Optical Measurements

phytoplankton pigments

absorption characteristics

cal/val of ocean colour sensors

Why study pigments?

Phytoplankton (microscopic plant life) are the basis of the food web within the oceans.

Phytoplankton are composed of pigments, the most dominant being chl *a*, which via photosynthesis converts light energy into chemical energy.

Chl *a* is an indicator of phytoplankton biomass and can be estimated on a global or regional scale using remotely sensed ocean colour techniques.

Important to know the quality of the data you may use in algorithm development, cal/val work etc..

Algal classes

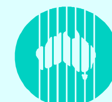
- Bacillariophyta - diatoms - *Chaetoceros sp.*
  Skeletonema sp.
- Dinophyceae - dinoflagellates - *Amphidinium sp.*
  Gymnodinium sp.
- Prymnesiophyceae - *Emiliania sp.*
  Phaeocystis sp.
  Pavlova sp.
- Cyanophyta - cyanobacteria, blue-green algae -
  Mycrocystis sp.
  Synechococcus sp.
  Trichodesmium sp.

Algal classes

- Prochlorophyta - prochlorophytes - *Prochlorococcus sp.*
- Chlorophyceae - chlorophytes, green algae - *Dunaliella sp.*
- Cryptophyta - cryptomonads - *Chroomonas sp.*
  Rhodomonas sp.
- Rhodophyta - red algae - *Porphyridium purpureum*
- Eustigmatophyta - yellow-green algae - *Nannochloropsis sp.*
- Chrysophyceae, Euglenophyta, Prasinophyceae and Raphidophyceae.

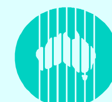
Distribution of pigments in algal classes

	Chlorophylls										Xanthophylls													
	chl a	chl b	chl c1	chl c2	chl c3	MgDVP	DV a	DV b	β,ε – car	β,β – car	Allo	19 BF	Diadino	Dino	Fuco	19HF	Lut	Neo	Per	Pras	Viola	Zea	P/cyanin	P/erythrin
Cyanophyta	●									●												●	●	●
Prochlorophyta							●	●	●	●												●		
Rhodophyta	●								●													●	•	●
Cryptophyta	●			●					●		●												●	●
Chlorophyceae	●	●							•	●							●	●			●	•		
Prasinophyceae	●	●				●			•	●							●	●		●	●			
Euglenophyta	●	●								●			●					•						
Eustigmatophyta	●									●											●	•		
Bacillariophyta	●		●	●						•			●		●									
Dinophyta	●			●						•			●	●					●					
Prymnesiophyceae	●		●	●	●				•	•		●	●		●	●								
Chrysophyceae	●			●	●					•		●	●		●									
Raphidophyceae	●		●	●						●			●		●									



Diagnostic marker pigments for algal classes

• Diatoms	fucoxanthin and diadinoxanthin
• Dinoflagellates	peridinin*
• Prymnesiophytes	19'-HF
• Prochlorophytes	DV chl *a* and DV chl *b*
• Chlorophytes	lutein
• Cryptophytes	alloxanthin
• Chrysophytes	19'-BF
• Prasinophytes	prasinoxanthin
• Eustigmatophytes	vaucheriaxanthin ester
• Cyanobacteria	(zeaxanthin and phycobilliproteins)



NATO/IOCCG Ocean Colour Training Course, Erdemli, Turkey, September 2000

How to measure pigments (1)

Sample collected on GF/F filter

- extracted in solvent

- analysed using High Performance Liquid Chromatography (HPLC)

  with gradient elution

  and photo-diode array detection

Samples need to be kept in the cool and the dark to avoid pigment degradation during the filtration and extraction processes.

If samples can't be analysed immediately after collection, store in liquid nitrogen.

How to measure pigments (2)

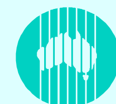
There are several methods in the literature that can be used:

- Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton

Wright et al. (1991), Marine Ecology Progress Series, 77, 183-196.

- uses a ternary gradient system with a reversed-phased polymeric column (ODS, C_{18})

- claims to separate > 50 pigments; used in global studies such as JGOFS and is the SCOR recommeded method
- disadvantage: cannot separate divinyl and monovinyl chl *a* or chl *b*



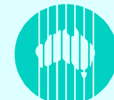
How to measure pigments (3)

- **Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C_8 column and pyridine-containing mobile phase**

Zapata et al. (2000), Marine Ecology Progress Series, 195, 29-45.

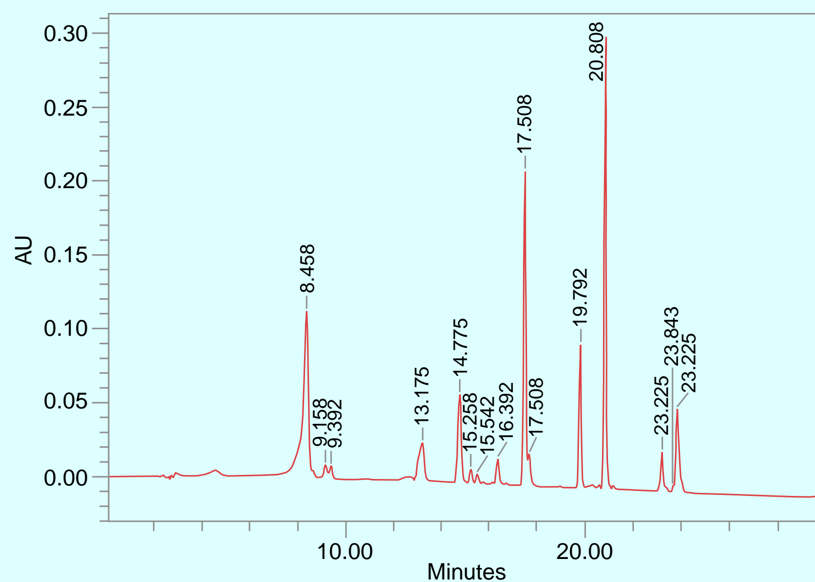
- uses a binary gradient system with a reversed-phase monomeric C_8 column maintained at 25°C

