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Experiments for continuous chlorophyll fluorescence measurements in a mesocosm

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1. Introduction

The workshop „Primary Production in Aquatic Systems“ at the FTZ-Westküste (Büsum) on October 5-8, 1998 gave us the opportunity to measure chlorophyll fluorescence responses over 27 hours with different fluorometers in a mesocosm, filled with natural pond water. We looked for the variation in chlorophyll-*a* concentrations, and for fluorescence parameters acting as a measure for the physiological state of the phytoplankton in the mesocosm. A comparison of chemical and fluorometric analytical methods to determine chlorophyll-*a* concentrations was one goal of the experiments; a second goal was to investigate the behaviour of the fluorescence parameters, which act as a measure for the photochemical efficiency of the phytoplankton. These parameters were influenced by environmental changes in the mesocosm.

2. Material and Methods

Mesocosm

Water from the freshwater pond in front of the FTZ building was transferred into a plastic tub with a final volume of about 190 litres (inner dimensions 0.72 x 0.53 m, depth 0.5 m). The walls of the mesocosm were transparent to allow light penetration. The mesocosm was exposed to natural conditions to create maximum variability in light and temperature changes over the day (see Fig. 1). Sedimentation of the algae was reduced by a little pump (35W), which continuously mixed the water column.

Figure 1. Mesocosm with measuring equipment on a canvas-top trailer.

Chlorophyll fluorescence parameters

Many theoretical and empirical equations have been formulated in the past to describe the primary production rate or the photosynthetic fitness of algae by chlorophyll fluorescence parameters (Genty et al., 1989; Moldaenke et al., 1995; Vanselow et al., 1997, Hartig et al., 1998).

Chlorophyll fluorescence competes with the electron transport processes in the photosynthetic apparatus. Parameters based on fluorescence measurements do not directly mirror the photosynthetic electron transport, but give a good estimation of the photochemical efficiency of Photosystem (PS) II (Table 1). The “photochemical efficiency” is defined as a quotient of Q_A reduction (turnover) and the irradiance available for the algae. The instruments do not monitor the total chlorophyll-*a* fluorescence emission, but the so-called fluorescence yield resulting from an intensity modulated LED.

Table 1: Parameters used to measure photosynthetic efficiency. The fluorescence yield is defined by the total fluorescence emission divided by the intensity of the illuminating light.

F_0	Minimum fluorescence yield, measured after 10 min of dark adaptation
F_m	Maximum fluorescence yield, measured after 10 min of dark adaptation
F	Fluorescence yield, measured with background light
F_m‘	Maximum fluorescence yield, measured with background light
$F_{1\text{-}Hz}$	This fluorescence yield signal is stimulated by an actinic rectangular irradiance in the Hz domain. It enables a distinction of active and inactive chlorophyll fluorescence by its kinetic behaviour.
$\phi_0 = (F_m - F_0) / F_m$	Potential photochemical efficiency of PS II of a dark adapted sample.
$\phi = (F_m‘ - F) / F_m‘$	Photochemical efficiency of PS II of a light adapted sample.
$\eta = F_{1\text{-}Hz} / F$	Quotient of 1-Hz signal ($F_{1\text{-}Hz}$) and whole fluorescence signal (F).

Fluorometers

We used three online fluorometers (bbe Moldaenke, Kiel) and two cuvette-analysers (bbe Moldaenke, Kiel; Walz, Effeltrich), as listed in Table 2. The online fluorometers were placed directly next to the mesocosm on a canvas-top trailer. They were equipped with a peristaltic pump, which transported the water sample from the centre of the mesocosm to the measuring chamber every 30 minutes. The measuring protocols of the different fluorometers are given in Table 1. Irradiance was measured by a 4 π sensor from Zemoko (The Netherlands).

Table 2: Specific parameters of the used fluorometers in these experiments.

Fluorometer	Company	Owner	Efficiency
Xe-PAM	Walz	Walz	$\phi_0 = (F_m - F_0) / F_m$
Algae Online Analyser	bbe	FTZ	$\phi_0 = (F_m - F_0) / F_m$
Online fluorometer	bbe	IGB	$\phi = (F_m‘ - F) / F_m‘$
1-Hz online fluorometer	bbe	BBE	$\eta = F_{1\text{-}Hz} / F$
1-Hz cuvette fluorometer	bbe	FTZ	$\phi_0 = (F_m - F_0) / F_m$ and $\eta = F_{1\text{-}Hz} / F$

1-Hz online fluorometer: This fluorometer detects the chlorophyll-*a* concentration by measuring the direct chlorophyll fluorescence. The photochemical efficiency is detected by the 1-Hz method as $F_{1\text{-}Hz}$ signal, described in detail by Moldaenke et al. (1995) and Vanselow et al. (1997).

