A new automated plant pigment analysis system

Application Note

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Introduction

The concentration of photosynthetic pigments is extensively used as an index of algal and phytoplankton biomass (i.e. the productivity of the water masses) and provides information on the quantity and potential photosynthetic activity of algae and algal "blooms". Ratios between various plant pigments possibly indicate the taxonomic composition or the physiological state of that community. The most widely used methods in either fresh or marine waters are based on spectrophotometric techniques[1] after plant pigment extraction in acetone, methanol or ethanol.

It is known that no one solvent or mixture of solvents extracts with 100% efficiency from all algal and phytoplankton groups[2,3,4]. There is conflicting evidence as to the efficiency of each solvent and the stability of the pigments in them. However, it is now generally accepted that extraction by alcohol is superior to acetone for some green and blue algae[5,6]. On the other hand, pigments may easily be extracted from diatoms with either acetone or alcohol[7] and in fact slightly higher figures were obtained for acetone than methanol[2,3,8,11]. Many support the use of acetone for several reasons, e.g. pure chlorophyll is more stable in it, the extinction coefficient is higher in it, the chlorophyll absorption band in the red is sharper in it, the straight forward acidification procedures for estimating chlorophyll a and phaeopigments, etc.

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Australian Water Technologies Trading, Science & Environment (AWTT) formerly Scientific Services Branch of Sydney Water Board) prefer using acetone as extraction solvent, other water and environment authorities nationally and internationally may prefer either methanol or ethanol. In AWTT plant pigments determination has been carried out for hundreds of samples per week over 20 years by the acetone/spectrophotometric method[9] and modification by Jeffrey & Humphries[10]. The method involved filtration followed by overnight chlorophyll extraction in cold 90% aqueous acetone. Then these solutions were scanned before and after acidification using a UV-Vis Shimadzu spectrophotometer; the absorption values were transferred manually to an Olivetti data system to carry out the calculations[9] and modification by Jeffrey & Humphries[10] equations for calculating the chlorophyll a, b, c, phaeopigment and lorenzen chlorophyll values. This method is found to be too slow, so we set about streamlining the method to produce same day results. The original method took 2-3 days to produce results for the water supply managers. The new method will produce hundreds of results the same day and will improve the accuracy and precision.

The two main goals of this work were the first to improve extraction efficiency by using a combined pre- and post-sonication freezing technique to achieve the maximum extraction in the shortest time and least chlorophyll degradation, the second was to introduce automation to the plant pigment determination and measurements by using acetone, methanol and ethanol. The first part of this combined technique was published elsewhere[2], the second part is presented here.

This new hardware/software combination technique makes it possible to execute the analysis of hundreds of samples in the same day and will improve the accuracy and precision. Hence it will increase the efficiency of river and dam management by producing an early warning of potentially toxic algal “blooms” or taste and odour problems thus giving the opportunity to change offtake levels and greatly reduce their toxic potential.

The new method will also be considerably more cost effective. Moreover, this task makes possible the automation of several analytical methods used in the Australian Water Technology Trading (AWTT).

Experimental

This system consists of a double beam scanning Cary 1 spectrophotometer, equipped with an SPS-5 Auto Sampler and fully controlled by a computer data system (see figure 1). A new software programme was written to scan (Table 2, see Appendix), record the absorption values of the different wavelengths (480, 510, 630, 647, 649, 664, 665, 750 nm), then automatically acidify the sample with the required volume (from 0.1-2 mL) of hydrochloric acid and repeat the scanning and recording of the absorption values. Then the system retrieves the stored data from the disk file and executes the calculations using the UNESCO/SCOR trichromatic equations and the modifications by Jeffrey & Humphries[10] and Lorenzen[12] for acetone extracts (See Appendix 1 for the complete equations used).

Method, results and discussion

The complete details of methods of sampling, storage, filtration and extraction are documented elsewhere[2,3]. Briefly, a one or two liter Ruttner bottle or similar sampling device is used for collecting samples from different depths in the storage reservoirs, lakes, rivers and marine sites. The samples to be transported to the laboratory at 4 °C and to be filtered within a maximum of eight hours after sampling using 47 mm glass fiber filter paper if 1.2 µm pore size. Hanna & Horkan used acetone for the plant pigment extraction[3,4]. Ethanol and methanol could be used also as extractants.