- can separate divinyl and monovinyl chl *a*



CSIRO MARINE RESEARCH

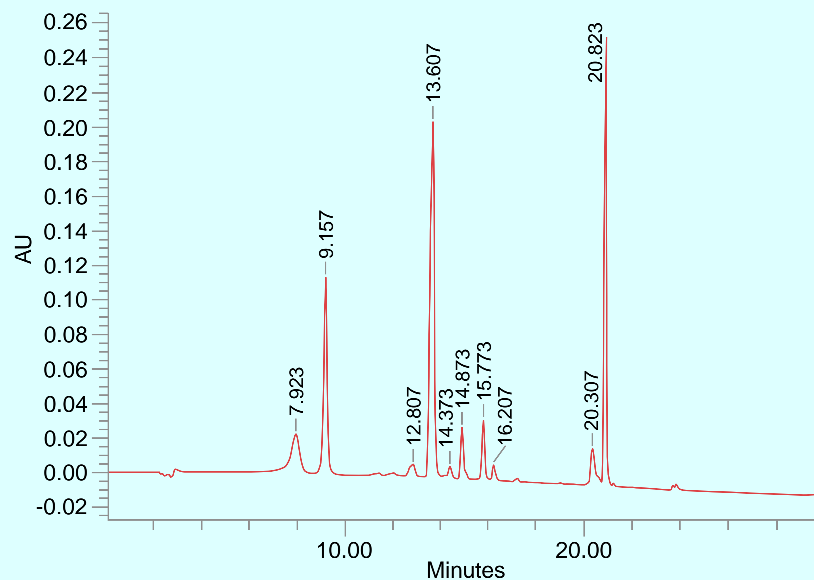
How to measure pigments (4)



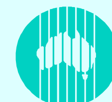
Dunaliella tertiolecta

CSIRO MARINE RESEARCH

How to measure pigments (5)



Emiliania huxleyi



Identifying pigments (1)

Pigment identification is made through the combination of retention time and absorption spectra

The easiest and most accurate method to identify individual pigments is to use a photo-diode array detector (PDA) with the HPLC system.

PDA detection involves scanning the sample continuously from 350 - 700 nm throughout the chromatographic run time.

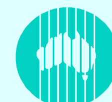
For each peak recorded at a single wavelength there is a full absorption spectra available.

These spectra can be matched to those in the literature for identification.

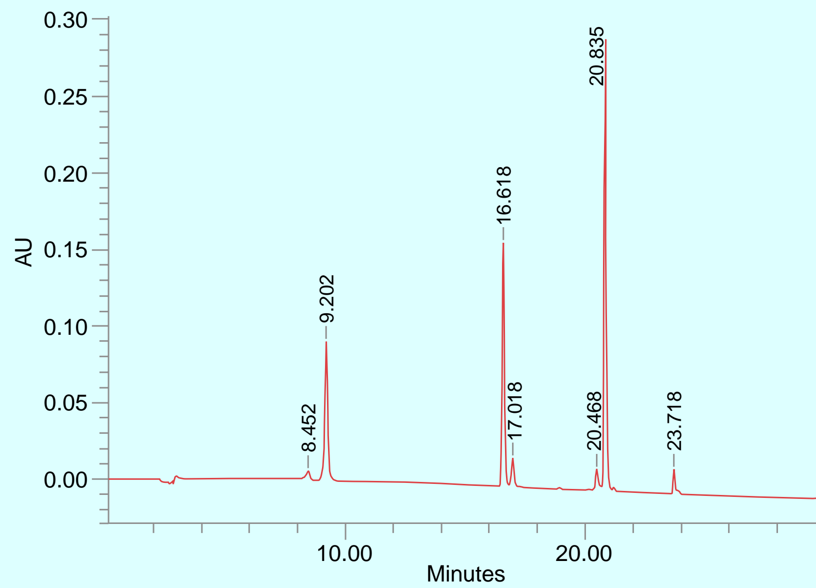
- **Phytoplankton pigments in Oceanography**

  Jeffrey, S.W., Mantoura, R.F.C. and Wright, S.W. (editors)

  UNESCO Publishing (1997)

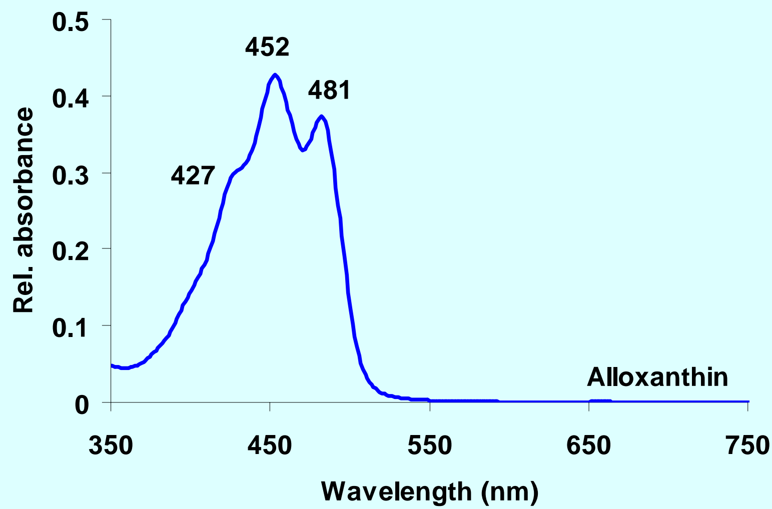


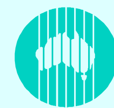
Identifying pigments (2)



Chroomonas salina

Identifying pigments (3)





Identifying pigments (4)

- Chromatograms are recorded at one or more single wavelengths. ie: 436 or 440 nm for reasonable retrieval of most chlorophylls and carotenoids.

- Using algal cultures where the pigment composition is known, you can compile a spectral library within your HPLC/PDA software

- More than one pigment can have the same absorption spectra, so retention time becomes important in discriminating between pigments.

Alloxanthin	λmax = 426, 454, 483 nm
Diatoxanthin	λmax = 427, 454, 482 nm
Zeaxanthin	λmax = 428, 455, 483 nm
β,β-carotene	λmax = 433, 455, 482 nm

Quantifying pigment concentration (1)

Some pigment standards are commercially available:

Chl *a*, Chl *b*, β,β-carotene	Sigma
several pigments	The International Agency for ^{14}C Determination, Denmark

A cheaper alternative is to isolate the pigments from cultures

CSIRO Microalgal Research Centre Australia

(full collection of SCOR cultures)

Centres in U.S.A., U.K., Japan & others

Isolation of pigments is done by
- preparative HPLC with fraction collection
- HPTLC

Quantifying pigment concentration (2)

Using spectrophotometric analysis, confirm the purity of the pigment by absorption spectra and determine the concentration using extinction coefficients from the literature.

Prepare a set of standards

- run each standard on the HPLC
- determine a calibration curve
- determine response factors

Standards should be run at regular intervals and whenever some part of the HPLC system has changed; ie: new lamp.

