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Comparison of chlorophyll-fluorescence-based measuring systems for the detection of algal groups and the determination of chlorophyll-*a* concentrations

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1. Introduction

The rise of the CO_2 amount in the atmosphere is one of the facts which makes research on measurement biomass production of increasing importance. Microalgae in the oceans (phytoplankton) are one of the most important primary producers, and their large surface to volume ratio and high growth rates make them good indicators for eutrophication and toxic stress (Vanselow 1998).

In general, there is a wide spectrum of possible uses of algae in water management, e.g. as indicators for toxicity and eutrophication. There is a large interest to detect imbalances in aquatic biological systems. Shellfish or fish can accumulate dangerous concentrations of toxins from a bloom-forming toxic alga. Also, although not toxic, a member of the algal class of the prymnesiophytes in the North Sea *(Phaeocystis)* regularly forms nuisance blooms, which can be negative for at least the coastal tourism industry. Consequently, it is not only important to have information about the amount of phytoplankton, but also (or even more importantly) how its composition is.

Here, we report the first inter-comparison between three different measurement systems that use chlorophyll fluorescence for algae group detection. The instruments used were: the Algae Online Analyser (AOA; bbe Moldaenke) described by Beutler (1998), a Phyto-PAM (Walz) presented by Kolbowski and Schreiber (1995), and a Flow Cytometer (FCM; Becton Dickinson), which is described extensively by Hofstraat et al. (1991), or briefly by Hintze et al. (1999) in this issue. A chemical method, the HPLC analysis to separate group-specific photosynthetic pigments (Mantoura & Llewellyn, 1983) was also included. The standard methods of Jeffrey & Humphrey (1975, 1997) and Lorenzen (UNESCO, 1966; Lorenzen, 1967) were used for a quick

determination of chlorophyll-*a* concentrations. This work was performed at the PriPro-Workshop at the FTZ Büsum from October 5th - 8th, 1998.

There are different ways to get information about phytoplankton. An important parameter, which is most widely determined due to its simplicity, is the concentration of chlorophyll-*a* (Chl.a). Chl.a is related to biomass and the registered species. Counting cells in preserved water samples by microscopy is one of the oldest, albeit still most exact method. However, a proper identification to the species (or even the genus) level requires a well trained investigator, and the low number of possible counts make it a statistical insecure method. During the workshop, we tested the above mentioned new methods in comparison to the well known chemical methods of Chl.a detection.

2. Material and Methods

Pigment analysis

Since 1966 standard methods exist to measure chlorophyll-*a* concentrations as a proxy of algal biomass. They are all based on a chemical method, in which the water samples are filtered onto glass fibre filters, the filters are homogenised, the cells extracted, and the absorption of the extract is measured at discrete wavelengths (UNSECO, 1966). Further developments of this method have been made (Jeffrey & Humphrey, 1975, 1997; Lorenzen, 1967), and also the fluorescence emission came to use (Yentsch & Menzel, 1963; Holm-Hansen, 1965; Yentsch & Yentsch, 1979; Yentsch & Phinney, 1985). The disadvantages are based on the fact that sample processing is time consuming and the samples have to be handled quite carefully, as the chlorophylls are easily destroyed by light and/or heat. Nowadays, the HPLC-analysis used here, is considered the best method. In addition to Chl.a, it allows the determination of the most important photosynthetic pigments and their derivates (Mantoura & Llewellyn, 1983). However, the disadvantages mentioned before are the same (sample processing, no online-measurements possible).

Algae group detection by Flow Cytometry (FCM), Algae Online Analyser (AOA) and Phyto-PAM

For a better understanding of the theoretical background of the analytical methods, we first give a short introduction to algal taxonomy and the classification by pigments and spectra.

Classification of algae

The algal classification system by Hoek (1993) and Hoek et al. (1995) is given below. The authors used the differences in pigment composition, cell wall structure, shape of the thylakoids, and other distinguishing characteristics to form 9 divisions (-phyta) of algae, with their related classes (-phyceae), as shown in Table 1. The bold marked classes in Table 1 are mainly form the phytoplankton in the North Sea and will be specially treated in the next chapters.

Table 1: Classification of the algae by Hoek (1993) and Hoek et al. (1995) with the number of known genus and species world-wide.

