Phytoplankton Analyzer
PHYTO-PAM-II
and
PhytoWin_3
Software V 3

Principles of Operation

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PhytoPamII_3.doc

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1   Safety instructions

1.1   General safety instructions

1.   Read the safety instructions and the operating instructions first.

2.   Pay attention to all the safety warnings.

3.   Keep the device away from water or high moisture areas.

4.   Keep the device away from dust, sand and dirt.

5.   Always ensure there is enough ventilation.

6.   Do not put the device anywhere near sources of heat.

7.   Connect the device only to the power source indicated in the operating instructions or on the device.

8.   Clean the device only according to the manufacturer's recommendations.

9.   If the device is not in use, remove the mains plug from the socket.

10.   Ensure that no liquids or other foreign bodies can find their way inside the device.

11.   The device should only be repaired by qualified personnel.

1.2   Special safety instructions

1.   PHYTO-PAM-II Multiple Excitation Wavelength Phytoplankton and Photosynthesis Analyzer is a highly sensitive research instrument which should be used only for research purposes, as specified in this manual. Please follow the instructions of this manual to avoid potential harm to the user and damage to the instrument.

2.   PHYTO-PAM-II employs high intensity LED-array light sources which may cause damage to the eye. Avoid looking directly into these light sources during continuous illumination or saturation pulses.

3.   Do not cover the ventilation grille rear side of the instrument.

2   PHYTO-PAM-II

PHYTO-PAM-II represents the progress of established WALZ fluorometers dedicated to aquatic research like **XE-PAM, WATER-PAM**, previous **PHYTO-PAM** and **MULTI-COLOR-PAM**. Choice of several measuring light wavelengths at variable measuring light settings (intensity and frequency) offered by XE- and 4-wavelengths PHYTO-PAM are combined with the high time resolution of MULTI-COLOR-PAM. Thus, enabling classical PAM analysis, deconvolution of phytoplankton and analysis of fast induction kinetics.

The PHYTO-PAM-II incorporates a multi-color Chip-On-Board (COB) LED Array featuring 5 measuring light colors and 6 colors for actinic light illumination. The 440 nm, 480 nm, 540 nm, 590 nm, and 625 nm measuring light facilitate online differentiation of 4 different pigmented groups therefore deconvolution of green algae, cyanobacteria, diatoms/ dinoflagellates and phycoerythrin containing organisms like cryptophytes. As in the first generation PHYTO-PAM deconvolution bases on fluorescence excitation reference spectra. But for the first time these spectra are not instrument specific but universal. Due to spectral calibration of PHYTO-PAM-II instruments reference spectra can be shared between users and instruments.

The PHYTO-PAM-II software PhytoWin_3 features classical PAM analysis like the estimation of the effective photochemical quantum yield of PS II and the determination of photochemical and non-photochemical quenching parameters. For extended applications a special fast kinetics mode of operation is added for measuring the wavelength-dependent O-I_1 fluorescence rise kinetics upon onset of pulses of strong actinic light information. Thus, enabling evaluation of the functional absorption cross-section of PS II (σPSII), determined by light color and the pigment composition of photosynthetic organisms (Klughammer and Schreiber, 2015)

Two versions of the PHYTO-PAM-II Phytoplankton Analyzer are available. A PHYTO-PAM-II Compact version with integrated emitter-detector unit and a PHYTO-PAM-II Modular version with separate emitter, detector and control unit in a modular design.

Both versions of the PHYTO-PAM-II are operated by PhytoWin_3 software.

2.1   Compact Version

The PHYTO-PAM-II Compact Version is a lightweight and easily portable instrument. The splash-proof cast aluminum housing incorporates the control unit as well as all essentials of the emitter-detector unit: the optical unit, the measuring and actinic LED-array, the photomultiplier-detector and the cuvette retainer. For automated sampling these instruments can be equipped with a flow through cuvette (

Fig. 1: PHYTO-PAM Compact Version

Front:

- **Power** switch and green power indicator lamp

- **USB** socket to connect external PC for instrument control

- **Charge** socket, to connect Battery Charger MINI-PAM/L

- **Fuse** plug, containing 3.15 AT main fuse of internal power circuit

- **Light Sensor** socket, to connect the **Spherical Micro Quantum Sensor** (US-SQS/WB) for PAR list calibration.

Top:

- **Measuring Head** with optical port for inserting sample cuvette

- **PVC centering ring** with o-ring sealing against the inner wall of the Measuring Head, serving as a guide for the cuvette and as an adapter for mounting the optional Miniature Stirring Motor **Water-S** and the optional Spherical Micro Quantum Sensor **US-SQS/WB.**

- **Cup-shaped perspex inset** sealing against an inner o-ring of the Measuring Head, thus protecting the opto-electronical components from spilled water samples.