The phycobilliproteins are water soluble and do not extract into organic solvents.

Quantifying pigment concentration (3)

Conc.in sample (ug/L) = (PA/RF) x (v/V) x (Std. I/Sam. I) x dil. Factor

PA = peak area

RF = response factor

v = volume of extract

V = volume of sample

Std. I = injection volume of std.

Sam. I = injection volume of sample

Chlorophyll *a*

For determination of chl a alone, spectrophotometric or fluorometric analysis is quicker and easier.

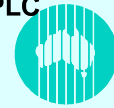
Spectrophotmetric analysis - use equations determined by Jeffrey and Humphrey (1975)

- **need to ensure range is in the linear part of the curve**
- **acidification of extract allows determination of the phaeopigments.**

Fluorometric analysis

- **more sensitive than spectrophotometric analysis - lower detection levels**
- **no interference from carotenoids**

- ** **Need spectrophotometric methods to calibrate fluorometric and HPLC methods.**



Absorption by in-water components (1)

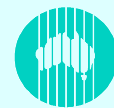
$$a(\lambda) = a_{ph}(\lambda) + a_d(\lambda) + a_{CDOM}(\lambda) + a_w(\lambda)$$

where $a_{ph}(\lambda)$ **is the absorption due to phytoplankton**

$a_d(\lambda)$ **is the absorption due to detritus or non-algal particulate matter**

$a_{CDOM}(\lambda)$ **is the absorption due to the chromophoric dissolved organic matter**

$a_w(\lambda)$ **is due to the absorption of the water**



Absorption by in-water components (2)

$$a_p(\lambda) = a_{ph}(\lambda) + a_d(\lambda)$$

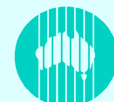
where $a_p(\lambda)$ is due to the absorption of all particulate matter

Direct measurements of $a_p(\lambda)$, $a_d(\lambda)$ and $a_{CDOM}(\lambda)$

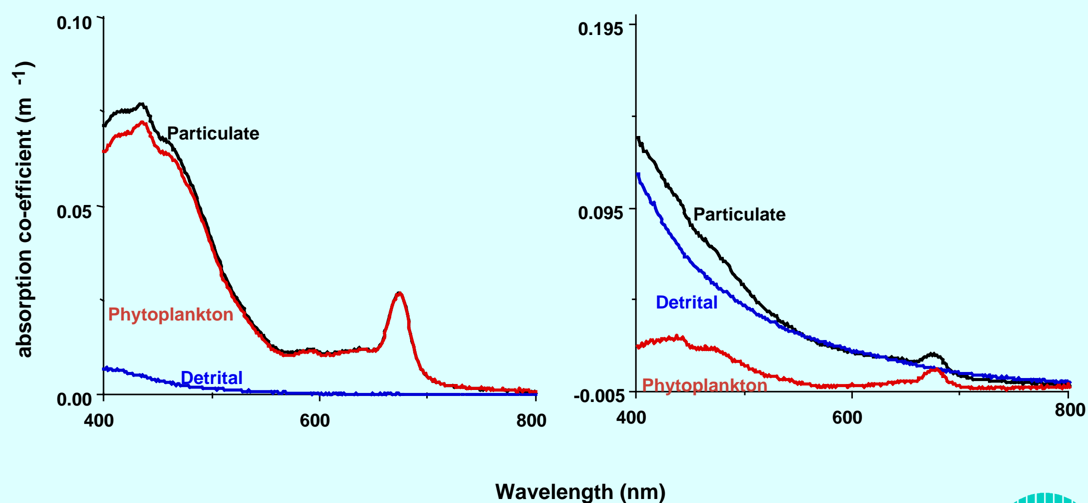
Literature values of $a_w(\lambda)$ - Smith and Baker (1981)

- Pope and Fry (1997)

Indirect measurement of $a_{ph}(\lambda)$ $[a_{ph}(\lambda) = a_p(\lambda) - a_d(\lambda)]$



Absorption by in-water components (3)



Absorption by phytoplankton (1)

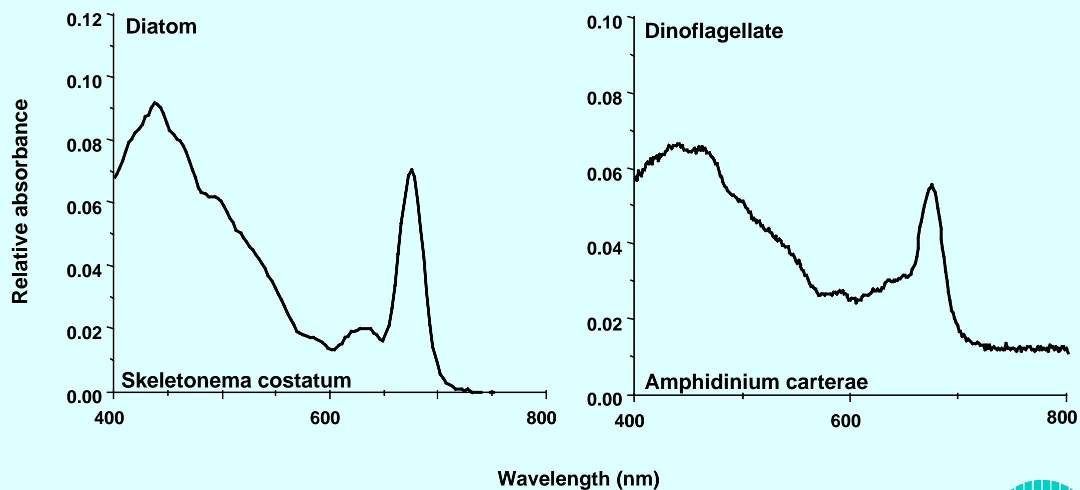
Factors which can affect the shape of the absorption spectra due to phytoplankton are:

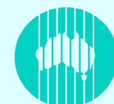
- **pigment composition and concentration**
- **shape and size of cells**
- **light adaption**
- **physiological state of cells**

However the basic shape of the absorption spectra of any algal group is a reflection of its pigment composition.