Online fluorometer: This fluorometer detects the chlorophyll-*a* concentration by measuring the chlorophyll fluorescence from LED-stimulation at wavelengths of 585 nm and 450 nm. It has a low irradiance intensity for F_0 detection. The parameters to create ϕ were measured at an irradiance of 50 $\mu Em^{-2}s^{-1}$, after an adaptation period of 30 seconds.

Algae Online Analyser (AOA): This fluorometer detects the chlorophyll-*a* content by measuring the chlorophyll fluorescence from LED-stimulation at five different wavelengths (Beutler 1998; Johnson et al., 1994). Light intensity was 10 $\mu Em^{-2}s^{-1}$. In addition, the contribution of different algal classes was analysed by an adequate selection of excitation and detector wavelengths, in correlation with the algal excitation fluorescence spectra (see also Ruser et al., 1999, this issue). The fluorescence parameters F_0, F, and F_m can be measured to calculate the photochemical efficiency ϕ_0.

1-Hz cuvette fluorometer: This fluorometer detects the chlorophyll-*a* concentration by measuring the chlorophyll fluorescence (F_0) from LED-stimulation at wavelengths of 585 nm and 450 nm. The irradiance level for F_0 detection was below 1 $\mu Em^{-2}s^{-1}$, and thus below the sensitivity range of the Zemoko light sensor. The fluorescence parameters F_0, F, and F_m were measured to calculate the photochemical efficiency ϕ_0. In addition, the $F_{1\text{-}Hz}$ signal was used to give a value for η (see Table 1; Moldaenke et al., 1995; Vanselow et al., 1997). All these parameters were measured once for each sample. The fluorometer was placed next to the other online fluorometers near the mesocosm on a canvas-top trailer (see Fig. 1). Samples were taken by a bottle from the centre of the mesocosm. Adaptation times were as in Table 1.

Xe-PAM: Bottle samples were dark adapted for 30 min in the water column of the mesocosm to minimise temperature effects. For the experiments, three samples of 2.5 ml were used, each of which was measured 5-fold for F_0, and once for F_m. The fluorescence signals were zero-point compensated by the fluorescence signals of 7.5 ml filtered (0.2 µm) water samples (blanks) according to $(F_m - F_0) / (F_m - \text{Offset})$. For the experiments, the fluorometer was placed in the working hall of the FTZ (25 meters away from the mesocosm).

Flow Cytometer (FCM):

The FCM allows the quantification and characterisation of phytoplankton by its optical properties. The cells are aligned in a very thin liquid thread, so that they pass the excitation laser beams one by one. The resulting fluorescence and scatter light signals are focused, split up into different wavelengths by colour filters and mirrors, and detected by photomultipliers.

The resulting electronic signals are then processed in a computer and can be analysed by special software (for more information see Hofstraat et al., 1991).

The FCM of the FTZ (Becton Dickinson FacsVantage, USA) is equipped with 2 excitation wavelengths (488 and 633 nm), 6 photomultipliers for fluorescence and perpendicular light scatter detection, and a photodiode for forward light scatter detection (FSC). The excitation and fluorescence parameters used in the experiments are listed in Table 3. The instrument is able to differentiate algal groups by their characteristic fluorescence and scatter signals. This is in accordance to the theory used for the AOA. The FCM is also equipped with a sorting module, allowing the physical separation of individual cells from the community.

Table 3: Fluorescence and Scatter signals detected by the Flow Cytometer and the respective algal groups that can be discriminated. Ex: Excitation wavelength, Em: Wavelengths reaching the detectors (Emission wavelengths), FSC: forward scatter.

Signal	Ex	Em	Pigment	Characterisation
FL3	488 nm	675 ± 20	Chlorophyll-*a*	All phytoplankton groups
FL2	488nm	575 ± 20	Phycoerythrin	Characteristic for some Cryptophyceae and Cyanophyceae
FL4	633 nm	660 ± 20	Phycocyanin	Characteristic for some Cryptophyceae and Cyanophyceae
FSC	488 nm	490 ± 10	-	Indicator for cell size

Physical parameters:

Global irradiance and air temperature was measured automatically every 6 minutes. The water temperature in the mesocosm was measured manually at irregular intervals.

