algorithm can be checked by repeating measurements on one sample using different reference water or sample water temperatures. Such effects arise because of small changes in the absorption of pure water with temperature. These changes are well described in the literature [15] and can influence the curvature of an absorption spectrum near 675 nm.

The influence of the temperature and the salinity differences between the spectrum of the sample and reference spectrum shall be considered if concentrations around or below 1 µg/l need to be determined accurately. These changes are well described in the literature [15] and can influence the shape of an absorption spectrum near 675 nm.

## 6.2 Blank Measurement

For any instrument system, the standard deviation of the determination of $C$ in a series of measurements on a clean water sample should be determined by statistical methods. This will indicate the contribution of instrument and algorithm fitting noise to the determination of minimum detectable chlorophyll concentration. For each new reference water, a second measurement of that water used as a sample to determine estimated chlorophyll $C$ should be made. This estimate should not exceed $\pm$ two standard deviations of zero for the

instrument. If this is not the case, flushing, calibration (6.1) and blank measurement (6.2) should be repeated until this condition is fulfilled. Further clean samples based on the same reference water should also meet this condition in > 95 % of the measurements.

### 6.3 Sample measurement

Load and measure the sample. After filling the well-mixed sample in the measuring chamber, the transmitted light signal $I_s(\lambda)$ should be measured within one minute to avoid mistakes which are caused by sedimentation of algal cells

Care should be taken to include appropriate flushing operations when successive samples show substantially decreasing chlorophyll-a concentrations or turbidity effects. The chlorophyll-a estimate should be calculated from $I_s(\lambda)$,$I_w(\lambda)$, and $d$ to yield $a_s(\lambda)$ (see Formula (1)). When carried out using a curve fitting algorithm, the system unit chlorophyll-a response should be used and a modelled absorption should be adjusted to fit the measurement $a_s(\lambda)$. The value of $C$, the concentration of chlorophyll-a in the model at time of fit, is the system output. The sample spectrum and other information derived from it (dissolved organic matter, DOM, total visible absorption, etc.) can then be computed and recorded.

## 7 Calculation and Expression of Results

### 7.1 General

The inclusion of some or all of the effects of scattering can be tolerated. A sample absorption spectrum should be recorded according to Formula (1):

$$a_s(\lambda) = (1/d)(-\ln(I_s(\lambda)/I_w(\lambda))) \quad (1)$$

where

$a_s(\lambda)$ is the sample absorption spectrum ($m^{-1}$);

$I_s$ is the signal observed through the sample;

$I_w$ the signal observed through clean water, free of chlorophyll;

$d$ is the optical path length of the light through the sample, in metres (m).

### 7.2 Datum

The datum describes the unit response spectrum for chlorophyll-a absorption $a_{chl}(\lambda)$ in the region around 675 nm for the spectrophotometer in use. See Annex B for suggested methods of determining this and Annex D for examples.

A multiple of the datum of chlorophyll-a should be recorded according to Formula (2):

$$a_{chl}(\lambda) = a_0 + a_1(\lambda - \lambda_0) \quad (2)$$

where

$a_{chl}(\lambda)$ is the unit response spectrum for chlorophyll-a absorption effective over a range of wavelengths from approximately 600 nm to 710 nm.

### 7.3 Mathematical routine

The mathematic routine used shall be capable of determining the quantity $C$ of unit chlorophyll-a spectra present in the sample spectrum. For example, a minimisation routine capable of finding the best fit between a sample absorption spectrum $a_s(\lambda)$ and the model spectrum $a_0 + a_1(\lambda - \lambda_0) + c \times a_{chl}(\lambda)$, by varying $C$ until sample and model spectra have minimum differences over the range 650 nm to 710 nm (see Annex E).

### 7.4 Other factors influencing the chlorophyll-a estimation

The following differences that may arise should be assessed:

a) breakdown pigments with similar spectral features;

b) the package effect;

c) the presence of other pigments whose absorption features extend to some extent into the 675 nm region; principally phycocyanin and chlorophyll-b.

The effects that these factors can have on a measurement are described in Annex C.

NOTE This method yields an estimate based on the chlorophyll-a-like absorption feature at 675 nm as it appears *in vivo*. This is not necessarily the same as the chlorophyll-a absorption effect produced by the chlorophyll-a molecules alone when extracted from the *in vivo* cells. While this *in vivo* estimate is a good measure of the effective solar harvesting capability of the chlorophyll-a within the cell at the time of measurement, the differences that may arise when compared with extracted chlorophyll-a estimates should be clearly understood.

## 8 Quality Assurance

### 8.1 Repeatability

The absorption characteristics may change if the samples remain in the measuring chamber for a longer time (e.g., with multiple measurements, by sedimentation, agglomeration or buoyancy of algae cells). Therefore, the measuring cell should be equipped with a stirrer; or else the measuring chamber shall be filled again after each single measurement.

### 8.2 Uncertainty

The measurements should be accompanied by a statement of uncertainty, prepared by taking full account of natural factors which may influence or interfere with the estimation.

Uncertainty estimation requires clear identification of:

a) the measurand, and

b) the uncertainty sources in accordance with ENV 13005.

Once the uncertainty sources are identified, they should be quantified and combined into a total uncertainty.

NOTE 1 Further useful guides to the estimation of uncertainty can be found in the bibliography, [13] and [14].

The measurand here is the chlorophyll-a estimate. Tracing backwards, the main sources of uncertainty are:

1) the uncertainty of the performance of the mathematical algorithm in determining the concentration $C$ of chlorophyll-a, i.e. in determining the amplitude of the system unit chlorophyll-a response present in the measured absorption spectrum;

2) the uncertainty in determining the system unit chlorophyll-a response from the a priori unit chlorophyll-a response (Annex A) which is used in 1 above;

NOTE 2 Annex A contains the specific chlorophyll-a absorption spectrum for a high resolution instrument. This uncertainty is associated with the process described in Annex B for converting the high resolution specific chlorophyll-a absorption spectrum into a system-related specific chlorophyll-a absorption spectrum for a reduced resolution system.

3) the uncertainty arising from temperature or salinity difference between reference water and sample water [15];

4) the additional uncertainty arising from the loss of signal to noise in the case of high turbidity water;

5) the uncertainty arising if the Beer-Lambert Law linearity no longer applies;

6) the uncertainty arising from spectrometer relative spectral absorption calibration in the 675 nm region;

7) the uncertainty arising from spectrometer wavelength calibration in the 675 nm region;

8) any uncertainty arising from non-uniformity or settling of sample;

9) uncertainty arising from knowledge of cell path length.