- **A green LED** shows the status of the internal **Photomultiplier Detector**. The photomultiplier automatically is switched off at excessive light impact.

- **Quartz Cuvette** with 15 mm outer and 13 mm inner diameter; height 46 mm.

- **Darkening Hood** covering the part of the Measuring Head protruding from the housing.

- **Photomultiplier Detector** The Photomultiplier supply voltage is automatically switched off when it sees too much light (red status LED on housing lights up).

- **COB LED-Array** The light emitting diodes (LED) are densely arranged on a 10 x 10 mm area. PHYTO-PAM-II provides 5 differently colored measuring lights (440 nm, 480 nm, 540 nm, 590 nm, 625 nm), and 5 actinic light sources (440 nm, 480 nm, 540 nm, 590 nm, 625 nm); the latter are complemented by white (440-640 nm) and far red (735 nm) LEDs (for measuring light and

actinic light spectra see Fig. 2). To compensate for varying brightness of the different types of light emission diodes, more LEDs are used when the intensity of the individual LED is lower.
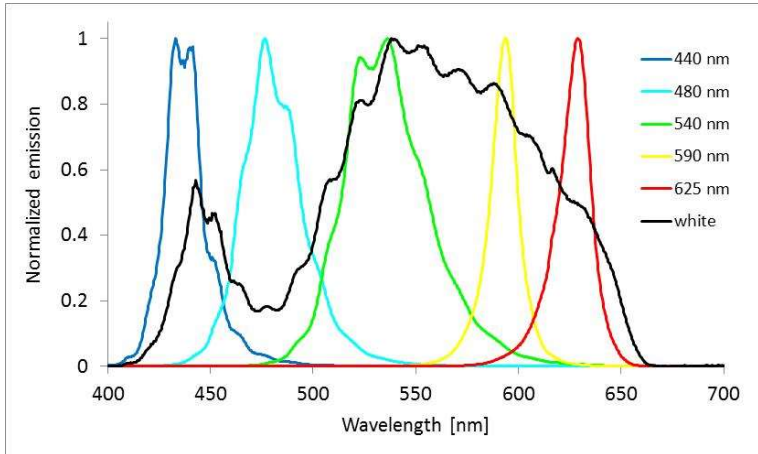
Fig. 2: Typical normalized emission spectra of PHYTO-PAM-II light sources. Spectra are not corrected for spectral response of the spectrometer.

2.1.1 Spherical Micro Quantum Sensor US-SQS/WB

Fig. 3: Spherical Micro Quantum Sensor US-SQS/WB

The Spherical Micro Quantum Sensor US-SQS/WB is included in the system package as **calibration of the PAR-List is essential for correct assessment of all PHYTO-PAM-II functions**.

The US-SQS/WB device consists of a submersible spherical quantum sensor, US-SQS/L, and a small amplifier unit which is connected to the light sensor socket. The amplifier is tuned to yield correct PAR readings within the automated PAR-List calibration (see chapter 3.1) and **needs to be set to 1x**.

The 3.7 mm diffusing sphere of the US-SQS/WB sensor picks up photosynthetically active radiation (PAR) with an angular response error of less than ± 5% from -100° to 100° angle. Hence, the sensor is ideally suited to measure light conditions in the suspension cuvette where reflections and scattering results in randomization of light direction. For stability of calibration, it is important to keep the diffuser clean. If needed the diffuser can be gently cleaned by using a cotton tip applicator moistened with some ethanol.

The position of the light sensor is adjustable and will be determined for PHYTO-PAM-II Compact version applications by a 15.3 mm spacer above the sensor hood.

2.1.2 Battery Charger MINI-PAM/L

The Battery Charger MINI-PAM/L is provided for recharging the internal lead-acid battery (12V/2Ah) of PHYTO-PAM-II. It is connected to the Charge-socket on the front panel of the Power-and-Control-Unit. The charger, which operates at input voltages between 100 and 240V AC, features overload protection. Battery voltage is displayed on the Settings window of PhytoWin_3 Software.

2.1.3 Flow-Through Cuvette PHYTO-II/FT (Add on)

The compact unit can be equipped with the special cuvette PHYTO-II/FT for flow through applications.

The flow through cuvette can be mounted to the PHYTO-PAM-II Compact version instead of the standard cuvette holder. Tubes can be mounted at the input and output to flush the cuvette between measurements. For software controlled automated sampling the use of Universal Pump Control PHYTO-II/FT/I for 0-10 V control of peristaltic pumps is recommended.