Chemical analysis of chlorophyll:

Parallel to the experiments, bottle samples from the centre of the mesocosm were taken for the following analyses:

- chlorophyll-*a* detection by the method of Jeffrey & Humphrey (1975, 1997; J&H),
- chlorophyll-*a* detection by the method of Lorenzen (1967),
- pigment analysis by the High Performance Liquid Chromatography (HPLC) method.

3. Results and Discussion

The different methods and fluorometers will be compared and evaluated in the following categories:

- determination of chlorophyll-*a* concentrations,
- differentiation of algal groups by pigment analysis,
- determination of the photochemical efficiency.

Determination of Chlorophyll-*a* concentrations

The different methods for chlorophyll-*a* determination are compared in this chapter. Especially the different spectral actinic light sources and light intensities implemented in the fluorometers could lead to different results.

The chlorophyll-*a* concentrations determined by the methods of J&H, Lorenzen and HPLC are shown in Fig. 2A. The results from the chemical methods show the same trends like the fluorometric results, as shown in Fig. 2B. The chlorophyll concentrations decreased until 18:00 o'clock in all cases, thereafter, the concentrations nearly stayed constant. The mean values of the five parallels taken at different times were taken from the chemical and from the fluorometrical methods and then divided for a better comparison (Table 4). The ratios vary strongly. Chlorophyll-*a* fluorescence competes directly with the photochemical processes of the electron transport system. Therefore, environmental changes could influence the fluorescence yield per chlorophyll-*a*. This should result in a decrease of the fluorescence yield per chlorophyll-*a* with decreasing photochemical efficiency (compare Table 4 and fig. 4B, C). This was found for the relation between chlorophyll-*a* concentration determined by fluorescence and J&H respectively Lorenzen, but not for the chlorophyll-*a* concentration determined by the HPLC method. An alternative explanation is given in the following chapter about algal group detection.

A problem was the time delay between the sampling for the fluorometric measurements and for the chemical analysis. To compare the methods, it is assumed, that the fluorescence values change linearly with time between two data points. Thus, fluorescence values have been calculated from two data points surrounding the data point resulting from the chemical analysis. This could result in a large calculation error at the beginning of the experiment, when the chlorophyll-*a* values decrease drastically.