Uncertainty 1 above may be established by statistical methods. Uncertainties 2 to 9 may be quantified by measurement and modelling. The expanded uncertainty should be determined from these by the root sum of squares of individual uncertainties and quoted as an expanded uncertainty using a coverage factor multiplier of 2 representing uncertainty at the 95 % level.

NOTE 3 An entire assessment of uncertainty of the determination of the chlorophyll a concentration of a water sample also requires the inclusion of the uncertainties derived from sampling, storage and sampling handling, etc.

NOTE 4 Taylor and Kuyatt, NIST 1994 [16] describe the combination by the root sum of squares of individual uncertainties as the ‘combined standard uncertainty’. A measure of uncertainty that defines an interval about the measurement result in which the value of the measurand is confidently believed to lie is termed the ‘expanded uncertainty’ and is obtained by multiplying the combined standard uncertainty by a coverage factor. In the case of a normal distribution, a coverage factor of 2 describes an interval having a level of confidence of approximately 95 %. Coverage factors between 2 and 3 can be used. The uncertainty reported should clearly state either the combined standard uncertainty or the expanded uncertainty and the coverage factor.

## 9 Test report

This test report shall at least contain the following information:

a) a reference to this European Standard (EN 16161:2012);

b) a description of the water samples, including sampling site name, position, sampling depth, date of sampling, date of analysis, name of sampler and analyst;

c) expression of the results;

d) sample pre-treatment;

e) any deviation from this method or any other circumstances which may influence the results.

# Annex A
(normative)

# Published *in vivo* specific chlorophyll a absorption spectrum

**Table A.1 — *In vivo* spectral absorption coefficient of 1 mg $m^{-3}$ of chlorophyll-a [3]** *(1 of 2)*

| Wavelength nm | Absorption 1/m | Wavelength nm | Absorption 1/m | Wavelength nm | Absorption 1/m |
|---|---|---|---|---|---|
| 404 | 0,013 2 | 520 | 0,000 5 | 636 | 0,003 8 |
| 406 | 0,013 9 | 522 | 0,000 5 | 638 | 0,003 6 |
| 408 | 0,014 8 | 524 | 0,000 6 | 640 | 0,003 4 |
| 410 | 0,015 9 | 526 | 0,000 6 | 642 | 0,003 2 |
| 412 | 0,017 | 528 | 0,000 7 | 644 | 0,003 |
| 414 | 0,018 | 530 | 0,000 7 | 646 | 0,002 9 |
| 416 | 0,018 8 | 532 | 0,000 8 | 648 | 0,002 9 |
| 418 | 0,019 3 | 534 | 0,000 8 | 650 | 0,003 |
| 420 | 0,019 5 | 536 | 0,000 9 | 652 | 0,003 2 |
| 422 | 0,019 3 | 538 | 0,000 9 | 654 | 0,003 6 |
| 424 | 0,019 1 | 540 | 0,000 9 | 656 | 0,004 2 |
| 426 | 0,018 9 | 542 | 0,000 9 | 658 | 0,005 2 |
| 428 | 0,019 1 | 544 | 0,000 9 | 660 | 0,006 6 |
| 430 | 0,019 9 | 546 | 0,000 9 | 662 | 0,008 2 |
| 432 | 0,021 2 | 548 | 0,000 9 | 664 | 0,010 6 |
| 434 | 0,022 9 | 550 | 0,000 9 | 666 | 0,013 7 |
| 436 | 0,024 7 | 552 | 0,000 9 | 668 | 0,015 5 |
| 438 | 0,026 1 | 554 | 0,000 9 | 670 | 0,018 2 |
| 440 | 0,026 5 | 556 | 0,000 9 | 672 | 0,019 6 |
| 442 | 0,025 5 | 558 | 0,000 9 | 674 | 0,020 3 |
| 444 | 0,022 9 | 560 | 0,000 9 | 676 | 0,019 9 |
| 446 | 0,019 3 | 562 | 0,000 9 | 678 | 0,018 2 |
| 448 | 0,015 1 | 564 | 0,000 9 | 680 | 0,015 4 |
| 450 | 0,011 1 | 566 | 0,000 9 | 682 | 0,012 7 |
| 452 | 0,007 7 | 568 | 0,001 | 684 | 0,010 4 |
| 454 | 0,005 2 | 570 | 0,001 1 | 686 | 0,007 6 |
| 456 | 0,003 4 | 572 | 0,001 2 | 688 | 0,005 8 |
| 458 | 0,002 2 | 574 | 0,001 3 | 690 | 0,004 5 |
| 460 | 0,001 5 | 576 | 0,001 4 | 692 | 0,003 3 |
| 462 | 0,001 | 578 | 0,001 6 | 694 | 0,002 |
| 464 | 0,000 7 | 580 | 0,001 7 | 696 | 0,001 3 |

**Table A.1** (*2 of 2*)

| Wavelength nm | Absorption 1/m | Wavelength nm | Absorption 1/m | Wavelength nm | Absorption 1/m |
|---|---|---|---|---|---|
| 466 | 0,000 6 | 582 | 0,001 8 | 698 | 0,001 |
| 468 | 0,000 5 | 584 | 0,002 | 700 | 0,000 8 |
| 470 | 0,000 4 | 586 | 0,002 1 | 702 | 0,000 6 |
| 472 | 0,000 4 | 588 | 0,002 2 | 704 | 0,000 5 |
| 474 | 0,000 3 | 590 | 0,002 2 | 706 | 0,000 4 |
| 476 | 0,000 3 | 592 | 0,002 3 | 708 | 0,000 4 |
| 478 | 0,000 3 | 594 | 0,002 3 | 710 | 0,000 3 |
| 480 | 0,000 3 | 596 | 0,002 3 | 712 | 0,000 3 |
| 482 | 0,000 3 | 598 | 0,002 2 | 714 | 0,000 2 |
| 484 | 0,000 3 | 600 | 0,002 2 | 716 | 0,000 2 |
| 486 | 0,000 3 | 602 | 0,002 1 | 718 | 0,000 2 |
| 488 | 0,000 3 | 604 | 0,002 1 | 720 | 0,000 2 |
| 490 | 0,000 3 | 606 | 0,002 1 | 722 | 0,000 1 |
| 492 | 0,000 3 | 608 | 0,002 2 | 724 | 0,000 1 |
| 494 | 0,000 4 | 610 | 0,002 4 | 726 | 0,000 1 |
| 496 | 0,000 4 | 612 | 0,002 6 | 728 | 0,000 1 |
| 498 | 0,000 4 | 614 | 0,002 9 | 730 | 0,000 1 |
| 500 | 0,000 4 | 616 | 0,003 1 | 732 | 0,000 1 |
| 502 | 0,000 5 | 618 | 0,003 4 | 734 | 0 |
| 504 | 0,000 5 | 620 | 0,003 6 | 736 | 0 |
| 506 | 0,000 5 | 622 | 0,003 8 | 738 | 0 |
| 508 | 0,000 5 | 624 | 0,004 | 740 | 0 |
| 510 | 0,000 5 | 626 | 0,004 1 | 742 | 0 |
| 512 | 0,000 5 | 628 | 0,004 1 | 744 | 0 |
| 514 | 0,000 5 | 630 | 0,004 1 | 746 | 0 |
| 516 | 0,000 5 | 632 | 0,004 | 748 | 0 |
| 518 | 0,000 5 | 634 | 0,003 9 | 750 | 0 |