2.1.3.1 Mounting of the Flow-Through Cuvette

1. Please remove the plastic screws of the PHYTO-PAM-II ED cuvette:

2. Remove PHYTO-PAM-II cuvette holder - the Perspex cuvette retainer (red arrow) remains within the control unit

3. Please mount the metal screws of the Flow-Through-Cuvette to the screw holes of the plastic screws

Note:
To guarantee water tightness and bubble free operation the cuvette needs to be filled before inserting the flow through cuvette in the PHYTO-PAM-II! Max 0.5 bar!

4. Insert the glass cuvette all the way to the Flow Through adapter (in the top is an O-ring seal)

5. Fill the cuvette in upright position to obtain air bubble free filling (for better visibility here ink dyed solution) – check for bubbles and leaks

6. Insert the filled flow through cuvette to the PHYTO-PAM-II Control unit. The cuvette outlet needs to point to the left (red arrow)! Secure the cuvette by two knurled screws.

2.1.3.2   Light sensor adapter for light list calibration

For determinations of the instrument light characteristics within the flow-through cuvette a light sensor adapter for SQS-L/WB is provided within the delivery package. For light list calibration please mount

Please fill the cuvette with distilled water and insert it all the way to
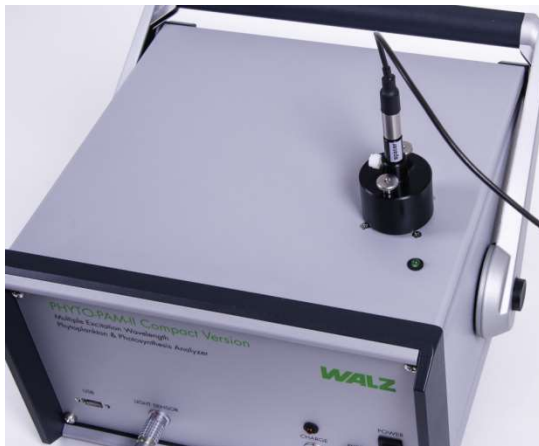
the light sensor adapter (in the top is an O-ring seal) — mount the light sensor with the spacer and secure it by the plastic screw. Insert the light sensor with the adapter to the control unit, secure it with the two knurled screws and start the light list calibration routine as described in chapter 3.1.

2.1.3.3   0-10 V Universal Pump Control

Together with the 0-10 V Universal Pump Control PHYTO-II/FT/I common laboratory peristaltic pumps can be used for software controlled automated sampling.

If you obtain the interface box in conjunction with a peristaltic pump from Walz all connectors will be pre-mounted, and the interface box will be ready for pump control.

If you obtain the interface box without a pump, one connection features an USB plug, the other a four-pin cable to fix pump specific 0-10V plug.

brown cable	→	0-10 V in/out
white cable	→	ground
green and yellow cable	→	without use

Please setup the pump according to the pump description. We recommend positioning the pump behind the flow through cuvette.

2.1.3.4   Flow-Through Cuvette specific software features
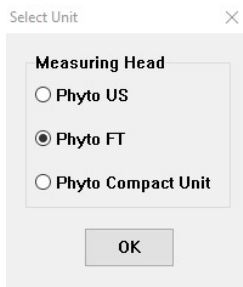
For pump control the flow-through cuvette needs to be initiated as Phyto FT unit. This configuration gives access to pump specific control commands. As the flow-through cuvette is an add-on to the PHYTO-PAM-II Compact version the corresponding data folder for this configuration is the Data_Cu6 folder.

So light lists, references and data (report and export) are stored in the Data_Cu6 folder.

During experiments with the flow-through cuvette, the flow must be stopped for the duration of the measurement. All other functions are analog to the standard cuvette.

If a pump is connected via universal pump control box, the menu item pump speed is available on the settings page. 0 corresponds to 0 V, 500 corresponds to 10 V analog signal.
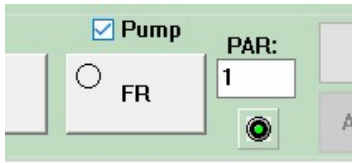
Always in direct access a Pump on/off check box is included above the FR button. When the checkbox is activated the voltage-signal is given and the pump switched-on.

All pump commands are also available as script file commands.

2.1.4 Stirring Device WATER-S (optional)

Fig. 4: Stirring Device WATER-S

WATER-S stirring device can be particularly useful for measurements of **rapidly settling samples.**

A special adapter ring is provided for mounting the optional Stirring Device WATER-S to the PHYTO-PAM-II Compact version in standard configuration.