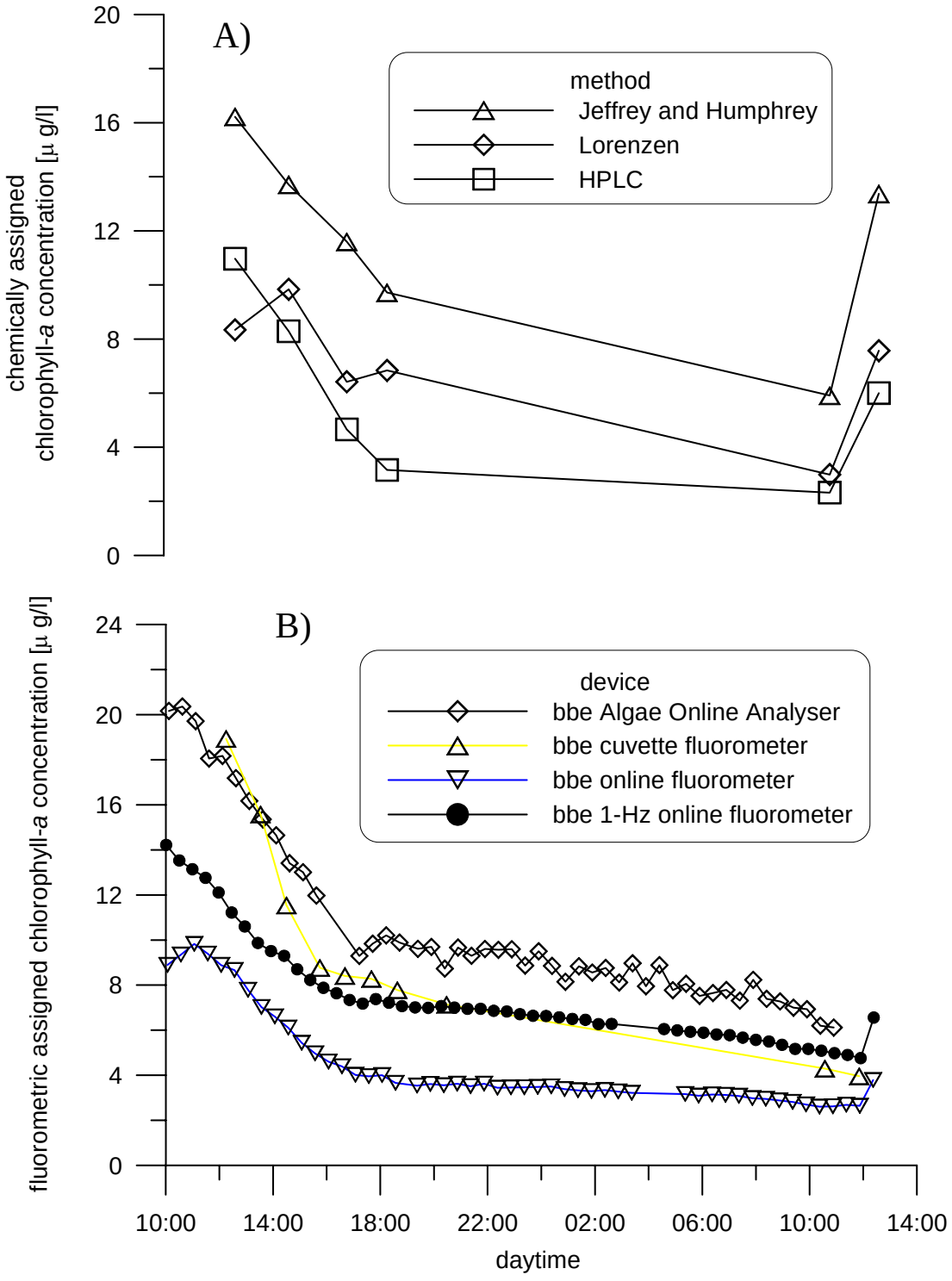

Figure 2: Measured chlorophyll-*a* concentrations in the mesocosm. A: Results by the methods of J&H, Lorenzen and HPLC. B: Results of the available fluorometers.

Table 4: Temporal development of the quotient of fluorometric and chemical determined chlorophyll-*a* concentrations. Standard-deviation is represented by σ. Quotients of HPLC, J&H, and Lorenzen give the values in parenthesis in the sequence mean value, σ, mean value/σ to show the daily variance: HPLC/J&H (0.48, 0.14, 0.29); HPLC/Lorenzen (0.82, 0.28, 0.34), and J&H/Lorenzen (1.72, 0.25, 0.15).

	1-Hz cuvette fluorometer (FTZ)			**Online fluorometer (IGB Berlin)**		
Time	**HPLC**	**J&H**	**Lorenzen**	**HPLC**	**J&H**	**Lorenzen**
12:35	1.73	1.17	2.27	0.80	0.54	1.05
14:35	1.48	0.89	1.24	0.76	0.46	0.64
16:45	1.86	0.75	1.35	0.95	0.38	0.69
18:15	2.53	0.82	1.17	1.26	0.41	0.58
10:45	1.85	0.73	1.44	1.13	0.44	0.87
Mean value	1.89	0.87	1.49	0.98	0.45	0.77
σ	0.39	0.18	0.45	0.21	0.06	0.19
σ/ mean value	0.21	0.20	0.30	0.22	0.14	0.25

	1-Hz online fluorometer			**Algae Online Analyser**		
Time	**HPLC**	**J&H**	**Lorenzen**	**HPLC**	**J&H**	**Lorenzen**
12:35	1.04	0.71	1.37	1.57	1.06	2.06
14:35	1.13	0.68	0.95	1.62	0.98	1.36
16:45	1.62	0.65	1.18	2.17	0.87	1.57
18:15	2.32	0.75	1.07	3.20	1.04	1.48
10:45	2.19	0.86	1.70	2.63	1.03	2.04
Mean value	1.66	0.73	1.25	2.24	1.00	1.70
σ	0.59	0.08	0.29	0.69	0.08	0.33
σ/ mean value	0.35	0.11	0.23	0.31	0.08	0.19

Differentiation of algal groups by pigment analysis

During the experiments, we also took samples for the algal group detection by the AOA and the FCM. Both instruments use spectral light signals for the detection of the different phytoplankton groups. The data are compared with the results of the HPLC analysis.