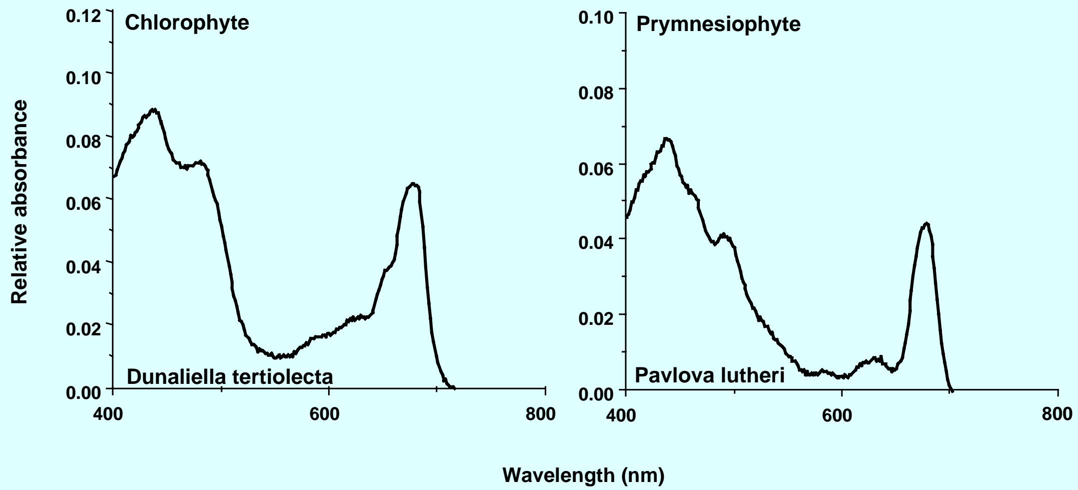
Scan algal cultures in suspension directly in a spectrophotometer

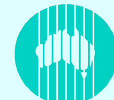
Absorption by phytoplankton (2)



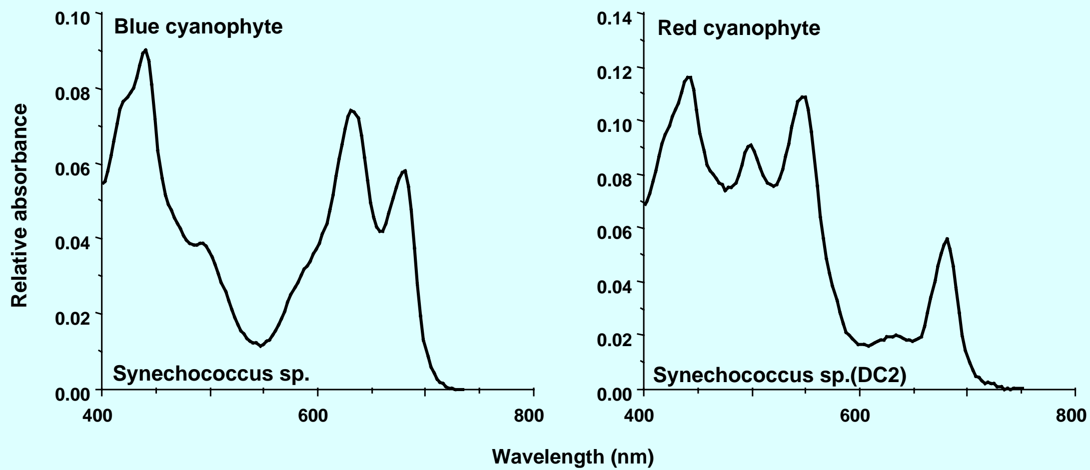


Absorption by phytoplankton (3)





Absorption by phytoplankton (4)



Absorption by phytoplankton (5)

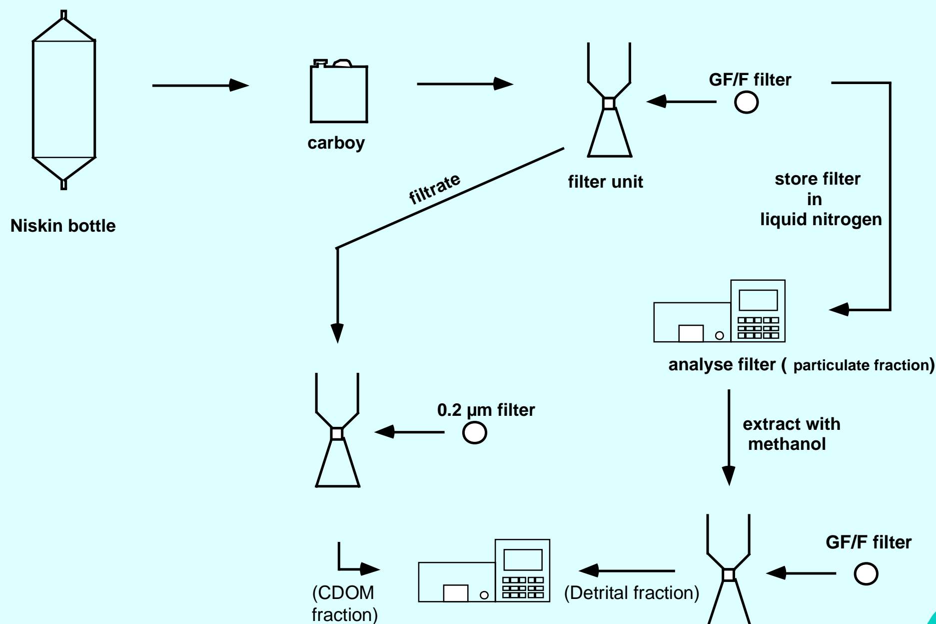
For field samples this direct form of measurement is not available as most waters contain such a dilute concentration of suspended particulate matter.

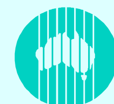
Concentrate particulate matter on a glass-fibre filter (GF/F) and scan the filter in a spectrophotometer.

Ideally the spectrophotometer should be equipped with an integrating sphere or a diffuse reflectance attachment to avoid the loss of forward scattered light.