# Annex B
(informative)

# Determination of the appropriate chlorophyll-a specific spectral absorption coefficient for the IVP system

The following two separate specific absorption coefficients have been much studied in open ocean science:

a) the specific absorption coefficients of individual pigments, such as chlorophyll *a*, *b* and *c*, and

b) the chlorophyll weight-specific absorption coefficients of algae, $a_{ph}$.

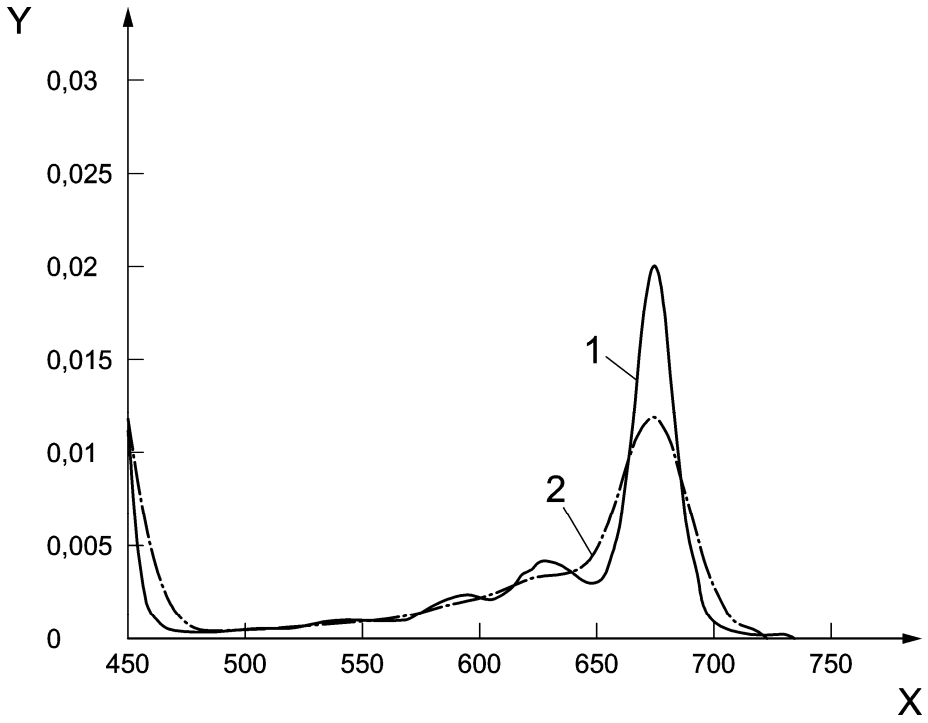
There have been many studies published showing how $a_{ph}$ can be derived from the sum of the coefficients of individual pigments, using laboratory derived (HPLC) individual pigment concentrations to spectrally reconstruct composite absorption spectra, (or the reverse, where algal absorption spectra are spectrally decomposed into constituent pigment concentrations). Although it has been shown that in the blue part of the spectrum this composition/decomposition process requires modification to take account of the package effect, it has also been demonstrated that this effect is much less significant or even negligible at the 675 nm chlorophyll-a feature, (see [1] [4] [5] [7] [12]).

A specific chlorophyll-a absorption spectrum that is often cited is the work of Bidigare et al. [3], who determined the relative specific absorption spectra of individual pigments within a complete specific algal absorption spectrum $a^*_{ph}$. Their chlorophyll-a specific spectrum is designated $a^*_{chl\_pub}(\lambda)$ here and given in Table A.1. Standard methods for the determination of chlorophyll-a in extract recommend that spectrometer resolution should be ≈ 1 nm to fully resolve the chlorophyll-a peak. For their work, Bidigare et al used an instrument resolution of 2 nm. This resolution might not be achievable or necessary for *in vivo* measurements. A resolution of nearer to 5 nm can be used.

The effect of reduced resolution on the specific absorption spectrum is easy to simulate and validate. That is done as follows: the $a^*_{chl\_pub}(\lambda)$ spectrum is forward and reversed filtered with a Gaussian instrument function using successively poorer resolution until the degraded spectral shape matches a measured IVP spectrum of a chlorophyll containing sample in the region around 675 nm. This process corresponds to the convolution of the full resolution spectrum by a Gaussian instrument function. The matching process takes account of possible offset and sloping background and leads to an amplitude difference as a result of the reduced resolution.

The degraded published spectrum now represents the unit chlorophyll-a spectrum for all subsequent IVP measurements $a^*_{chl\_sys}(\lambda)$ and the peak height at 675 nm, $a^*_{chl\_sys}(675)$ is the required bio-optical conversion factor for use with the IVP system. (The term bio-optical conversion factor is used here to indicate the absorption peak height at 675 nm measured with an IVP system for one unit of chlorophyll-a (1 mg $m^{-3}$).) While $a^*_{chl}$ (675), the published chlorophyll peak height at 675 nm is 0,020 3 $m^2mg^{-1}$; the degraded peak height for a working system of reduced spectral resolution can be of the order of 0,01 $m^2mg^{-1}$. The published and degraded chlorophyll-a spectra for one such system are shown in Figure B.1 along with fitted chlorophyll curves measured with such IVP systems in Figures B.2 and B.3.

The process described above does not affect the linearity of the subsequent chlorophyll-a estimations. Linearity can be shown to hold up to Beer-Lambert limits; and certainly up to concentrations greater than 100 µg/l in moderate turbidity waters.