Algae Online Analyser (AOA)

The AOA allows the differentiation of algal groups by analysing their fluorescence excitation spectra, and is also able to determine their respective chlorophyll-*a* concentrations. An overview over the different groups is given in Table 5.

Table 5: Overview of the different algae groups by their spectral behaviour.

green spectral group	Chlorophyta
blue spectral group	Cyanophyta
brown spectral group	Heterokontophyta, Haptophyta, Dinophyta
red spectral group	Rhodophyta
mixed spectral group	Cryptophyta

The AOA was able to divide the algae in the mesocosm into the green, brown, blue, and the mixed spectral group, as demonstrated in Fig. 3. The figure shows, that the decrease in chlorophyll-*a* is mainly caused by the cryptophyceaen group. The green algae remained relatively constant in contrast to the cryptophyceae, which decreased dramatically shortly after the beginning of the experiment (Fig. 3). The measured concentrations from the blue and brown spectral groups can be neglected. More results from AOA experiments are given by Ruser et al. (1999, this issue).

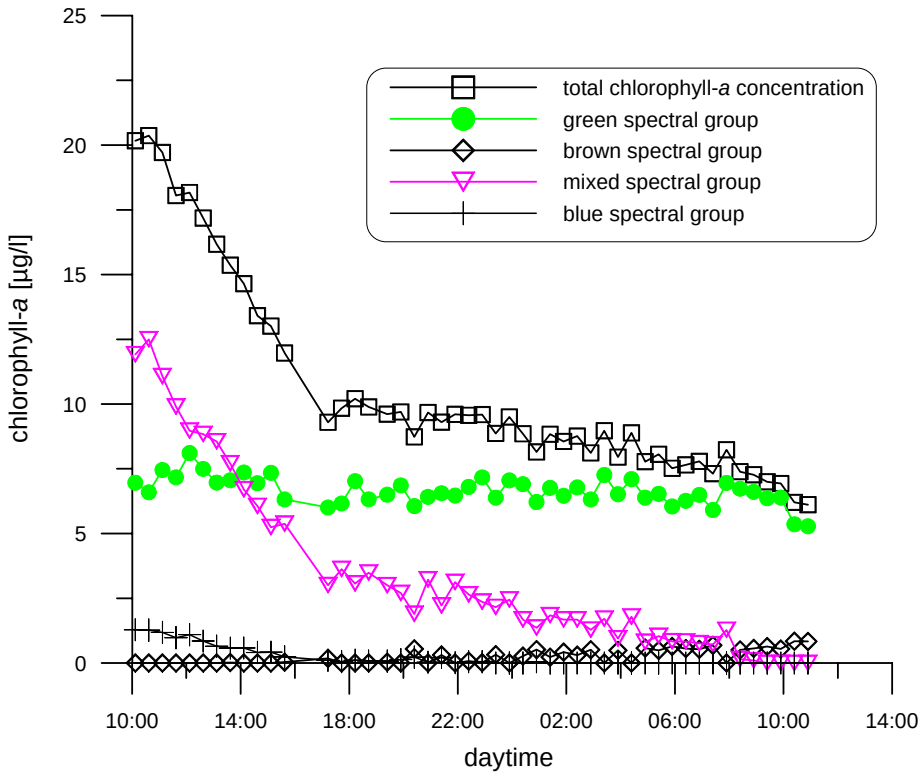

Figure 3: AOA results of the chlorophyll-*a* distribution of the different spectral algal groups in the mesocosm over the day.

HPLC analysis

Alloxanthin, the pigment marker for the cryptophyceae, decreased within the first hours of the experiment, as indicated in Table 6. At the beginning of the experiment, the phytoplankton mostly consisted of cryptophyceae, but at the end, the green spectral group dominated. The main marker for this group is chlorophyll-*b*, but it was not found in the samples by HPLC. Algae of the brown spectral group (Table 5) often contain fucoxanthin, but this pigment could not be detected in appreciable concentrations. So we do not believe, that the green group and possibly some algae of the brown group are present at the end of the experiment in the AOA results. The constant ratio of chlorophyll-*a* to alloxanthin (4:1) indicates that a high percentage of the phytoplankton in the mesocosm consisted of cryptophyceae. In the cryptophyceaen group, the pigment phycoerythrin plays an important role for the spectral curve shape due to its typical orange fluorescence; however, not all cryptophyceae necessarily contain it. The AOA was calibrated with a phycoerythrin-containing species (*Pyrenomonas sp.*). The differences in the results of the analyser and the HPLC data can possibly be explained in the very different cryptophyceae fluorescence excitation spectra (with or without phycoerythrin).