WATER-S runs on a long life 3 V Lithium battery (size CR 123A). It features an on/off switch and a potentiometer knob for stirring rate adjustment. In the bottom center, a stirring paddle is mounted on the motor axis (via split brass-tube adapter). The **disposable paddle** can be removed by gentle pulling. The other way around, a replacement paddle can be mounted by pushing its cylindrical end all the way into the holder. For replacement of the battery the housing has to be opened by pulling the white and grey halves apart. Separation of the two halves is facilitated by forcing gently a thin flat body into the slit (like fingernail or thin screwdriver).

It should be noted that at high photomultiplier gain the paddle of the WATER-S will cause some increase of noise. This is due to the fact that some measuring light is reflected from the paddle towards the photodetector, such that the background signal is approximately doubled, and the electronic noise is correspondingly increased. Furthermore, there is an increase of sample noise caused by the movement of cells or cell groups.

2.2   Modular Version

The PHYTO-PAM-II Modular version bases on the well proven optical geometry applied in Walz instruments since 1993 (XE-PAM Schreiber et al. 1993, MULTI-COLOR-PAM Schreiber et al. 2012, Schreiber and Klughammer 2013). It is the more flexible and extendable PHYTO-PAM-II system. Next to exchangeable detector filters and two additional optical ports this version provides options for software triggered stirring and temperature control.

The basic system includes following components:

1. Power-and-Control Unit PHYTO-II-C

2. Multi-Color-Emitter Unit PHYTO-II-E

3. Photomultiplier-Detector Unit PHYTO-II-D

4. Optical Unit ED-101US/MP

5. US-SQS/WB

Accessories are:

6. Miniature Magnetic Stirrer PHYTO-MS

7. Temperature Control Unit US-T

The instrument is mounted to the Stand with Baseplate 101. The assembly of the stand is described in Fig. 5. Necessary tools are provided in the PHYTO-PAM-II transport box.
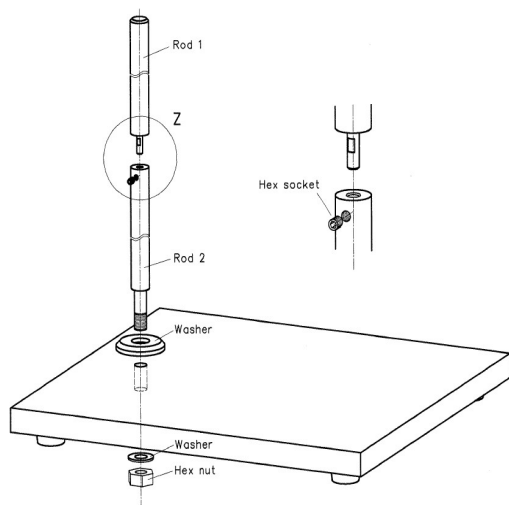
Fig. 5: Assembly of the Stand with Baseplate 101

The Optical Unit ED is mounted to the stand´s rod. The optical unit features a central 10 x 10 mm quartz cuvette interfaced with four horizontal light ports (one pre-mounted with the detector unit light guide) and a port for the Miniature Magnetic Stirrer PHYTO-MS at the bottom.

Please insert the 10 x 10 mm quartz cuvette and remove a horizontal light port located in a 90° angle to the pre-mounted detector light guide.

Insert the Multi-Color-Emitter Unit PHYTO-II-E to this light port till the Perspex rod gently touches the quartz cuvette. Please fix PHYTO-II-E by the white knurled screw.

The delivery package includes a detector filter which needs to be placed to the filter slot between photomultiplier detector and light guide (see Fig. 6: Photomultiplier-Detector Unit PHYTO-I-D with detector filter). Please pay attention to the orientation as labelled on the detector filter.

Please insert the Photomultiplier-Detector Unit PHYTO-II-D light guide till the Perspex rod gently touches the detector filter and fix the PHYTO-II-D light guide by the knurled screw.
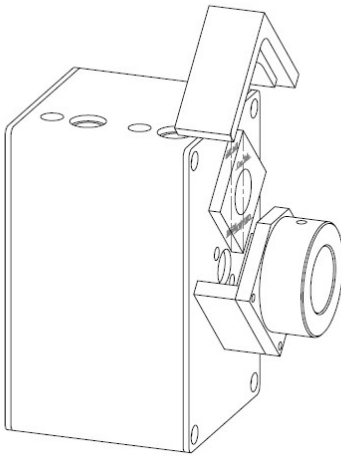
Fig. 6: Photomultiplier-Detector Unit PHYTO-I-D with detector filter