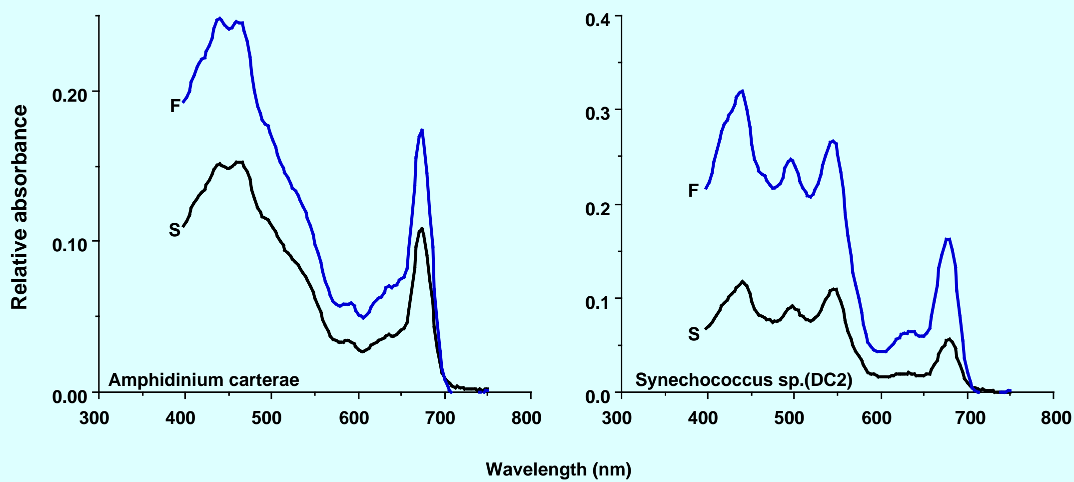
The detritus scan is obtained by extracting the pigments from the particulate matter on the filter, generally using the method of Kishino et al. (1985) and then re-scanning the filter.

Absorption measurement of water components





Absorption by phytoplankton (6)



Absorption by phytoplankton (7)

Find no major spectral differences in the absorption by the particulate material, whether it be in suspension or on a filter.

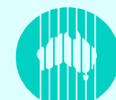
The light absorption is greater on the filter than in suspension due to the scattering of light within the GF/F filter - pathlength amplification.

$$\beta = l_o/l_g$$

where l_o is the optical pathlength and l_g is the geometric pathlength

$l_g = V/A$ **where V is the volume of sample filtered and A is the clearance area of the filter**

$$a_p(\lambda) = 2.3\ A_p(\lambda)/\beta\ (\lambda)$$



Absorption by phytoplankton (8)

Mitchell (1990) using the "quantitative filter technique - QFT"

developed a quadratic equation to determine the equivalent optical density in solution of the particulate matter on the filter.

$$OD_S(\lambda) = aOD_F(\lambda) + b(OD_F(\lambda))^2$$

where a = 0.392 and b= 0.665 (Mitchell, 1990)

or a = 0.378 and b = 0.523 (Cleveland and Wiedemann, 1993)

Both sets of coefficients are recommended in the SeaWiFS protocols.

$$a_p(\lambda) = 2.3\ OD_S(\lambda)/l_g$$

Absorption by phytoplankton (9)

The equations of both Mitchell (1990) and Cleveland and Wiedemann (1993) are general and have been developed from a range of different algal species.

Moore et al. (1995) looked specifically at *Prochlorococcus* and *Synechococcus* species, which are dominant in oligotrophic waters.

Prochlorococcus marinus

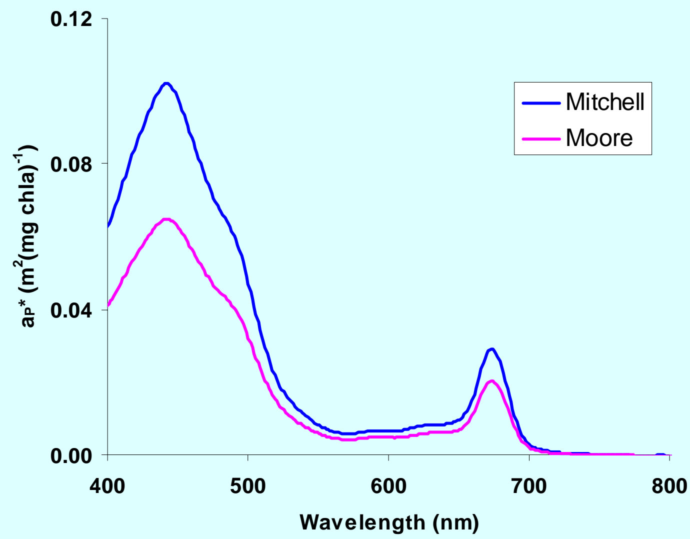
$$OD_S(\lambda) = 0.291OD_F(\lambda) + 0.051(OD_F(\lambda))^2$$

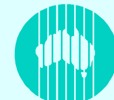
Synechococcus WH8103

$$OD_S(\lambda) = 0.304OD_F(\lambda) + 0.450(OD_F(\lambda))^2$$

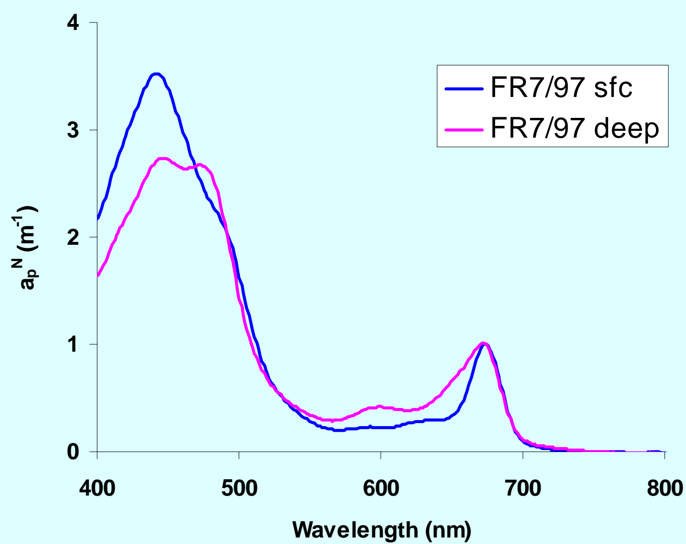
which are 50% and 30%, respectively, lower at $OD_F = 0.4$ than that of Mitchell (1990).

Absorption by phytoplankton (10)





Absorption by phytoplankton (11)



Absorption by phytoplankton (12)

Don’t overload filters with particulate material

- maximum $OD_F(\lambda)$ should not exceed 0.4

Filtering of samples and preparation of samples for analysis should be done under dimmed light.

During sample preparation, filters should be kept in the cool and the dark.

Sample and blank filters should come from the same box/batch.

If samples can’t be analysed immediately after collection, store flat in liquid nitrogen.

Absorption by phytoplankton (13)

Disadvantage of the GF/F technique

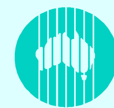
pore size of GF/F is 0.8 - 1.0 µm

- therefore only measuring absorption due to particles > 0.8 µm

CDOM is a measurement of absorption due to particles < 0.2 µm

Therefore the fraction between 0.2 and 0.8 µm is not accounted for.

If this fraction is considered important, may want to use the technique of Allali et al. (1995) Limnology and Oceanography, 40, 1526 - 1532.