The measurements of the optical densities of either aqueous 90% acetone[9], or 90% methanol[13,] or 95% ethanol[14] solutions containing the extracted plant pigment, have been carried out by using this newly developed hardware/software combination system and technique.

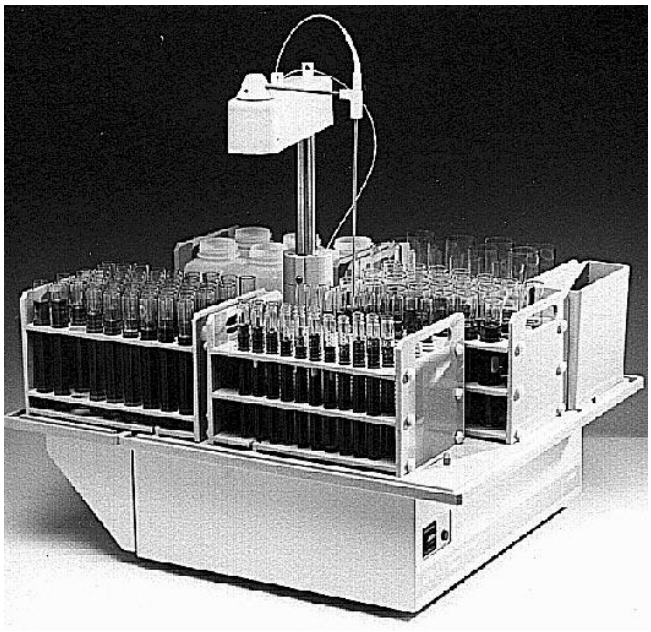

Figure 1. SPS-5 Auto Sampler

The system can produce and print a complete data sheet for the calculated results of chlorophyll a, b, c, phaeopigments a, Carotenes, total chlorophyll before acidifications and then again the corrected values after acidification (Table 1, see Appendix). If the user decides to use methanol as an extractant, it is easy to switch to the methanol programme and the system will execute the calculations using the equations given by Marker et.al.[13] (Appendix 2) for 90% methanol to obtain the chlorophyll a and phaeopigment values in mg/m^3. The system executes measurements before and after acidification considering acidification time to be less than 3 minutes.

If the user decides to use ethanol as an extractant, it is still possible. By choosing one of the two exonol programmes, the system will execute the calculations (Appendix 3) according to the equations given by Wintermans & de Mots[14] which give the chlorophyll a and b values in mg/m^3. By using the equations given by ISO (1992), the corrected chlorophyll a phaeopigment values are obtained by scanning and measuring the absorbance before acidification and then after 3 minutes (but before 30 minutes). This method of measurement of chlorophyll a and phaeopigments may be used semiquantitatively.

The ISO method uses phaeopigments to correct the interference with chlorophyll a determination and to indicate the portion of inactive algal biomass.

A series of pure mono and mixture of algal cultures and natural samples (from rivers, reservoirs, lakes, creek, sea, etc) have been used to compare both the old manual and new auto methods. The results obtained during these tests were recorded together with the time required to get the final results (Table 3, see Appendix). The results obtained during these tests were as follows:

1. Samples were scanned with the old system, then all absorption values were transferred to the old Olivetti. The time required was 8-12 minutes per sample, and;
2. The sub-samples were scanned on the new automated Cary 1, the calculations were carried out automatically and simultaneously within the system. The time required was about one minute. Moreover, the new system executed scanning, acidifying, the scanning again, then calculating automatically unattended. The old system must be attended and manually operated for all stages.

Ten replicate absorption values were obtained from an unacidified and an acidified aliquot of a 90% aqueous acetone pigment solution extracted from different natural samples and pure mono cultures to determine precision. The coefficients of variation were 2.2, 6.5, 10.3 and 7.6% for chlorophyll a, b, c and phaeophytines, respectively.

Analytical accuracy was confirmed (for chlorophyll) by analyses of a series of chlorophyll, a standard prepared from pure standard chlorophyll (Sigma Chemical Co, U.S.A) in 90% acetone. The accuracy for the new system was better than ± 5%.

Conclusion

We conclude that the saving in time, expense and the expected quality assurance benefits from the new automated system are very significant, and fully justify the cost of the development work and that of the new instrument.

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Appendix

Table 1. A model of the full report page

Report Page Header Line 18th Feb, 1993.