Overall the core of the optical system is a powerful Chip On Board (COB) multi-color LED array (1), which serves for pulse-modulated measuring light (ML), actinic light (AL), which can be applied either quasi-continuously or in form of strong ms pulses, far-red light (FR) for preferential excitation of PS I and saturating single-turnover flashes (ST) for instantaneous, quantitative closure of PS II reaction centers and determination of the so-called I1-level.
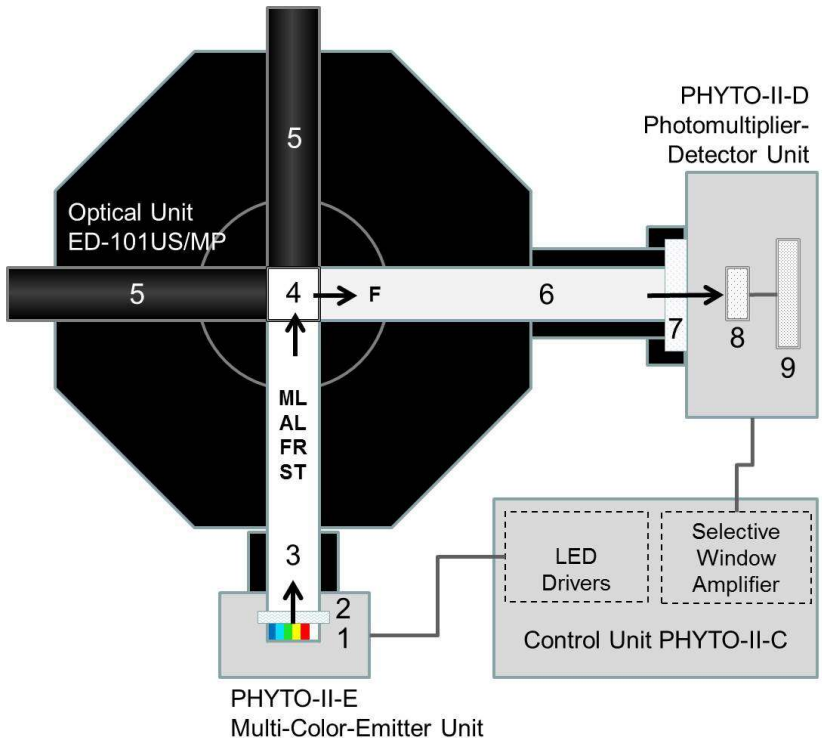
Fig. 7: PHYTO-PAM-II Modular Version optic schema

The light passes a short-pass dichroic filter (2) before it enters a 10x10 mm Perspex rod (3) that guides it to the 10x10 mm cuvette (4), optically mixing the various light qualities by multiple reflections. The suspension within the cuvette (4) is continuously stirred with the help of a small magnetic "flea". Push-in rods with mirror front surfaces (5) are inserted at 90° and 180° angles to incident light, thus increasing both the effective light intensities and the amount of fluorescence picked up by a 10x10 mm Perspex rod (6) at 90° angle to incident light. The fluorescence (F) passes a special low-background bandpass filter (650-750 nm) (7), which absorbs scattered incident light, so that only fluorescence reaches the photomultiplier or photodiode detector (8). The pulse-modulated fluorescence signal selectively is amplified by a pulse-preamplifier (9) within the detector unit and then further processed by a special selective window amplifier within the main control unit PHYTO-II-C.

2.2.1 Battery Charger MINI-PAM/L

see chapter 2.1.2

2.2.2 Miniature Magnetic Stirrer PHYTO-MS (optional)

The stirrer has an inductive drive system which results in its compact size and facilitates its integration into the Optical Unit ED-101US/MP. The stirrer moves a miniature stir bar placed inside a cuvette in the optical unit. Stirrer action is software-controlled (Settings window chapter 6.4) and stirrer speed is adjusted manually by a control knob at the front panel of the PHYTO-PAM-II control unit.

2.2.3　Temperature Control Unit US-T (optional)

The US-T unit is placed on top of the ED-101US/MP Optical Unit. The device controls temperature of the cuvette by a Peltier-heat-transfer rod which is immersed in the upper part of the sample suspension. Temperature is measured by a PT-100 sensor which is dipped in the cuvette. Temperatures of 15 K above and below ambient temperature can be achieved.

Please follow separate instruction manual.

3   PhytoWin_3 Software installation / bringing PHYTO-PAM-II into operation

PhytoWin_3 and manual versions can be downloaded from Walz web page http://www.walz.com/downloads/overview.html. The PhytoWin_3 program needs to be installed on the PC going to be used in conjunction with the PHYTO-PAM-II. At the end of the guided installation procedure a **PhytoPam_3 folder** is created on the PC with **Data-directories** for the different PHYTO-PAM-II Versions (Compact (Data_Cu6) and Modular (Data_US6). Into these directories all measured data will be saved automatically. Next to these directories a script folder, the Phyto.exe file and, after bringing into operation, a Phyto.cfg, light lists, and reference files are stored.