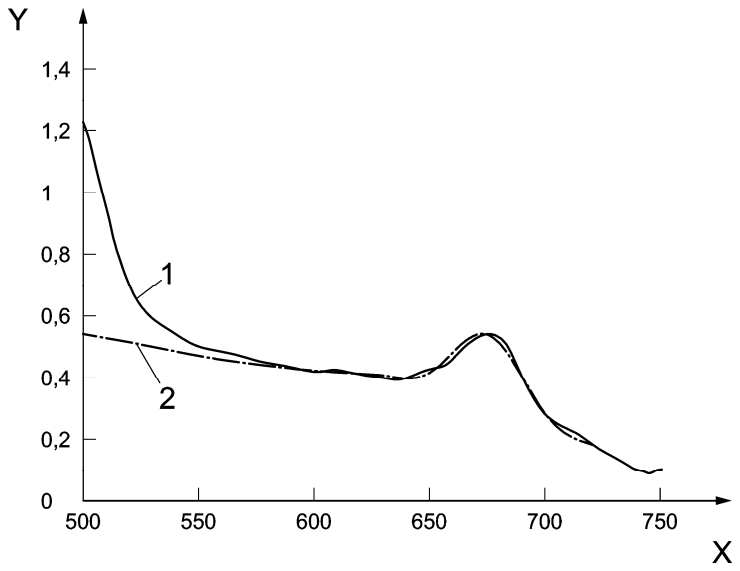

**Key**

X wavelength, nm

Y absorption, m-1

NOTE The high resolution spectrum is the published chlorophyll-a spectrum. The smoothed spectrum represents the chlorophyll-a spectrum that would be observed by an IVP instrument with reduced resolution and is calculated for each specific instrument.

**Figure B.1**




**Key**

1 sample *in vivo* absorption

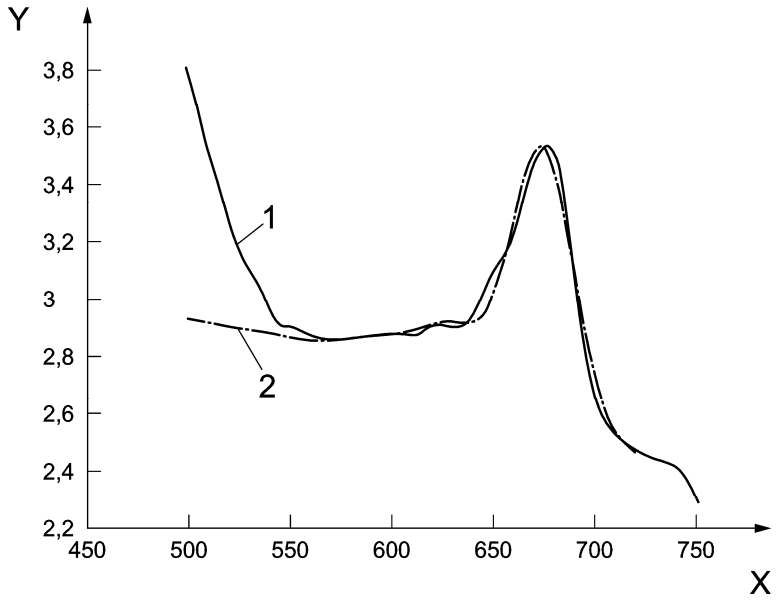
2 model

X wavelength, nm

Y absorption, $m^{-1}$

NOTE The instrument chlorophyll-a response included in a model of absorption and fitted to a measured spectrum of a chlorophyll containing suspension.

**Figure B.2**




**Key**

1 sample *in vivo* absorption

2 model

X wavelength, nm

Y absorption, m-1

NOTE As for Figure B.2, but for a different instrument and a humic and turbid sample.

**Figure B.3**

# Annex C
(informative)

# Factors influencing the chlorophyll-a estimation

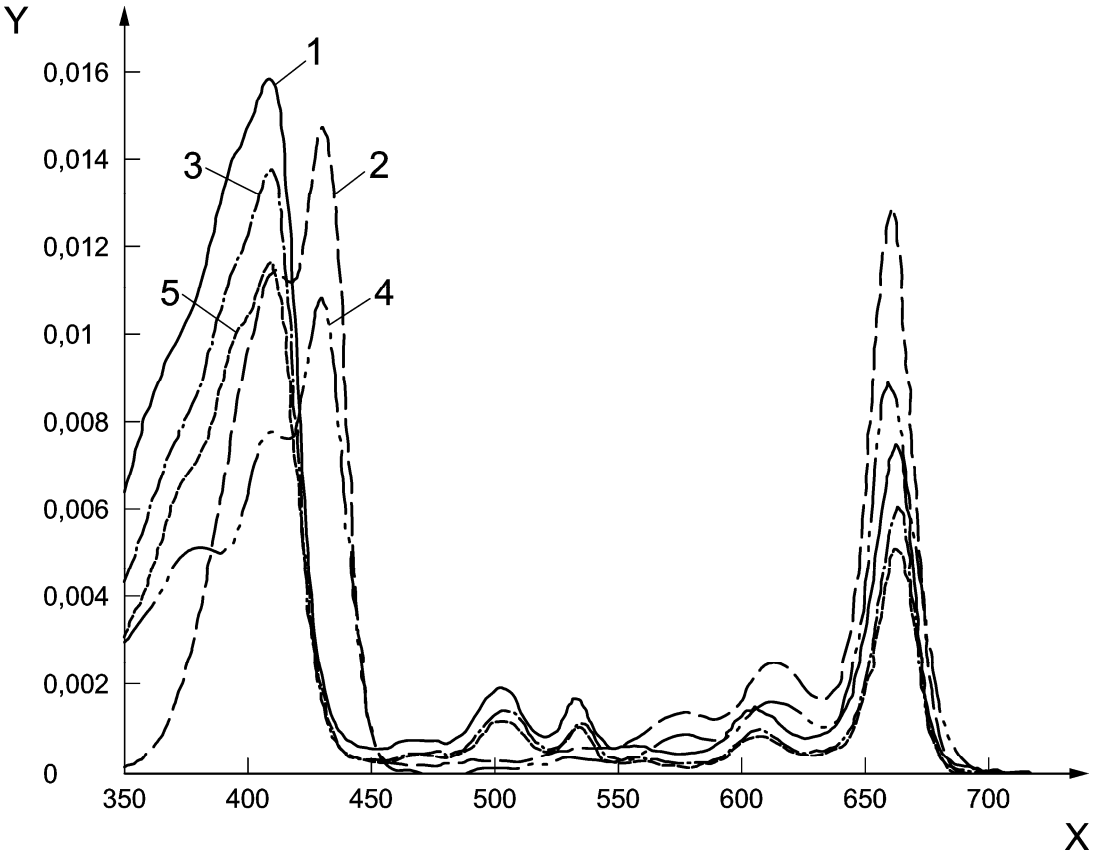
## C.1 Breakdown pigments

Chlorophyll-a molecules can break down, either as a result of senescence in algae or as a result of conditions during storage or extraction. The usual breakdown pigment is pheophytin. It has an absorption peak close to chlorophyll-a (about 2 nm shorter wavelength) and a smaller specific absorption coefficient (63 % of chlorophyll-a coefficient). A sample fully broken down will therefore be estimated to have up to 60 % more pigment concentration than it actually contains.