Table 6: Pigment data measured by HPLC. Pigment values below the traceable limit of HPLC are marked by a "-". The upper line gives date and time, separated by a semicolon.

Pigments	**Unit**	**6.10; 12:35**	**6.10; 14:35**	**6.10; 16:45**	**6.10; 18:15**	**7.10; 10:45**	**7.10; 12:35**
Peridinin	**µg/l**	-	-	-	-	-	-
Fucoxanthin	**µg/l**	-	-	-	-	-	0.23
19-Hex-fuco	**µg/l**	-	-	-	-	-	-
Diadinoxanthin	**µg/l**	-	-	-	-	-	0.18
Alloxanthin	**µg/l**	2.52	2.32	1.24	0.90	0.53	1.35
Lutein	**µg/l**	-	-	-	-	0.16	0.38
Chlorophyll-*a*	**µg/l**	10.97	8.29	4.66	3.16	2.32	6.00
Chlorophyll-*b*	**µg/l**	-	1.32	-	-	-	1.09
Chlorophyll-*c*	**µg/l**	0.84	0.59	0.35	0.22	-	0.23
Carotene-*a*	**µg/l**	0.25	0.23	0.10	-	0.02	0.13
Carotene-*b*	**µg/l**	0.12	0.13	0.05	-	-	0.16
Chlorophyll-*a* / Alloxanthin		4.34	3.56	3.77	3.53	4.37	4.43

Flow Cytometry (FCM)

Two samples were taken for FCM from the centre of the mesocosm, at 10:30 and 12:45 (Oct. 7th). The second sample was taken after agitation to evaluate the possibility of algal sinking at

the bottom. The FCM gave ratios of three different fluorescence signals and the forward scatter signal, making it possible to distinguish between 8 phytoplankton groups (Table 7).

Table 7: Results of the FCM measurements. 8 different algae groups could be distinguished. The signals FL2, FL3, FL4 and FSC are explained in Table 3. The columns are sorted by the values of the FSC values, which are proportional to cell size. The mesocosm was mixed strongly at the end of the experiment. Quotient = Cell number not mixed / Cell number mixed.

Group	**1**		**2**		**3**		**4**	
possible classification	**Cryptophyceae, Cyanophyceae**		**Cryptophyceae, Cyanophyceae**		**Cryptophyceae, Cyanophyceae**		**Not identified**	
Sample	**not mixed**	**mixed**	**not mixed**	**mixed**	**not mixed**	**mixed**	**not mixed**	**mixed**
Cell number /ml	1203	1146	20508	21616	119	136	1890	2056
Quotient	0.95		1.05		1.15		1.09	
FSC/Cell	48.92	48.70	81.31	79.15	311.99	310.59	596.20	604.30
FL2/Cell	2.28	2.27	2.37	2.39	3.38	3.52	4.07	4.10
FL4/Cell	5.05	5.42	1.04	1.04	43.13	38.89	1.44	1.46
FL3/Cell	0.02	0.02	0.09	0.10	0.22	0.21	0.60	0.62
FL2/FL3	114	103	25	25	15	17	7	7
FL4/FL3	253	245	11	11	197	184	2	2
FL3*cell number	24	25	1915	2073	26	29	1136	1280
Group	**5**		**6**		**7**		**8**	
possible classification	**Not identified**		**Cryptophyceae**		**Cryptophyceae**		**Cryptophyceae**	
Sample	**not mixed**	**mixed**	**not mixed**	**mixed**	**not mixed**	**mixed**	**not mixed**	**mixed**
Cell number /ml	1639	2042	241	400	9	75	102	2090
Quotient	1.25		1.66		8.26		20.40	
FSC/Cell	749.89	777.37	2007.86	2653.56	2425.30	4450.79	2763.16	3751.62
FL2/Cell	4.74	5.33	82.42	105.07	121.88	181.06	791.48	1485.51
FL4/Cell	1.64	1.76	9.26	9.69	1.11	1.89	103.66	81.31
FL3/Cell	0.61	0.63	7.55	8.39	0.52	0.63	35.61	43.50
FL2/FL3	8	8	11	13	235	285	22	34
FL4/FL3	3	3	1	1	2	3	3	2
FL3*cell number	992	1285	1818	3355	53	1326	322	3247