After download double click the file Setup.exe

After start of Setup.exe the Install Wizard is called up.

This wizard guides through the installation (including installation of the Phidget library for pump control), at the end of links to **PhytoPam_3 Folder**

and **PhytoWin_3.exe** are established on the desktop.
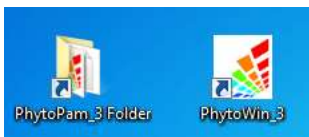
Connect PHYTO-PAM-II to a computer via USB-cable and start PhytoWin_3 program by clicking the **PhytoWin_3** icon on the desktop. The Start window is displayed, showing the number of the current PhytoWin_3 version.

Following query appears:
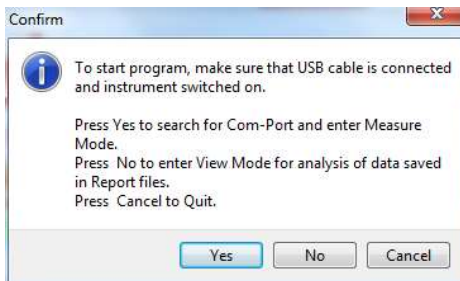
 to search for Com-Port and enter MEASURE mode

 to view and analyze data acquired by the PHYTO-PAM-II. Starts the program in the viewing mode.

 to quit the program.

In both software operation modes (**MEASURE mode** and **VIEWing mode)** the selection of the applied measuring head is essential, as each measuring head features individual parameters (e.g. relating to photomultiplier sensitivity) that are stored in separate **Data directories**. These parameters are essential for correct storage and analysis of the measured data. After definition of the measuring head the actual program is started with the 5-channels excitation window being displayed.

NOTE:

When a computer installs the PhytoWin_3 user software first time for a selected PHYTO-PAM-II measuring head, the **internal PAR list needs to be measured before bringing the instrument into operation**. Calibration of the PAR-Lists and a valid .par file in the measuring head folder is essential for correct assessment of PHYTO-PAM-II functions.

The default.par file is only for startup and not suitable for functional operation.

The features of the PhytoWin_3 user software described in the following sections apply to all measuring heads. Exceptions are indicated.

3.1   Measure PAR Lists

To bring the PHYTO-PAM-II to operation first the LED array light parameters (PAR lists) need to be calibrated. The light list measurements include calibration of the light sensor offset and readout of the complete LED array done in an automated calibration procedure.

- Switch on PHYTO-PAM-II and start PhytoWin_3 software. Please plug in the Spherical Micro Quantum Sensor US-SQS/WB to the PHYTO-PAM-II light sensor connector (or the AUX connector for the MODULAR version), the light sensor will be recognized and the **Measure PAR Lists** button in the **Settings window** enabled.

   At first use the light sensor might show an offset. This offset will be eliminated during light sensor calibration within the Measure PAR Lists routine.

- Fill distilled water into the quartz glass cuvette and insert it in the PHYTO-PAM-II cuvette retainer.

- Insert the light sensor. The position of the light sensor is critical for correct detection of PAR values. Therefor the light sensor/darkening hood geometry is fixed by a spacer (Compact version 15.2 mm, Modular Version 19 mm) and should not be altered.

- Open the measure PAR routine by clicking the Measure PAR Lists button (Settings window) and start light calibration process.
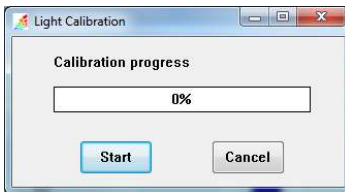
- The light sensor offset, and all LED array parameters will be calibrated. When the Measure PAR List routine is finished a .par file will be stored within the Data-directories of the related measuring head folder.

- Disconnect light sensor and restart PhytoWin_3 to establish functionality of PHYTO-PAM-II.