NOTE 1 The nearest equivalent to the chlorophyll-a estimation derived from this method is the determination according to the ISO 10260 method without carrying out the acidification step in which the absorption of all chlorophyll-a-like pigments is determined.

NOTE 2 An examination of a full summer programme of fresh water sampling analysed by the ISO extractive technique with acidification indicates that a 20 % over-estimate due to the presence of detected, but un-differentiated breakdown products would be more typical. The acidification step specified in ISO 10260 is designed to measure the influence of breakdown products, but is not always implemented. (The precision of measurement of chlorophyll-a using the acidification procedure is reduced as the chlorophyll-a content is estimated from a difference of two absorption measurements.)

The precision of measurement of chlorophyll-a using the acidification procedure is reduced as the chlorophyll-a content is estimated from a difference of two absorption measurements.




**Key**

1 pheophorbide-a
2 chlorophyllide-a
3 pyropheophytin-a
4 chlorophyll-a
5 pheophytin-a
X wavelength, nm
Y specific absorption coefficient

NOTE Absorption spectra of chlorophyll-a related pigments in acetone extract expressed as decadic absorption in $m^{-1}/mg/m^3$ (after Jeffrey et al, UNESCO 1996).

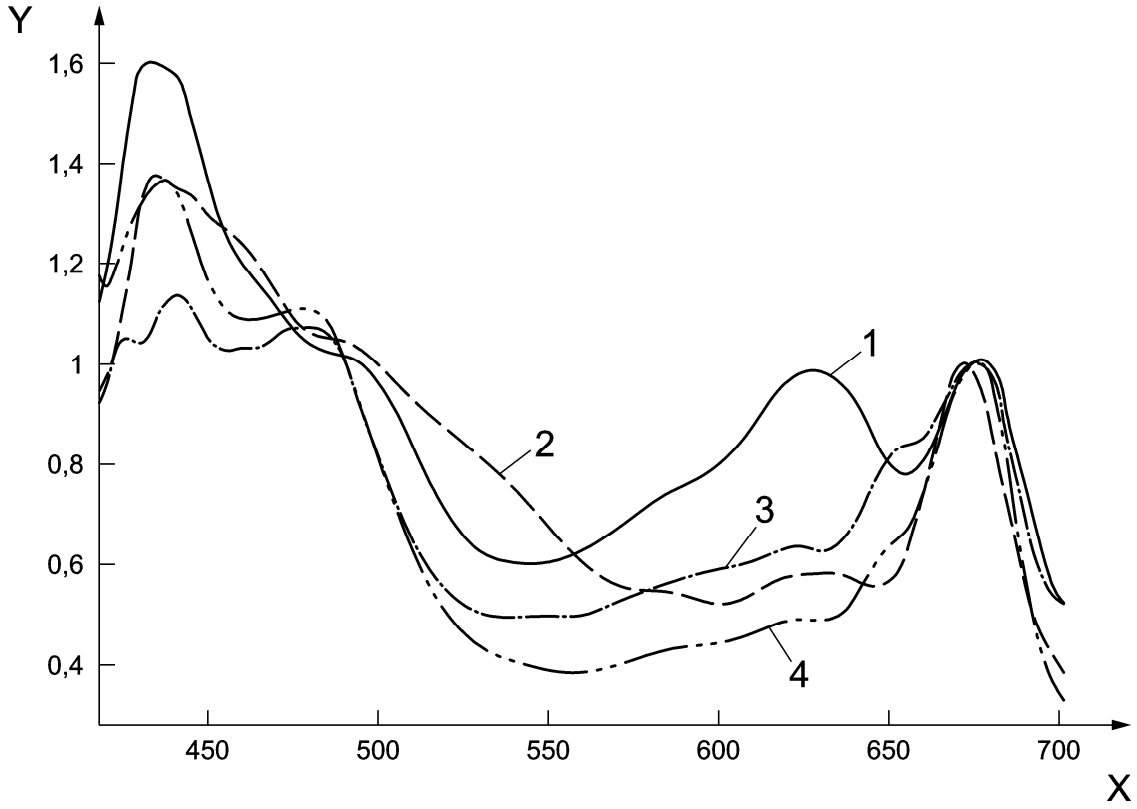
**Figure C.1 — Absorption spectra of chlorophyll-a related pigments**

## C.2 Package effect

The package effect may result in the net absorption effect in an algal system being reduced from what would be predicted on the chlorophyll content alone. This reduction has been shown to affect mainly the blue absorption features of chlorophyll. Despite the impression given by the theoretical modelling of the package effect in earlier work (e.g. Kirk 1976), the influence of the package effect at 675 nm in causing a discrepancy between component chlorophyll-a absorption and packaged chlorophyll absorption in natural marine waters or between IVP and EP chlorophyll-a estimates is minimal (see for example [5] and Annex D). The IVP technique estimates packaged chlorophyll-a while the EP technique estimates unpackaged chlorophyll-a (see also Annex E).

## C.3 Chlorophyll-b

The IVP chlorophyll-a method is based on the assumptions that the curve-fitting algorithm will not be adversely affected by the presence of other pigments. While chlorophyll-b can be present, it tends to form a small shoulder on the short wavelength side of the chlorophyll-a peak without influencing the determination significantly.




**Key**

1 microcystis aeruginosa
2 phaeodactylum tricornutum
3 stichococcus bacillaris
4 nannochloris atornus
X wavelength, nm
Y normalised absorption, $m^{-1}$

NOTE *In vivo* absorption spectra of algal cultures normalised to the chlorophyll-a peak which is the basis of chlorophyll-a quantification in the *in vivo* absorption technique (IVP) and the laboratory extractive photometric technique (EP). Curves are normalised to one at 675 nm.