Absorption by phytoplankton (14)

Specific absorption is defined as the absorption coefficient of phytoplankton ($a_{ph}(\lambda)$) per unit concentration of a specific substance.

In most optical applications, the substance is the dominant pigment chl *a* or chl*a* plus its degradation products.

$$a^*_{ph}(\lambda) = a_{ph}(\lambda)/[\text{chl } a] \qquad (m^2(\text{mg chl } a)^{-1}$$

Use $a^*_{ph}(\lambda)$ to compare absorption characteristics (both magnitude and spectral shape) between regions.

The specific absorption coefficient indicates the amount of radiant energy the cells are able to utilise.

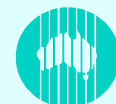
Absorption by phytoplankton (15)

Variation in specific absorption spectra is due to

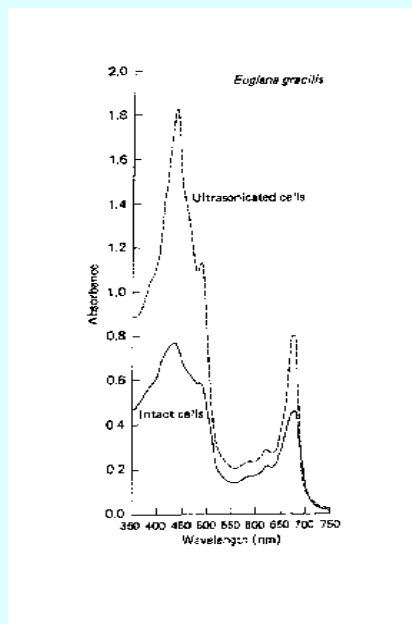
- differences in cell size
- intracellular pigment concentration
- the composition of the accessory pigments

The main variation occurs in the blue region of the spectrum where the accessory pigments have maximal absorption.

Differences in cell size and intracellular pigment concentration causes the “packaging effect” which leads to the “flattening effect”.



Absorption by phytoplankton (16)

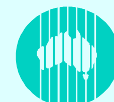


From Kirk (1994)

Duysens (1956) was the first to report that the absorption spectra per unit pigment of a suspension of intact cells will be "flattened" at all wavelengths in comparison to a homogeneous solution of the same cells.

The flattening effect is caused by the "packaging effect".

ie: the pigments are contained within discrete packages - within chloroplasts, cells and cell colonies.

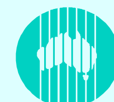


Absorption by phytoplankton (17)

That is an increase in either the average cell size or the intracellular pigment concentration results in a depressing or flattening of the absorption spectra.

Algal species have very different cell size and shape and this together with variation in the intracellular pigment concentration causes the different species to exhibit differing degrees of the packaging effect.

An increase in the packaging effect reduces the ability of the cells to collect radiant energy.



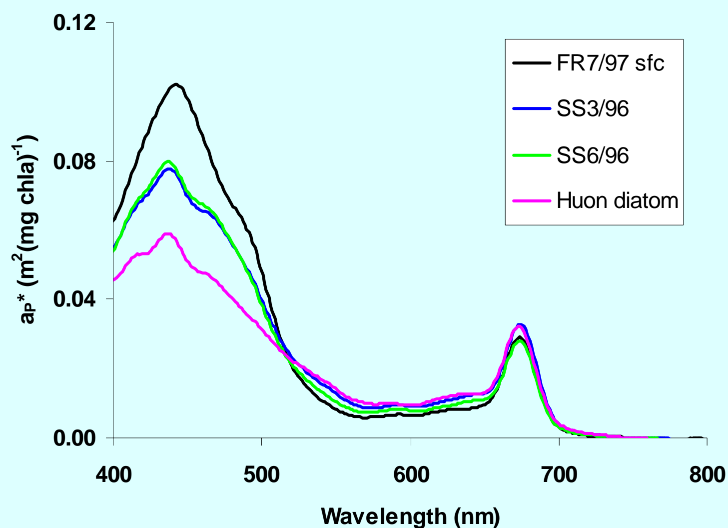
Absorption by phytoplankton (18)

Prochlorophytes	0.4 - 0.8 µm
Cyanobacteria	0.5 - 2.0 µm
Prymnesiophytes	5 - 20 µm
Chlorophytes	1 - 40 µm
Diatoms	2 - 200 µm
Dinoflagellates	10 - 200 µm

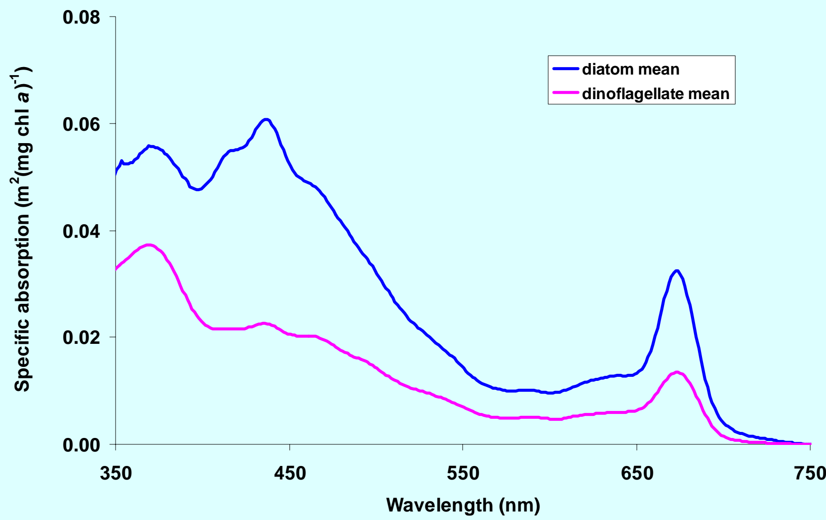
Generally find higher $a^*_{ph}(440)$ values in areas dominated by small cells, such as the equatorial region.

This is probably due to a weaker packaging effect due to the small size of cells and/or low intracellular pigment concentration.

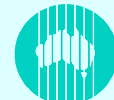
Absorption by phytoplankton (19)



Absorption by phytoplankton (20)



Diatoms 8-15 µm; Dinoflagellates 30 -120 µm plus chains



Absorption by phytoplankton (21)

$a^*_{ph}(\lambda)$ - separated into contributions from photosynthetic pigments (ps) and nonphotosynthetic pigments (nps).

$$a^*_{ph}(\lambda) = a^*_{ps}(\lambda) + a^*_{nps}(\lambda)$$

Photosynthetic pigments transfer excitation energy to chl a to be used in photosynthesis.