Division:	Class:		Genus / Species
Heterkontophyta	**Bacillariophyceae**	(diatoms)	200 / 6000
	Chrysophyceae	(gold algae)	200 / 1000
	Phaeophyceae	(brown algae)	250 / 2000
	Chloromondaophyceae		6 / 10
	Xanthophyceae	(yellow-green algae)	80 / 400
Dinophyta	**Dinophyceae**	(dinoflagellates)	120 / 1000
Cyanophyta	**Cyanophyceae**	(blue-green algae)	150 / 2000
Prymnesiophyta	**Prymnesiophyceae**		45 / 250
Cryptophyta	**Cryptophyceae**		12 / 120
Rhodophyta	Rhodophyceae	(red algae)	600 / 4500
Eustigmatophyta	Eustigmatophyceae		5
Chlorophyta	**Chlorophyceae**	(green algae)	500 / 8000
	Prasinophyceae		
	Charophyceae		
Euglenophyta	Euglenophyceae		40 / 800

Differences in the pigment composition of the photosynthetic apparatus

The pigment composition of the antenna system of photosystem II is of major importance for the fluorescence detection. The pigments are subdivided into chlorophylls, carotenoids, phycobiliproteins and xanthophylls. The pigments representative for the respective algal classes (marked by ‘x’) and the pigments found in some cases (marked by ‘-‘) are shown in Table 2.

The chlorophyll-*b* and the xanthophyll lutein are important pigments for the class of the chlorophyceae and can be used as markers for this class.

Table 2: Important pigments of selected algal classes.

Class	**Pigments**							
	Chlorophylls			**Carotenoids**		**Phycobiliproteins**		**Xanthophylls**
-phyceae						Phyco-		-xanthin
	Chl.a	Chl.b	Chl.c	α	β	-cyanin	-erythrin	
Bacillario-	x		x	-	x			Fuco-, Diadino-, Diato-
Dino-	x		x		x			Peridinin
Crypto-	x		x	x	-	x	x	Allo-
Cyano-	x			x		x	x	Myxo-, Zea-, Echinon
Prymnesio-	x		x		x			Fuco-,(Diadino-, Diato-)
Chloro-	x	x		-	x			Neo-,Viola-,Zea-, Lutein

Fluorescence excitation spectra

The differences in pigment composition of the antenna complexes of photosystem II can be determined because the shapes of the excitation spectra depend on it. Figure 1 shows the spectra of three algal species (from three different taxonomic classes).