Cells with a low FSC signal (= small cells) have a lower sinking rate, while cells with a high FSC signal (= large cells) seem to sediment out of the water column within the experimental time. The decrease in the chlorophyll-*a* concentration might be explained by this effect. The contribution of a specific algal group to total chlorophyll-*a* can be estimated by the product of the mean FL3 fluorescence and the number of cells of that group (lowermost line in Table 7).

Groups 6, 7, and 8 showed only low cell concentrations, but a large FL3 signal. It can be assumed that the sinking of the groups 6, 7 and 8 with time caused the decrease in the chlorophyll-*a* concentration. The high FL2/FL3 ratio, the low FL4/FL3 ratio, and the large cell size (strong FSC signal) characterise these groups as phycoerythrin-containing cryptophyceae. The decrease of this group with time correlates well with the decrease of alloxanthin measured by HPLC and the results of the AOA. The groups 1, 2, and 3 can be assumed to be small members of the cryptophyceae or cyanophyceae.

The total FL3 signal, which reflects the total chlorophyll-*a* fluorescence emission, amounts to 6286 for the unmixed sample and increases to 12620 for the mixed sample. The quotients between the total FL3 signal and the chlorophyll-*a* concentration determined by HPLC show similar values for the unmixed sample (2709, at 10:45 o'clock), and for the mixed sample (2100, at 12:35 o'clock) .

Determination of the photochemical efficiency

The variability of the photochemical efficiencies measured by the different fluorometers will be described in this chapter. Only the PAM- and the 1-Hz fluorometer were used in these measurements, as FCM is not able to detect these parameters, and the AOA needs higher algae concentrations than provided in this experiment. The PAM- and the 1-Hz fluorometer give values for ϕ or ϕ_0 by detecting F_m, F_0, and F, or by measuring the $F_{1\text{-Hz}}$ signal additionally to create η (see Table 1). In Figs. 4B and 4C the photochemical efficiencies vs. time are presented. All curves showed a depression, with an extreme at 17:00 o'clock. The high starting values of the photochemical efficiency at the beginning of the experiment were not attained in the course within the experiment again (Figs. 4B, 4C). A similar effect had been found at the workshop one year before in Zingst. It is well known that high irradiance levels lead to a decrease in the photochemical efficiency of the algae. A rise in ambient temperature stimulates this effect. So the depression at 17:00 o'clock may have its origin in the high light intensity (Fig. 4A). This could be due to photoinhibition.

A control sample directly from the pond was taken on Oct. 7th at 13:30, and measured by the Xe-PAM. It showed a photochemical efficiency of 0.67. The fact that the algae did not recover overnight can only be explained by irreversible stress factors of the containment in the mesocosm. These factors could be a higher temperature and/or irradiance in the mesocosm, or mechanical stress from the pump.