- chl *b*, chl *c*, peridinin, fucoxanthin, 19BF, 19HF, phycobilliproteins

Nonphotosynthetic pigments absorb energy but do not transfer it to chl a.

- violaxanthin, diadinoxanthin (DD), alloxanthin, diatoxanthin (DT), lutein, zeaxanthin, β,ε-carotene, β, β -carotene

NPS pigments protect the photosynthetic system from high irradiance and are often referred to as photoprotective pigments.

Absorption by phytoplankton (22)

$a^*_{ps}(\lambda)$ and $a^*_{nps}(\lambda)$ are reconstructed based on scaling the absorbance of each pigment (determined by HPLC) to its weight specific extinction coefficient at a given λ with the absorption maxima shifted to match the *in vivo* maxima.

Bidigare et al. (1987,1990); Babin et al. (1996)

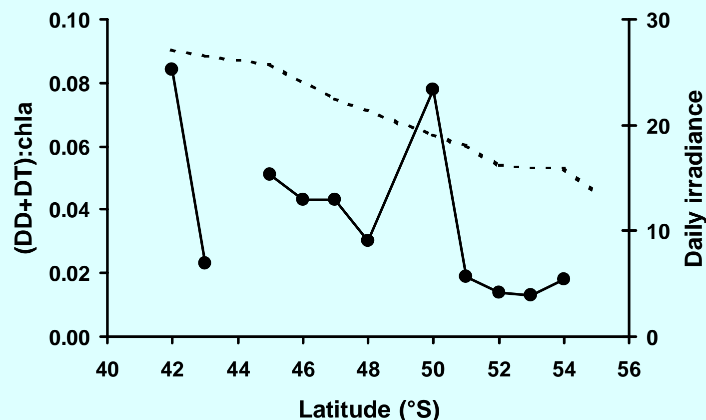
Pigments in vivo are complexed with proteins which cause a shift in the absorption maxima to the red end of the spectrum by up to 90 nm.

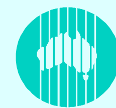
Pigment	In vivo maxima (nm)	
Chlorophyll *a*	435	675
Chlorophyll *b*	470—490	650
Chlorophyll *c*	450	630
Carotenoids	475 ———— 540	
Phycocyanin	580— 600	
Phycoerythrin	540-565	

Absorption by phytoplankton (23)

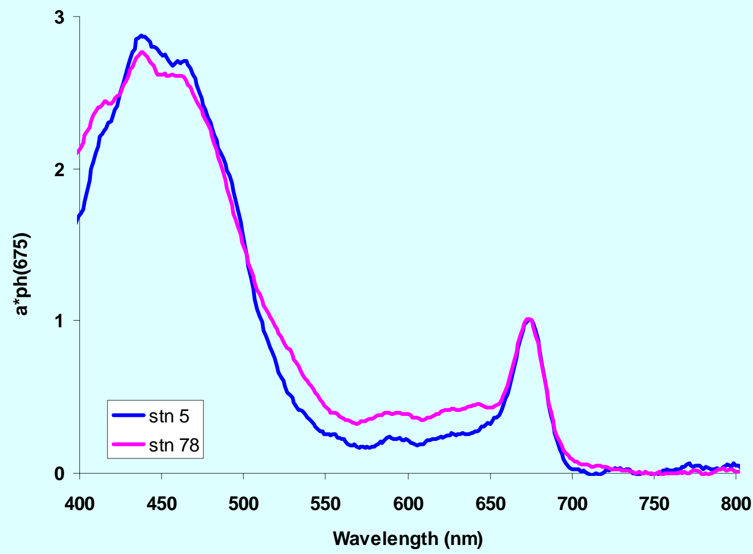
The relative amounts of individual pigments within a species pigment composition can vary with changes in irradiance.

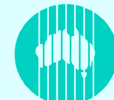
Increase in nps or photoprotective pigments with an increase in irradiance can cause small differences in the shape of the absorption spectra.



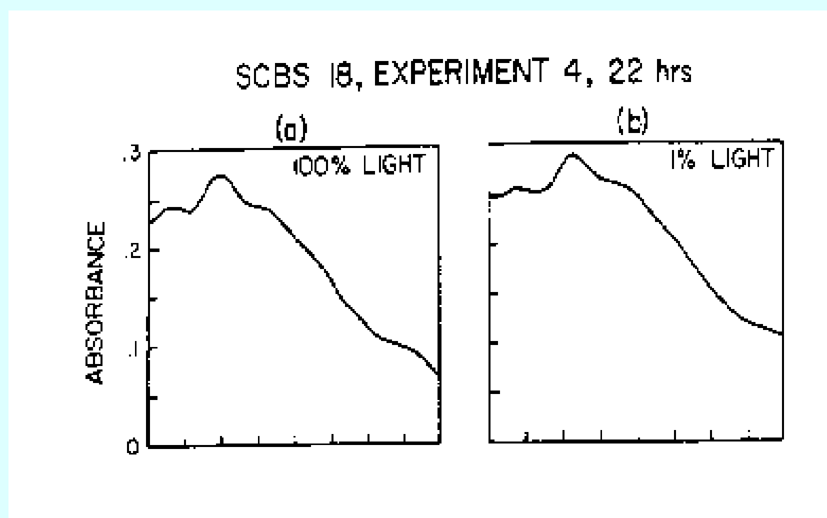


Absorption by phytoplankton (24)





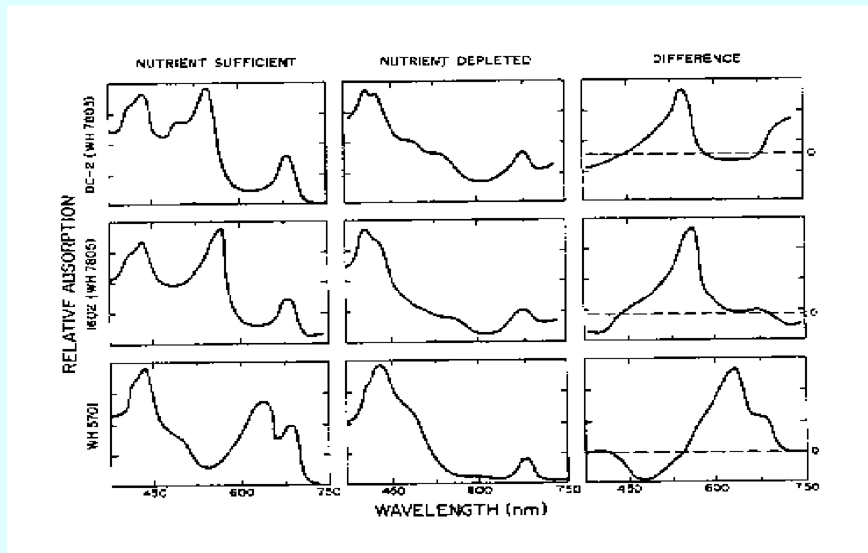
Absorption by phytoplankton (25)