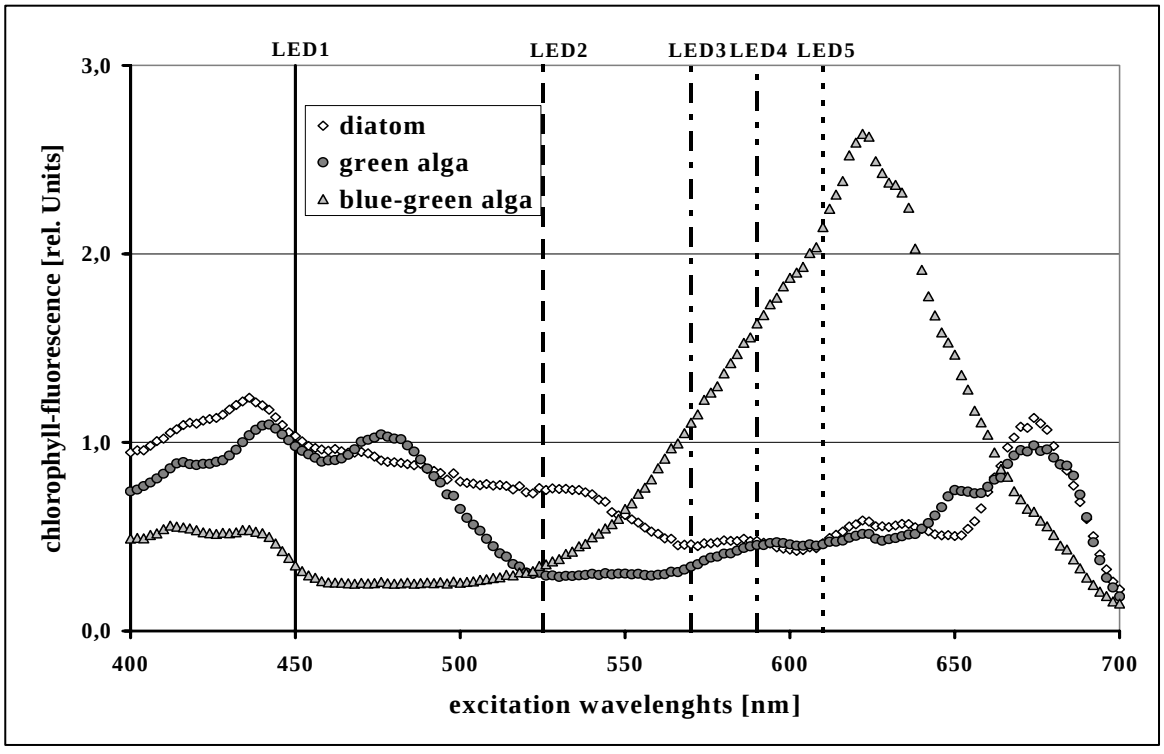

Figure 1: Fluorescence excitation spectra of the bacillariophyceae *Thalassiosira weissflogii* (diatoms), the chlorophyceae *Dunaliella salina* (green alga) and the cyanophyceae *Synechocystis sp*. (blue-green alga) (standardised to 100 µg chlorophyll-*a* / litre) at an emission wavelength of 720 nm. Also shown are the five excitation wavelengths (produced by LEDs) used by the AOA. Spectra measured with a HITACHI- Model F 4010-Fluorimeter.

For the analysis of the different spectra, the full range of excitation wavelengths is not necessary. A reduction to 3 to 5 wavelengths is sufficient for a classification (Johnson et al., 1994; Beutler 1998) and enables the construction of small chlorophyll-fluorescence-based measurement-systems for the determination of the chlorophyll-*a* concentration for outdoor-investigations (Kolbowski & Schreiber, 1995; Moldaenke et al., 1995; Vanselow et al., 1997) and algae group detection.

Preparation of the experiment

For the algae group detection experiment, an AOA, a Phyto-PAM, an FCM and the HPLC-Analyser should be compared with the results of the HPLC analysis. In addition to the determination of the chlorophyll-*a* concentration by HPLC, the faster methods of Jeffrey & Humphrey (J&H; 1975, 1997) and Lorenzen (1967) were used.
The following algal species were used in the experiment:

Diatom	(Bacillariophyceae)	*Thalassiosira weissflogii*	(salt-water)
Green alga	(Chlorophyceae)	*Dunaliella salina*	(salt-water)
Blue-green alga	(Cyanophyceae)	*Synechocystis sp.* PCC 6803	(fresh water)

The preparations first step was the determination of the algal cultures chlorophyll-*a* concentration. As abbreviation the blue-green alga *Synechocystis sp.* is indicated sometimes as 'blue alga'.

Table 3: Determination of the Chl.a concentrations by the chemical methods.

	J&H	**Lorenzen**	**HPLC**	**mean**	**deviation**	**max. error**
Species	µg Chl.a/l	µg Chl.a/l	µg Chl.a/l	µg Chl.a/l	µg Chl.a/l	%
diatom	106.69	87.67	101.84	98.74	9.88	11.27
green alga	487.85	485.42	390.15	454.47	55.72	14.28
blue alga	168.94	171.07	138.91	159.64	17.98	12.95

The maximum error is calculated by taking the deviation divided by the minimum of the samples. Due to the lack of time only one sample of each algal culture had been taken and Table 3 shows an error rate of about 15% for the mean value of the different -chemical methods. The experience shows that the most trustworthy results are measured by HPLC.

Calibration of the instruments

For the online-measurement systems the first results of the chlorophyll-*a* determination had to be performed for the calibration of the algae group detection. The results of the methods of Jeffrey & Humphrey (1975, 1997) and Lorenzen (1967) were available first. The Chl.a

contents of the green alga seemed to be to high and therefore a 1:3 dilution of the basic culture has been used for the determination.

Table 4: Chlorophyll-*a* concentrations by J&H and Lorenzen used for the AOA and the Phyto-PAM.

Mean of J&H and Lorenzen	diatom	green alga	blue-green alga
Chl.a- concentration [µg/l]	97,2	162,2	170,0

The AOA and the Phyto-PAM used the mean values of Table 4, especially for making the fingerprints of the three algae species.
For the flowcytometer analysis the HPLC-results of chlorophyll-*a* concentration of Table 3 were used for the offline-calculation of the algae group detection. The same HPLC-results were used for the mixtures which must be compared with the theoretical calculated HPLC-results for the mixtures (see later on “Results of the algae group detection”).