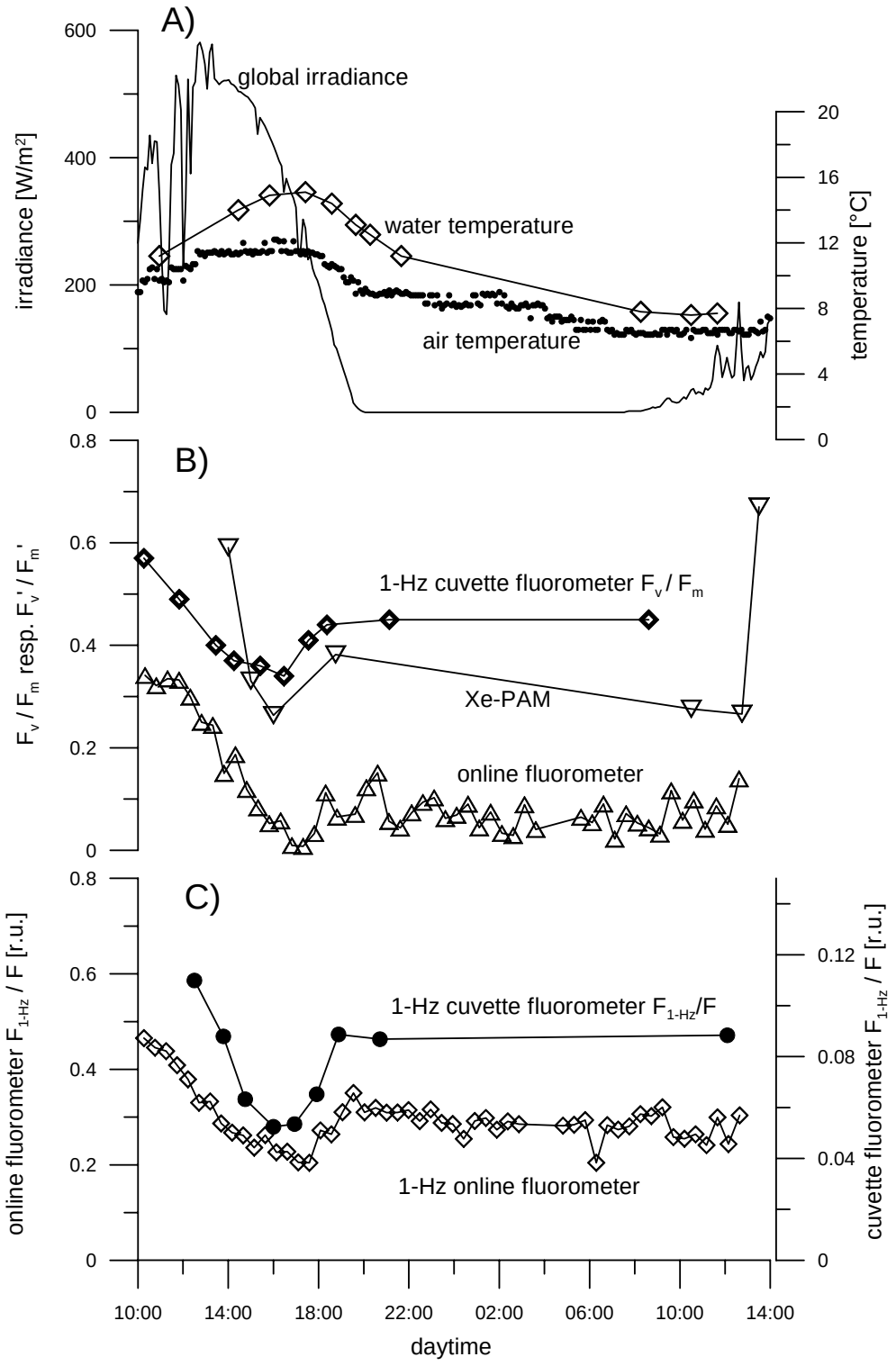

Figure 4: Daily behaviour of the measured parameters in the mesocosm. **A:** Physical parameters. **B:** Photochemical efficiency measured by F_0 and F_m. The last sample in the Xe-PAM curve was not taken from mesocosm but from the pond. **C:** Photosynthetic activity analysed by $F_{1\text{-}Hz}$ and F.

The correlation between photochemical efficiency and primary production is complex. A direct determination of primary production via photochemical efficiency is not possible without knowledge about the actual irradiance at the sample location in the mesocosm. The problem of measuring irradiance is discussed by Meyercordt et al. (1999a,b; this issue).
A comparison of the photochemical efficiency, calculated either on the basis of the $F_{1\text{-}Hz}$ signal, or of the F, F_0 and F_m signals detected with the online fluorometers, show a better signal to noise ratio in case of the $F_{1\text{-}Hz}$ signal. This could be due to the double correlation technique.

4. Conclusions

The present study demonstrates, that fluorescence measurements can be successfully used for continuous environmental monitoring. The technique may be useful to determine the development of chlorophyll-*a* concentrations and photochemical efficiency with time in a dynamic system.
The development of chlorophyll-*a* concentrations with time, as determined by fluorescence, is very similar to the chemical measurements. In contrast, the amount of fluorescence per chemical determined chlorophyll-*a* concentrations varies considerably. The differentiation of algal groups on the basis of their excitation fluorescence spectra is a promising new method.
Today, no standard method is available for the determination of primary production from fluorescence kinetic data. The measured photochemical efficiency shows a significant diurnal behaviour, independent of the method. Until now, a standardised technique doesn't exist to determine primary production only by chlorophyll fluorescence measurements. Further investigations are necessary.

5. Acknowledgements

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