NOTE:

The PAR lists need to be recalibrated:

• modifications of any current values (see chapter 6.12.2)

• using a different Computer

• MODULAR Version: after each change of optical geometry

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4   First fluorescence measurements

Fig. 8   Channels window

The 5-channels excitation mode is the standard mode of operation of the PHYTO-PAM-II. After start of the program, on the PC monitor screen the **"Channels" window** is displayed. This shows the current Chl fluorescence yield, Ft, measured continuously with 5 different excitation wavelengths. In addition, also the mean value of the 5 fluorescence signals is displayed. Normally, after program start the displayed Ft-values are close to zero, as the Gain (photomultiplier voltage) is set to a low setting by default,. As indicated by the status of the **ML-switch** (bottom, left), the measuring light is switched on upon program start. It is applied in LED-pulses, such that its actinic effect is relatively weak. This means that no electrons accumulate at the acceptor side of PSII and, hence, the minimal fluorescence yield, Fo, of a dark-adapted sample is assessed. When the **AL-switch** is activated also the intensity of the ML-LEDs is increased. This is due to an **automatic increase of the frequency of ML-pulses** during actinic illumination. In this way, the signal/noise ratio is increased, and the fluorescence changes induced by the actinic light are assessed. At the same time the ML at high frequency contributes to overall actinic intensity, which is displayed in the **PAR-field** in units of µmol quanta $m^{-2}s^{-1}$ (photosynthetically active radiation). A third

type of illumination is triggered by the **"Sat-Pulse" button**. But, please avoid looking directly into the LED-array source, as this light is very strong and may harm your eyes. This so-called **"saturation pulse"** can cause complete reduction of the PSII acceptor pool and, hence, induce an increase of fluorescence yield from its current level (**Ft**) to its maximal value (**Fm**). Based on such measurements, the effective quantum yield of photosynthetic energy conversion in PSII can be determined, using the simple relationship:

$$Yield = (Fm-F)/Fm = Fm-Fo/Fm$$

For some first fluorescence measurements fill the cuvette with a sample (which first may be pure water) and make sure that the **Photomultiplier-Detector** is switched on (green indicator LED on the right-hand side of the elements of system operation bar). Underneath the photomultiplier button is the **Gain** control box, showing setting 5 of photomultiplier gain upon program start. This gain is by far too low to show any fluorescence signal with a pure water sample. After clicking the Gain-button the Gain-setting is automatically increased until the channel with the largest signal shows about 700 units (**Auto-Gain** function). Even pure water samples will show a fluorescence signal, if the Gain is sufficiently high (ca. setting 26 by Automatic Gain control). This unavoidable **"background signal"** is due to stray fluorescence originating from various system components like the LED-array, cuvette and filters. You will find that an empty cuvette gives a much larger background signal than a cuvette filled with pure water. The unavoidable background signal can be digitally suppressed by the automatic **Zero-offset** function **(Zoff)**. But, please note that it will always cause a decrease in the signal/noise ratio.

In practice, natural surface waters often contain (besides phytoplankton) other fluorescing substances (like humic acids) in solution. To get rid of this contribution, together with the small background signal caused by system fluorescence, it is recommended to proceed as follows:

–   Make sure that the cuvette is clean, e.g. by washing with ethanol and rinsing with water.

–   Make sure that the cuvette is placed correctly into the measuring head. If the cuvette is not all the way down, this will cause an increased background signal.

–   Fill the cuvette with ca. 4 ml of the sample to be investigated and apply Auto-Gain to define the Gain-setting at which the measurements will be carried out.

–   Prepare a **filtrate of the sample** using a 0.2 μm millipore filter that will retain all phytoplankton.

–   Exchange the cuvette by a cuvette containing ca. 4 ml of the filtrate and measure its fluorescence using the same Gain-setting as found appropriate for the unfiltered sample. By giving a saturation pulse, you may convince yourself that the signal displayed by the filtrate really is not originating from active Chl. The Fm`-F and Yield-values will be zero or close to zero. Please note the little **indicator lamp** below the PAR-box, which lights up red as long as the signal is unstable. All measurements, including Zoff-determination, should be preferentially carried out after this lamp lights up green. After Zoff-determination, the signals of the 5 channels are close to zero. Fluctuating values of up to ca. 5 units may occur due to digital noise and are of no concern.

After Zoff-determination the filtrate is substituted by the sample and now the proper fluorescence measurements can start, as the fluorescence yields displayed for the 5 channels now are only due to the phytoplankton. The most fundamental measurement is the assessment of the **quantum yield of photochemical energy conversion** in PSII by application of a **saturation pulse**. With an active sample, the 5 channels will show values of **maximal PSII quantum yield** (Yield under quasi-dark-adapted conditions) in the order of 0.5 - 0.8. You may have a look at the **saturation pulse kinetics** (**View Pulse** check-box). For appropriate Yield-determination, it is important

that the maximal fluorescence yield is reached during the saturation pulse, which is the case when a distinct plateau is observed

Another fundamental measurement is the recording of the fluorescence changes upon transition from darkness to continuous light. Just switch on the actinic light (**AL-button**) and follow the changes of fluorescence yield with time, Ft. You will observe that fluorescence yield first rises to a peak level and then slowly declines towards a steady state level. This is the famous **Kautsky-effect**, which reflects the dark-light induction kinetics of photosynthesis. In the slow kinetic window, you can see the graphical displayed of the recorded values.