Table 5: Chlorophyll-*a* concentrations by HPLC used for the HPLC-results for the mixtures.

HPLC	diatom	green alga	blue-green alga
Chl.a- concentration [µg/l]	101,8	130,1	138,9

It is quite important to realise that there are two different reference points used for the instruments and therefore there is a partition towards this fact in the results (please remember this in chapter 3 (Results)).

Preparation of the mixtures
Different mixtures were prepared and then measured by the different systems.

Table 6: Mixtures used for the experiments**.**

Mixture	**diatom**		**green alga**		**blue alga**		**Chl.a content ratios**
A	1	+	3	+	0		1.0 : 3.8 : 0.0
B	4	+	1	+	0		3.1 : 1.0 : 0.0
C	1	+	1	+	1		1.0 : 1.3 : 1.3
D	1	+	1	+	4		0.8 : 1.0 : 4.0
E	1	+	1	+	1	+ humin acid	1.0 : 1.3 : 1.3

The Chl.a content ratio is calculated by using the mixture relation and the Chl.a concentration as follows:

$$\text{Chl.a contents (diatom)} = \frac{\text{Number of parts (diatom)} * \text{Chl.a concentration (diatom)}}{\text{Sum of all parts (diatom, green alga, blue alga)}}$$

$$\text{Chl.a ratio (diatom)} = \frac{\text{Chl.a contents (diatom)}}{\text{Constant}}$$

Mixture A and B (in Table 6) had been motivated by the fact that the blue alga was a fresh water species, so that mixture C and E contained a third part freshwater and mixture D was dominated by it. A change in the salinity could cause a (serious) damage of the species, what may have an effect on the results of the fluorometric measurement methods. The added humin acid in mixture E is a fluorescence emitting natural dye existing in lakes and in the seas. It should test the algorithm of the algae group detection for showing no change in the results for the algae group detection.

3. Results and Discussion

The measurements for all systems started at the same time. Only the measurements with the AOA had to be repeated five hours later due to an incorrect calibration, so that the AOA results stem from a later time.

Results of the algae group detection

In order to verify the results, the chlorophyll-*a* content ratios were calculated using the chlorophyll-*a* concentrations of the basic cultures (see Table 4 and 5) and the mixture ratios (see Table 6). Two reference values are important for comparison, the ‘Theo. Ratio HPLC’, calculated with the chlorophyll-*a* concentration measured by HPLC (see Table 4 and 6), and the theoretical ratio of J&H and Lorenzen, calculated with the value used for the calibration (see Table 5 and 6), named ‘Theo. Ratio J&H+L’ in Table 7. Both are marked bold in Table 7.

A differentiation of the algae by HPLC was possible by analysing the respective marker pigments (fucoxanthin for the diatom, chlorophyll-*b* for the green alga and zeaxanthin for the blue-green alga). The results of the AOA and the Phyto-PAM were available after a short measuring period of some few minutes (depending on the measuring mode, in- or exclusive the parameter for the photosynthetic activity). The FCM data represent cell counts multiplied by the Chl.a-normalised fluorescence intensity of each species.

Table 7: Chlorophyll-*a* content ratios between the species. For abbreviations see text above.

	Mixture	Diatom	green alga	blue alga	Mixture	diatom	green alga	blue alga
Theo. Ratio HPLC	**A**	**1.0**	**3.8**	**0.0**	**B**	**3.1**	**1.0**	**0.0**
FCM		1.0	3.8	0.0		3.1	1.0	0.0
HPLC		1.0	3.7	0.0		2.9	1.0	0.0
Theo. Ratio J&H+L		**1.0**	**5.0**	**0.0**		**2.4**	**1.0**	**0.0**
AOA		1.0	3.5	0.0		3.0	1.0	0.0
Phyto-PAM		1.0	3.8	0.1		4.4	1.0	0.0
Theo. Ratio HPLC	**C**	**1.0**	**1.3**	**1.4**	**D**	**0.8**	**1.0**	**4.3**
FCM		1.0	1.1	1.1		1.1	1.0	5.8
HPLC		1.0	1.3	0.9		1.1	1.0	3.8
Theo. Ratio J&H+L		**1.0**	**1.7**	**1.7**		**0.6**	**1.0**	**4.0**
AOA		1.0	1.5	1.8		0.1	1.0	3.5
Phyto-PAM		1.0	2.4	1.6		0.5	1.0	2.8
Theo. Ratio HPLC	**E**	**1.0**	**1.3**	**1.4**				
FCM		1.0	1.2	1.2				
HPLC		1.0	1.3	0.9				
Theo. Ratio J&H+L		**1.0**	**1.7**	**1.7**				
AOA		1.0	1.6	1.9				
Phyto-PAM		1.0	2.4	1.8				