From Neori et al. (1994)

Absorption by phytoplankton (26)

Physiological effects



From Lewis et al. (1986)

CSIRO
MARINE RESEARCH

Absorption by CDOM and detritus (1)

The detritus scan is obtained by extracting the pigments from the particulate matter on the filter, generally using the method of Kishino et al. (1985) and then re-scanning the filter.

$$a_d(\lambda) = 2.3\ OD_S(\lambda)/l_g$$

CDOM absorption is measured in 10 cm quartz cells in the normal cell compartment of the spectrophotometer with doubly distilled deionised water as the reference.

$$a_{CDOM}(\lambda) = 2.3\ (A(\lambda)/l)$$

where $A(\lambda)$ is the absorbance (normalised to zero at a particular wavelength) and l is the cell path length in meters.

CSIRO
MARINE RESEARCH

Cal/val of the satellite sensor (1)

Why make these optical measurements?

To determine how well global ocean colour algorithms predict phytoplankton biomass and associated conditions within a particular region.

Many of the components of the global ocean colour algorithms are based on temperate water conditions, particularly in the northern hemisphere.

Errors may arise in other regions where the conditions are routinely different to those of northern hemishere temperate waters.

Need to develop regional ocean colour algorithms.

Cal/val of the satellite sensor (2)

Ocean colour algorithms predict chl *a* concentration by comparing ratios of water leaving radiance (L_w) or reflectance (R) at two or more wavelengths.

Main assumption is that light attenuation by the dissolved and detrital components covaries with phytoplankton absorption.

True for case 1 waters where phytoplankton absorption is the dominant contributor to the total absorption.

Errors will arise for case 2 waters where either the CDOM or the detrital absorption or both are equal to or greater than the phytoplankton absorption.

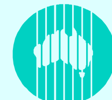
Cal/val of the satellite sensor (3)

In the SeaWiFS algorithm the ratio used is R(490):R(555)

Changes in this ratio are interpretated as changes in chl *a*.

The presence of other pigments and substances can change the ratio.

- high CDOM
- the presence of phycobilliproteins
- changes in species composition



CSIRO
MARINE RESEARCH

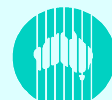
Cal/val of the satellite sensor (4)

- high CDOM - CDOM absorption increases exponentially with decreasing wavelength.

In areas where CDOM is a significant component of the total absorption the effect on the ratio will be to indicate an overestimate of the chl *a* concentration.

- the presence of phycobilliproteins

Pigment	In vivo maxima (nm)	
Chlorophyll *a*	435	675
Chlorophyll *b*	470—490	650
Chlorophyll *c*	450	630
Carotenoids	475—540	
Phycocyanin	580—600	
Phycoerythrin	540-565	



CSIRO
MARINE RESEARCH

Cal/val of the satellite sensor (5)

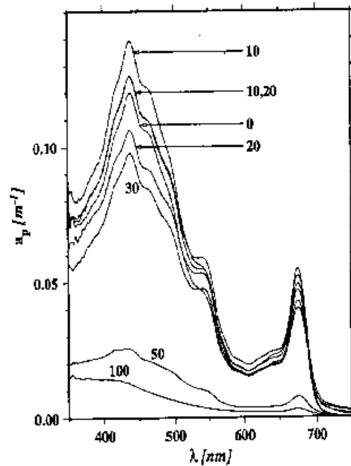


Fig. 3. Absorption spectra of the algal plus nonalgal particles, sampled at various depths as indicated, on 16 and 17 June.

Morel (1997)

A *Synechococcus* bloom showed high absorption capabilities, hence depressing the reflectance at both 443 and 555 nm. The R(443):R(555) ratio was lowered and produced an over estimate of chl *a* by a factor of 3.

Trichodesmium species also contain phycoerythrin, but have different reflective properties to *Synechococcus.*

(Coccolithophore blooms are another unique example.)

CSIRO
MARINE RESEARCH

Cal/val of the satellite sensor (6)

- changes in species composition

Hoepffner and Sathyendranath (1992) found a positive correlation (r = 0.86) between the relative absorption at 550 nm and the ratio of fucox:chl *a*.

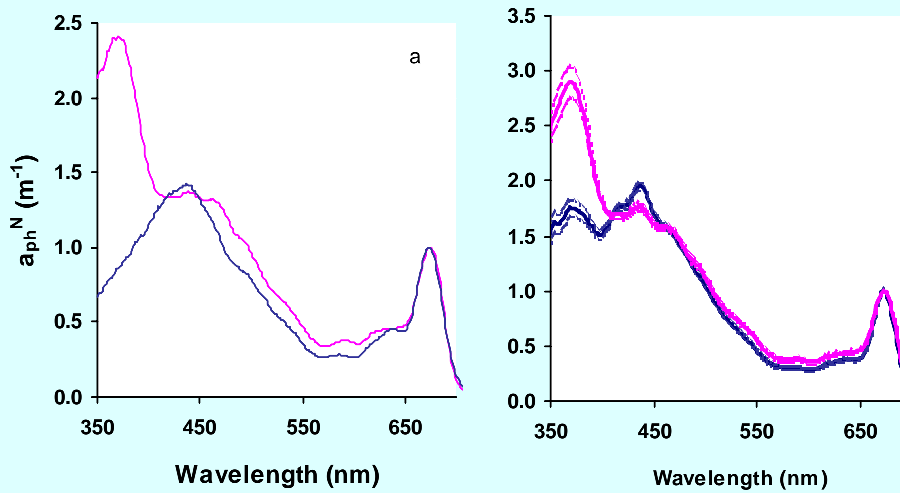
The ratio of pigments such as fucoxanthin to chl *a* can vary without there being any change in the chl *a* concentration.

CSIRO
MARINE RESEARCH

NATO/IOCCG Ocean Colour Training Course, Erdemli, Turkey, September 2000

Cal/val of the satellite sensor (7)

Other factors: species discrimination.



Cal/val of the satellite sensor (8)

Other factors: motility of algal species.