AWT Science & Environment
51 Hermitage Road
West Ryde NSW 2114

Chlorophyll
Date: 18th February, 1993.
Source: Warragamba
Client: Water Resources
Sample:

CHL A mg/m^3	CHL B mg/m^3	CHL C mg/m^3	LOR.CHL mg/m^3	PHAE mg/m^3	TOT.PIG mg/m^3
22.95	0.53	2.65	16.99	6.23	23.22
22.99	0.55	2.58	17.10	6.38	23.48

LOR/	PC1	A/B	A/C	LOR/B	LOR/C	TOT.CHL
PHAE		mg/m^3			Not Corr	

TOT CHLBIO PC2 PC3

Corr Mass

Table 2. A model of the first and second page - to execute the programme

CHLOROPHYLL -

1. Source:
2. Client Name:
3. Sample Label File Name:
4. Number of Samples (up to 60):
5. Cell path length (1 cm or 5 cm):
6. Volume of sample filtered (in litres):
7. Volume of Extract (in mL):
8. Volume of Acid (0.1 to 2 mL):
9. Supplementary Report (Y/N):
10. Flush SPS:
11. Select Analysis:

(When chose option 11, the second page appears)

1. Chlorophyll A, B, C in Acetone
2. Full range of plant pigment in Acetone
3. Full range of plant pigment + Carotenoids in Acetone
4. Chlorophyll A, B and Phaeoph in Ethanol
5. Chlorophyll A and phaeoph in Methanol

Table 3. Results of plant pigments using the old and new systems

Sites/Dates	Chlorophyll a		Chlorophyll b		Chlorophyll c		Phaeophytines	
	Old Mean ± SD	New Mean ± SD	Old Mean ± SD	New Mean ± SD	Old Mean ± SD	New Mean ± SD	Old Mean ± SD	New Mean ± SD
Dam Samples 16/9/92	9.4± 0.1 (4)	9.8±0.07 (4)	0.76± 0.3 (4)	1.1±0.07 (4)	0.62±0.01 (4)	1.0±0.1 (4)	-	-
	25.2 (2)	27.4 (2)	4.3 (2)	5.4 (2)	0.56 (2)	2.3 (2)	0.89 (2)	9.9
Urban Runoff Samples 22/992	3.4 (2)	3.2 (2)	0.3 (2)	0.3 (2)	0.37 (2)	1.1 (2)	N.D	3.6 (2)
	7.5 (2)	10.2 (2)	1.0 (2)	2.6 (2)	N.D (2)	1.5 (2)	N.D	2.1 (2)
	12.7 (2)	12.0 (2)	2.3 (2)	2.1 (2)	0.5 (2)	1.1 (2)	N.D	2.6 (2)
Dam Samples 12/11/92	7.5±0.0 (3)	10.1±3.6 (3)	0.3±0.0 (3)	0.0	0.3± 0.0 (3)	0.0	0.5±0.8 (3)	2±1.1 (3)
	6.5±0.2 (3)	7.4±3.1 (3)	N.D (3)	N.D.	0.3±0.2 (3)	N.D	0.9±0.8 (3)	2.3±1.6 (3)
Pure Culture (8)	90.4±0.4 (8)	88.4±0.4 (8)	N.D. (8)	N.D. (8)	1.67±0.7 (8)	2.6±0.1 (8)	N.D. (8)	13.5±1.0 (8)
(Anabena Cylindrica) (3) 15/12/1992	63.7±0.8 (3)	62.0± 0.9 (3)	N.D. (3)	N.D. (3)	N.D. (3)	2.2±0.2 (3)	N.D. (3)	30.0±3.2 (3)
Pure Culture	46.9±3.3 (2)	46.3± 2.2 (2)	N.D. (2)	N.D. (2)	1.1±0.7 (2)	2.2±0.8 (2)	N.D. (2)	6.8±2.0 (2)
16/12/92 (2)	85.9±8.2 (2)	83.4±6.3 (2)	N.D. (2)	N.D. (2)	1.3±0.5 (2)	3.7±0.1 (2)	N.D. (2)	56.3±1.0 (2)
(Mix)	48.4±1.2(10)	49.6±1.1 (10)	0.8±0.7 (10)	3.1±0.2 (10)	3.2±1.2 (10)	7.8±0.8 (10)	36.1±3.0 (10)	43.0±3.3 (10)
Sea Water	1.9±0.3 (6)	2.2±0.2 (6)	N.D	N.D	N.D.	N.D.	N.D.	N.D.