Determination of chlorophyll-*a* content per cell by FCM

As FCM yielded direct cell counts, and the FCM-measured fluorescence intensities of the respective species could be related to Chl.a in the basic cultures, it was possible to calculate a Chl.a content per cell:

Thalassiosira weissflogii 16.366 cells/ml (± 2.8%) corresponding to **6.2 pg Chl.a / cell**
Dunaliella salina 253.364 cells/ml (± 0.9%) **- " -** **1.5 pg Chl.a / cell**
Synechocystis sp. PCC 6803 7.172.223 cells/ml (± 8.6%) **- " -** **20.0 fg Chl.a / cell**

Determination of chlorophyll-*a* concentration

The problems of comparing the measurement systems are the same as in the chapter before. Even here the comparison is only relative to two reference values: the 'Theo. Contents HPLC' and the 'Theo. Contents J&H+L' given by the use of the different Chl.a concentrations of Table 5 and 6 for the calibration.

Table 8: Chlorophyll-*a* concentrations of the mixtures. Abbreviations see text above and Table 7.

Mixture:	**A**	**B**	**C**	**D**	**E**
	Total Chlorophyll-*a* concentration [µg / l]				
Theo. Contents HPLC	**123,0**	**107,5**	**123,6**	**131,3**	**123,6**
HPLC	153,5	132,5	126,1	113,9	129,2
FCM	133,4	118,0	115,1	109,3	123,0
Theo. Contents J&H+L	**146,0**	**110,2**	**140,7**	**151,6**	**140,7**
AOA	118,3	112,4	132,8	102,9	129,9
Phyto-PAM	137,0	107,0	159,0	171,0	141,0
	Deviation to the 'Theo. Contents HPLC' result [%]				
HPLC	24,8	23,3	2,0	13,3	4,5
FCM	8,5	9,8	6,9	16,8	0,5
	Deviation to the 'Theo. Contents J&H+L' result [%]				
AOA	18,9	2,0	5,6	32,1	7,6
Phyto-PAM	6,1	2,9	13,0	12,8	0,2

The highest deviations to the theoretical values are found in column D, which was the freshwater dominated mixture. A damage of the salt-water species is probably the reason for these large deviations; on the other hand, the deviations of the fluorometric measurements are not larger than those of the HPLC in Table 8, indicating that the fluorometric methods are not necessarily inferior to the HPLC method.

Determination of the photosynthetic activity and the actual quantum yield

The AOA and the Phyto-PAM are able to measure the actual quantum yield (photosynthetic activity), together with the algae group detection measurements (optional) as shown in Table 2.

The Phyto-PAM measurements (short named PAM in Fig. 2) show (Fig. 2) similar results for the quantum yield of the algae groups of the first four mixtures (A-C and E). The AOA show comparable values for mixture A and B (only salt-water), but an evident change of photosynthetic activity for the mixtures C and E, which are partly composed of freshwater. This change is probably related to a damage of the diatoms after the five hours, when the AOA measurements have been conducted. This could also be the reason for the detected Phyto-PAM yield of 0.2 in mixture D. The result of the AOA of about 0.9 has never been observed before for phytoplankton. Regarding Table 7 with the very low Chl.a-content for the diatom we concluded that the pigment concentration was too low for reliable measurements.