**Figure C.2 — *In vivo* absorption spectra of algal cultures**

## C.4 Phycocyanin

The major pigment types for four algal cultures are described in four absorption spectra in Figure C.2. While the chlorophyll-a feature at 675 nm is common to all, the phycocyanin pigment dominates the blue-green microcystis species in the red (around 630 nm) and some influences of other chlorophyll pigments are noticeable on the short-wavelength side of the chlorophyll-a peak, particularly in the Bacillariophyceae. The phycocyanin absorption peak occurs at about 630 nm and is broader than the chlorophyll-a peak at 675 nm. The presence of the phycocyanin peak in an absorption spectrum indicates the presence of blue-green algae.

The absence of such a peak does not necessarily mean that no blue-green algae are present: the relative height of the phycocyanin peak with respect to the chlorophyll-a peak is an indication of the availability of nitrogen within the blue-green algae. During blue-green growth stage, the phycocyanin peak can be of the same order of height as the chlorophyll-a peak, reducing during the senescence stage to negligible proportions. When compared with the chlorophyll-a peak, the phycocyanin peak can interfere with the process of estimating the chlorophyll-a peak height (and a reduction of up to 25 % in estimated chlorophyll-a may result). One solution regarding this effect is to first estimate the phycocyanin peak height and then subtract this effect from the measured absorption effect before submitting the spectrum for chlorophyll-a estimation.

# Annex D
(informative)

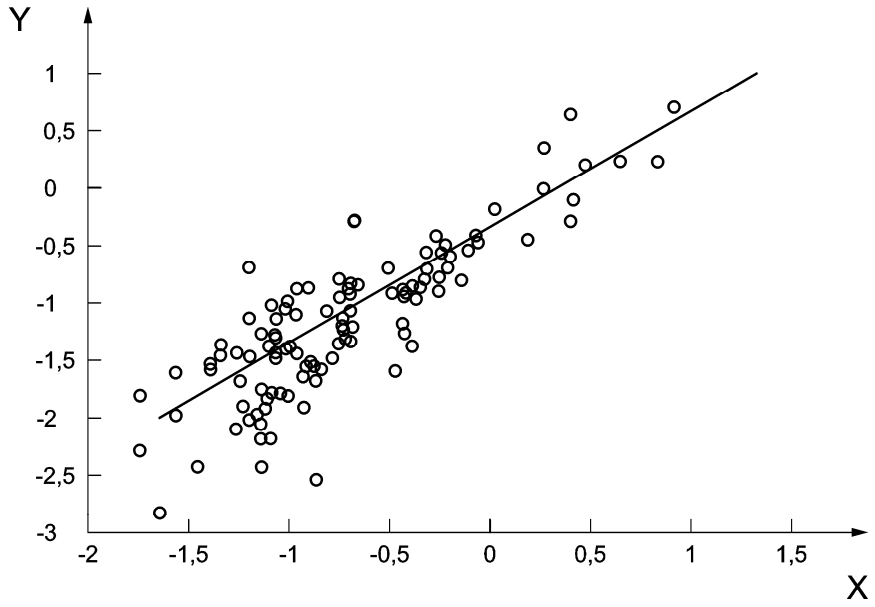
# Examples of paired sample method validation — Comparison of extraction and *in vivo* methods under operational conditions

## D.1 General

Two separate laboratory paired sample comparisons are relevant to this work: one carried out at IRH, Nancy, France in 2003 and the other at the laboratories of Adasa-Sistemas in Barcelona, Spain in 2004. This work was carried out under the E.U. funded Aquastew project: Contract No. EVK1-CT2000-00066 supporting IRH and UCD and Adasa Sistemas.

## D.2 The IRH Laboratory Data Set

During 2003, the normal river water sampling programme of IRH-Environment (Nancy, France) was augmented by the taking of additional duplicate samples for analysis by a commercial IVP system. Each sample pair underwent the same transport and storage regime. Of the 126 sample pairs gathered for analysis, 122 resulted in valid measurements by both EP and IVP techniques. Compared in Figure D.1 are the net absorption of the extract $a_0$ (where $a_0$ is the absorption at 665 nm less the absorption at 750 nm before acidification) and $a_{IVP} = c\, a^*_{chl\_sys}(675)$ is the height of the *in vivo* chlorophyll-a absorption feature at 675 nm estimated by the IVP system, as described in Annex B.




**Key**

X log (laboratory absorption ($a_o$))
Y log (*in vivo* photometric absorption ($a_{IVP}$))

NOTE *In vivo* Photometric (IVP) absorption versus Laboratory Extractive Photometry (EP) absorption for 122 river samples shown along-side a fitted line. The axes show Log[a($m^{-1}$)]. The root mean square error (Buckton et al. 1999) is –4,76 dB.

**Figure D.1 — *In vivo* Photometric (IVP) absorption ($a_{IVP}$) versus Laboratory Extractive Photometry (EP) absorption**

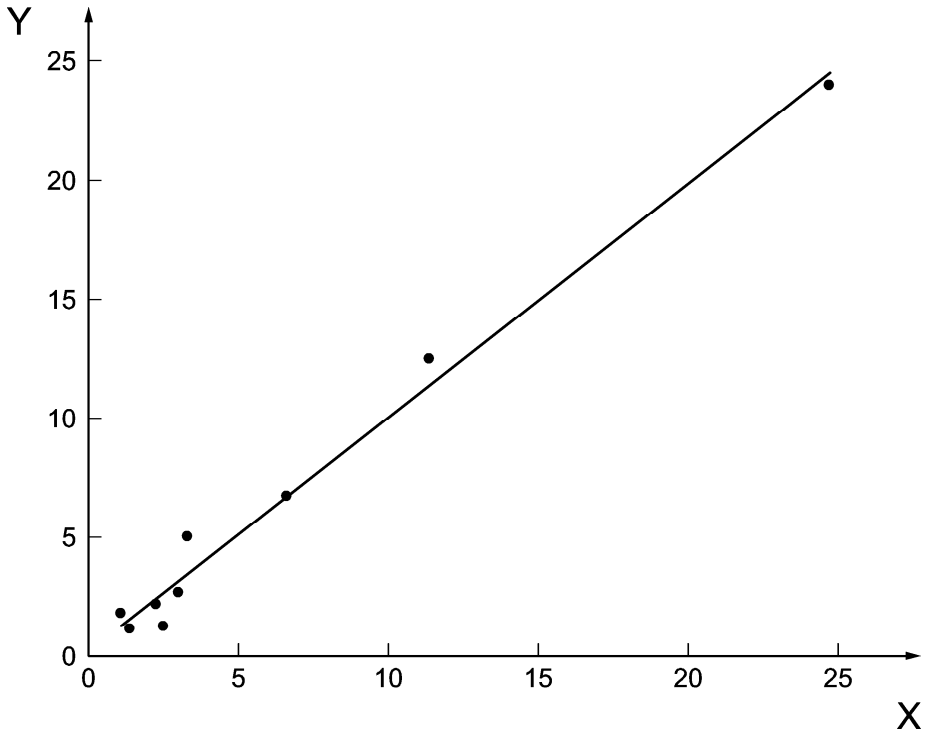
The data in Figure D.1 illustrates the correlation between laboratory extractive absorption measurements and IVP absorption measurements before conversion into units of chlorophyll-a. The slope of 0,465 here rather than 1,0 arises from the instrument bandwidth effect on the chlorophyll-a spectrum described in Annex B. A peak absorption height of 0,020 3 $m^{-1}$ per unit chlorophyll-a in the datum in Annex A reduces, as a result of the bandwidth effect, according to these results from the IRH data set to a 0,465*0,0203 or to approximately 0,009 4. This is consistent with the factor of just below 0,01 calculated by the method of Annex B applied to the instrument used for the IRH data set. Thus, the IRH data set verifies:

a) that over a complete season of water samples in natural and turbid waters laboratory and *in vivo* techniques correlate well;

b) that the bandwidth effect described in Annex B correctly describes the effective reduction in peak height of the chlorophyll absorption feature at 675 nm.

## D.3 The Adasa Sistemas Laboratory Data Set

The Adasa Sistemas Data Set consisted of eleven paired samples from different locations. These ranged in chlorophyll-a concentration from 1 µg/l to 38 µg/l and turbidity from 0,75 NTU to 59 NTU. Those nine data points with turbidity less than 10 NTU were selected for this analysis as those likely to be less affected by non-chlorophyllous absorption and scattering effects. This comparison was done at the level of computed chlorophyll-a, using these data to predict the correct peak height bio-optical factor for comparison with that derived by the method in Annex B.




**Key**

X lab chlorophyll-a

Y IVP chlorophyll-a

NOTE These samples were less turbid than those in the IRH data set. Regression slope adjusted to one to illustrate the value of bio-optical factor obtained: $a^*_{chl\_sys}(675)$ = 0,01 $m^2$/mg.

**Figure D.2 — Comparison of Laboratory (Extractive method) and *in vivo* chlorophyll-a (IVP Method)**

# Annex E
(informative)

# Validation of the spectrometric technique by determining the chlorophyll-a specific absorption of a set of algal samples

## E.1 Laboratory sample data for validation $a^*_{chl\_sys}$ the appropriate system unit chlorophyll-a response peak height

This process can be further validated by determination of chlorophyll-a specific absorption curves as seen by the IVP system obtained using the independent laboratory chlorophyll-a data (derived by EP method) and showing that these compare well with the $a^*_{chl\_sys}(\lambda)$ in Figure B.1.

Both the IRH and Adasa data have been used to determine the specific absorption as seen by an IVP instrument. A chlorophyll-a specific absorption spectrum can be derived for both data sets, while distinct specific curves for Pheo-pigments and total pigments can also be derived from the IRH data set. This involves performing a regression between IVP spectral absorption and laboratory chlorophyll-a at every wavelength. The slope of these regression relationships is the rate of change of absorption per unit chlorophyll-a or the specific absorption of chlorophyll-a *in vivo* as measured by the IVP system: $a^*_{ph\_sys}(\lambda)$ in $m^2\,mg^{-1}$.

The chlorophyll-a specific absorption of 122 samples of river water is derived from the *in vivo* absorption spectra of the samples obtained with the IVP system in two ways:

1) At each wavelength the *in vivo* absorption of the sample is regressed against the corresponding laboratory measured absorption $a_0$/(specific absorption of chlorophyll-a peak in extract):

$$a^i_{IVP}(\lambda) = a_{ph}(\lambda)\frac{a^i_0}{a^*_{chl}} + a_{resid}(\lambda) \quad \text{for samples } i = 1{:}122 \tag{E.1}$$

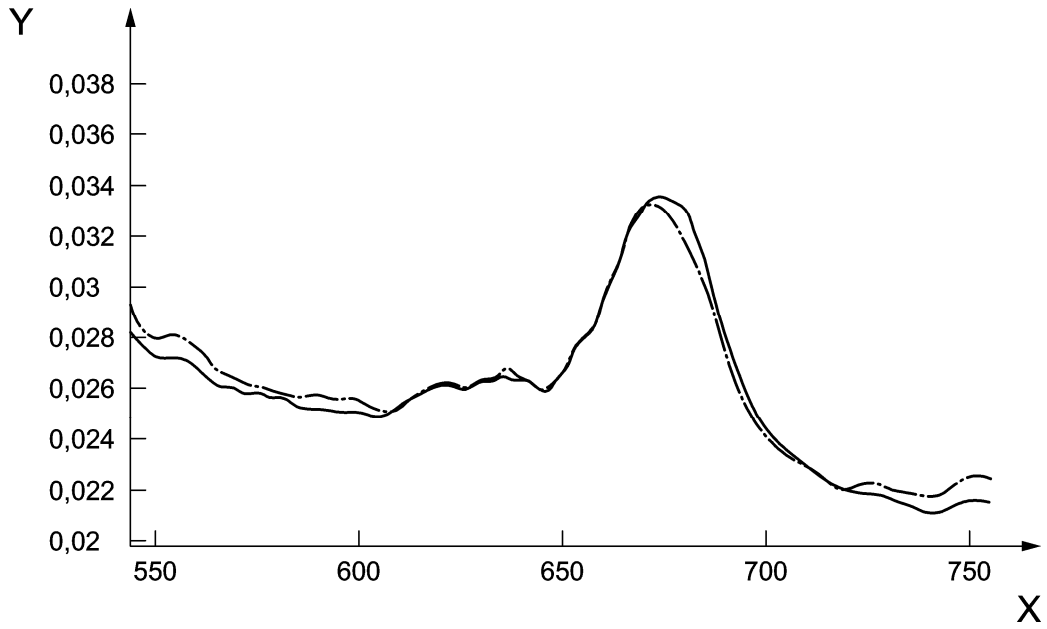
The $a_{ph}(\lambda)$ from this fit is plotted as the solid curve in Figure E.1.