( ) The number in brackets represents the number of samples

Appendix 1. SCOR/UNESCO[9], JJeffrey & Humphrey[10] and Lorenzen[12] trichromatic equations using 90% acetone for plant pigment calculations

Chlorophyll a = $\frac{Ca\ VE}{V_F * n}$ mg/m³

Chlorophyll b = $\frac{Cb\ VE}{VF * n}$ mg/m³

Chlorophyll c = $\frac{Cc\ VE}{VF * n}$ mg/m³

Lorenzen Chlorophyll = $\frac{26.73\ (E_{665b} - E_{665a})\ VE}{VF\ *\ N}$ mg/m³

Phaeopigments = $\frac{26.73\ (1.7\ E_{665b}) - E_{665a})\ VE}{VF\ *\ n}$ mg/m³

Plant Carotenoids (Without regard to the nature of the Crop) = $\frac{[7.6\ (E_{480} - 1.49\ E_{510})]\ VE}{VF\ *\ n}$ mg/m³

Plant Carotenoids (if crop predominantly Chlorophyta or Cyanophyta) = $\frac{[4.8\ E_{480}]\ VE}{VF\ *\ n}$ mg/m³

Plant Carotenoids (if Crop predominantly chrysophyta or pyrrophyta) = $\frac{[10.0\ E_{480}]\ VE}{VF\ *\ n}$ mg/m³

Where: VE = Volume of 90% acetone extract in ml
VF = Water sample filtered in L
n = Light Path length in cm

$Ca = 11.85\ E_{664} - 1.54\ E_{647} - 0.08\ E_{630}$
$Cb = 21.03\ E_{647} - 5.43\ E_{664} - 2.66\ E_{630}$
$Cc = 24.52\ E_{630} - 1.67\ E_{664} - 7.6\ E_{647}$

$E_{664} = OD_{664} - OD_{750}$
$E_{647} = OD_{647} - OD_{750}$
$E_{630} = OD_{630} - OD_{750}$
$E_{510} = OD_{510} - OD_{750}$
$E_{480} = OD_{480} - OD_{750}$
$E_{665b} = OD_{665b} - OD_{750b}$ Before acidification
$E_{665a} = OD_{665a} - OD_{750a}$ After acidification

Appendix 2. Marker et .al Equations[13] using 90%methanol for plant pigment calculations

$$chl\ a = \frac{37.5\ (Ab - Aa)\ VE}{VF * n} \quad mg/m^3$$

$$\text{Phaeopigment} = \frac{19.24\ [Ab - 3.0\ (Ab - Aa)]\ VE}{VF * n}$$

Ab = $OD_{665} - OD_{750}$ Before acidification
Aa = $OD_{665} - OD_{750}$ After acidification
VE = Volume of 90% methanol extract in ml
VF = Volume of water sample filtered in L
n = Light path length in cm

Appendix 3. ISO (1992)and Wintermans &Demots[14] using 95% ethanol for plant pigment calculations

This method for the determination of the chlorophyll a, b and phaeopigments concentrations only. Phaeopigments may be used to correct for interference with chlorophyll a determinations and to indicate the portion of inactive algal biomass.

1. According to Wintermans & de Mots (1965)

$$\text{chl a} = \frac{(13.7\ E_{665} - 5.76\ E_{649})\ VE}{VF * n} \quad mg/m^3$$

$$\text{chl b} = \frac{(25.8\ E_{649} - 7.6\ E_{665})\ VE}{VF * n} \quad mg/m^3$$

Where $E_{665} = OD_{665} - OD_{750}$
$E_{649} = OD_{649} - OD_{750}$

VE = Volume of 95% ethanol extract in ml
VF = Volume of Water Sample filtered in L
n = Light path length in cm

2. ISO (1992)

Considering the correction for interference with chlorophyll a from the presence of phaeopigments.

$$\text{chl a} = \frac{29.6\ (Ab - Aa)\ VE}{VF * n} \quad mg/m^3$$

$$\text{phaeopigment} = \frac{20.8\ Aa * VE}{VF * n} - \text{chl a} \quad mg/m^3$$

Ab = $OD_{665} - OD_{750}$ Before acidification
Aa = $OD_{665} - OD_{750}$ After acidification

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