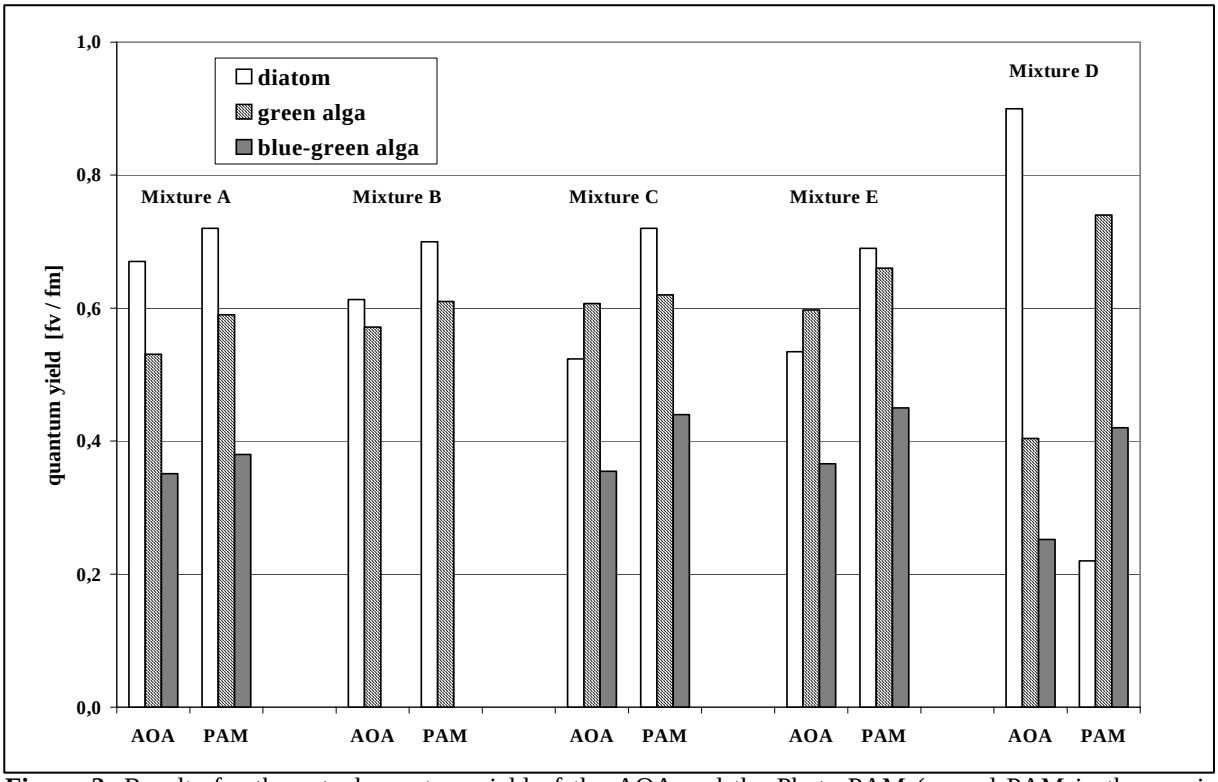

Figure 2: Results for the actual quantum yield of the AOA and the Phyto-PAM (named PAM in the x-axis legend) for the five mixtures (A to E). The mixtures contained the three algae *Thalassiosira weissflogii* (diatom), *Dunaliella salina* (green alga) and *Synechocystis sp.* PCC 6803 (blue-green alga).

Surprisingly, photosynthetic activity of the blue-green alga was detected in mixture A. The reason for this is a low number of cyanobacterial cells (blue-green algae) in this mixture, that could not be detected by the algae group detection measurement systems, except for the Phyto-PAM. Compared to the other species in these mixtures, their contribution to Chl.a was negligible, but nevertheless recognisable.

4. Conclusions

Scope of the workshop was to compare the different measuring systems, and to figure out the difficulties that emerge, when they are faced with mixtures of different algal species and taxonomic groups. The data and experience gathered during the PriPro98 workshop form an important basis for future research.

Due to a lack of time, no further investigations could be made to explore the reasons for the large deviations of the algae group detection analyses from the theoretical mixtures. However, one major reason could have been the fact that two salt-water and one fresh water species were combined within one mixture. The time delay of five hours for measurements with the

AOA relative to the measurements with the other instruments also could have caused a problem for a proper comparison.

Compared to a fluorometer with only one excitation wavelength, the AOA and the Phyto-PAM can be expected to be superior in the detection of chlorophyll-*a* concentrations. However, with only one wavelength, the calibration of the systems presents the biggest problem. The optimal detection of different taxonomic classes (i.e. suites of accessory pigments) requires different excitation wavelengths, so that in a mixture of different species (or in a natural water sample), the excitation wavelength will only be proper for the taxonomic class it is calibrated for, but suboptimal for the other groups (see Figure 1).

In conclusion, it can be stated that an algae group detection is possible, despite the problems we faced during this workshop. In general, the results of the fluorescence signal analyses are quite comparable to the chemical methods. The biggest advantage is that there is no need for 'first-to-do' preparation of the samples, it's a rather fast method, easy to run, and therefore highly suited for 'outdoor'- investigations.

5. Acknowledgements

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