2) This process is repeated at each wavelength using the *in vivo* chlorophyll-a estimate $C_{chl}(IVP)$:

$$a^i_{IVP}(\lambda) = a_{ph}(\lambda)C^i_{chl}(IVP) + a_{resid}(\lambda) \quad \text{for samples } i{=}1{:}122 \tag{E.2}$$

The $a^*_{ph\_sys}(\lambda)$ from this fit is plotted as the dotted curve in Figure E.1.

The fact that the curves in Figure E.1 are so closely aligned shows that the IVP chlorophyll-a estimate leads to the same chlorophyll-specific absorption spectrum in the 675 nm region as the laboratory EP estimate. Note that for these plankton the feature at 675 nm of amplitude < about 0,01 $m^{-1}$ is sitting on a background of chlorophyll-a related absorption of about 0,025 $m^{-1}$. This serves to highlight the difference between the IVP absorption peak estimate, which is a pigment feature shape based estimate, and the total height of the chlorophyll-a specific absorption, often designated $a_{ph}(675)$ in the literature. There is no certainty that this additional broadband "absorption" is not due to chlorophyll correlated backscattering rather than absorption effects as the IVP system used for this work includes a diffuser in the light path providing a measure nearer to 'absorption + backscatter' than to pure absorption.




**Key**

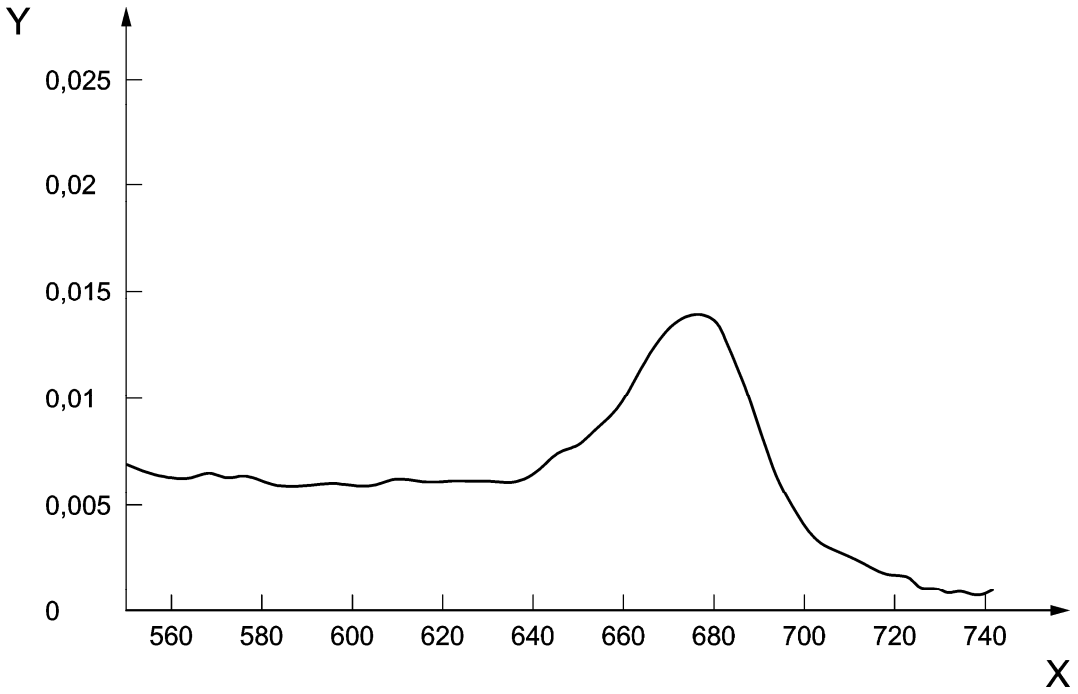
X wavelength, nm

Y chlorophyll-a specific absorption, $m^{-1}$

NOTE Specific spectral absorption of chlorophyll-a as seen by a typical IVP system, determined from IRH laboratory data (solid curve) and IVP data (dotted curve).

**Figure E.1 — Specific spectral absorption of chlorophyll-a from IRH laboratory data and IVP data**

Similarly, the Adasa data set has also been used to determine specific absorption as seen by an IVP instrument. Both of these spectral curves illustrate that the specific absorption feature of chlorophyll-a $a^*_{chl\_sys}(\lambda)$ is always detectable as a component of the total chlorophyll-a specific algal absorption $a^*_{ph}(\lambda)$. The latter should strictly be described in both cases as $a^*_{ph\_sys}(\lambda)$, since in this case it has been observed through the reduced spectral resolution of an IVP system. It is the chlorophyll-a specific featured portion of these spectral curves which the curve-fitting algorithm quantifies.




**Key**

X wavelength, nm

Y absorption, $m^{-1}$

NOTE Specific spectral absorption of chlorophyll-a as seen by a typical IVP system, determined from Adasa Sistemas EP laboratory data

**Figure E.2 — Specific spectral absorption of chlorophyll-a determined from Adasa Sistemas EP laboratory data**

## E.2 *In vivo* photometric chlorophyll-a and the package effect

The package effect arises when the concentration of chlorophyll molecules within cells rises so high that some chlorophyll molecules are effectively shielded from the measuring illumination. This effect is known to cause a flattening effect on the chlorophyll absorption features [10]. It has been suggested that the existence of the package effect, by flattening the absorption feature at 675 nm, may make the IVP chlorophyll-a estimation less valuable as a monitoring tool. There is no doubt that, if the effect were to be significant, the IVP estimate of chlorophyll-a would represent the photosynthetic photon harvesting capability of the suspension and that this would be smaller than the corresponding extracted chlorophyll-a estimate. It could be argued that the *in vivo* absorption gives a better indication of the algal growth potential than the estimate obtained after the chlorophyll-a is extracted from the cells.

The data shown in this annex, based on fresh water samples from rivers in France and Spain, and more recent papers cited in the bibliography below, based on measurements in marine waters in various parts of the world (e.g. [5] [12]), seem to indicate that the overall influence of the package effect on natural water samples at 675 nm is minimal. Indeed, the opposite effect is also observed, where the chlorophyll-a specific absorption $a_{ph}(675)$ is found to be greater than the known specific absorption of chlorophyll-a at 675 nm (e.g. [9]). The chlorophyll-a specific absorption spectra near 675 nm in Figures E.1 and E.2 show that there can be a substantial difference between the total apparent chlorophyll-a specific absorption at 675 nm ($a_{ph}(675)$) and the amplitude of the pigment spectral absorption feature which the IVP technique quantifies; and it is the latter which best correlates with the laboratory determination.

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