When a saturation pulse is applied during actinic illumination, the observed Yield-values are distinctly lower than after dark-adaptation. This reflects a decrease in the efficiency of energy transformation at PSII reaction centers due to two major factors: first, partial reaction center closure (primary acceptor Q_A reduced) and, second, increase of nonradiative energy dissipation.

Chl fluorescence carries information on the **effective quantum yield of PSII** under quasi-dark and light conditions. The product of quantum yield and quantum flux density of incident photosynthetically active radiation (PAR) provides a relative measure of **electron transport rate (ETR)**. Plots of quantum yield and ETR versus PAR (so-called **light response curves**) give valuable information on the photosynthetic performance and light saturation characteristics of a sample.

The PhytoWin-program provides a routine for automated recording of light response curves. For a first demonstration, open the **"Light Curve"** window and click the **"Start"-button**. There is an immediate Yield-determination of the sample adapted to the Measuring Light (at the given frequency of Measuring Light pulses). Then light intensity automatically is increased in a first step (see increase in displayed PAR-value) that extends over a defined time period, at the end of which Yield again is determined. Further steps of increased light intensity follow and at the end of each the Yield is determined,

thus resulting in light response curves of Yield and of the derived ETR. The PAR-values of the various steps, the illumination time during each step and the total number of steps can be defined by the user (via Edit, see 6.6.1). These **"Rapid Light Curves"** provide relevant information as outlined in more detail below (see 6.6).

The fluorescence information obtained will be automatically stored in the **Report-file** which can be accessed by clicking the corresponding "register card" (Report see 6.7). All data stored in the Report-file can be also recalled on the **Channels**- and **Algae windows** for further inspection with the help of the **VIEW-mode** (see 6.11). To continue with measurements, the user must return to the **MEASURE-mode**.

4.1   Principle of deconvolution

Well proven by the 1st generation PHYTO-PAM is the deconvolution of different groups of phytoplankton. The reliable assessment of fluorescence parameters using several different excitation wavelengths is the basis for this distinction and differentiation. PHYTO-PAM-II features now a new spectral composition using five excitation wavelengths that are chosen for optimal differentiation between cyanobacteria, green algae, diatoms/dinoflagellates and phycoerythrin-containing organisms, which differ substantially in the absorbance spectra of their antenna pigments.

An essential prerequisite for the differentiation between various types of phytoplankton is that the 5-channels fluorescence responses of the pure cultures are known. The measurements of such **"Reference Excitation Spectra"** are automated and, hence, quite simple to be performed with the PHYTO-PAM-II, (see 6.7). PHYTO-PAM-II normalizes measured reference spectra. Reference spectra measured with PYTHO-PAM II are **universal** and can be **shared among users**. More information about reference spectra are given in chapter 6.8.

Based on the "Reference Spectra" the PhytoWin-program deconvolutes the original 5-channels signals into the contributions of the corresponding algal classes. It should be emphasized that contrary to the unbiased fluorescence information displayed in the "Channels" window, the information on the "Algae" window is strongly biased by the information contained in the applied References. Hence, the quality of the obtained results depends on previous work invested by the user into the measurement of the References. Such work will profit from background knowledge on the likely presence of particular phytoplankton species in the investigated water sample. In this sense, the success of practical applications to a considerable extent depends on close interaction with basic research, not only using Chlorophyll fluorescence, but also alternative methods, like microscopy, flow cytometry and analysis by HPLC.

Deconvolution of the phytoplankton classes can be applied to data obtained by classical PAM analysis as outlined in chapter 5.

5   Saturation Pulse Analysis

Typically, five different types of fluorescence levels are acquired by Saturation Pulse analyses named Fo, Fm Fo' Fm' and F. In most cases, the PAM fluorescence signal is proportionally related the yield for chlorophyll fluorescence. Therefore, differences between these five fluorescence levels reflect variations in chlorophyll fluorescence yields.

Two of these levels (Fo and Fm) need to be determined with a dark-acclimated sample. The three remaining levels (Fo', F, and Fm') are repeatedly measured during subsequent sample treatments (e.g., exposure to actinic light; see Fig. 9).

5.1   Measurements with Dark-Acclimated Samples

Fo Minimum fluorescence level excited by very low intensity of measuring light to keep PS II reaction centers open.

Fm Maximum fluorescence level elicited by a pulse of saturating light (Saturation Pulse) which closes all PS II reaction centers.
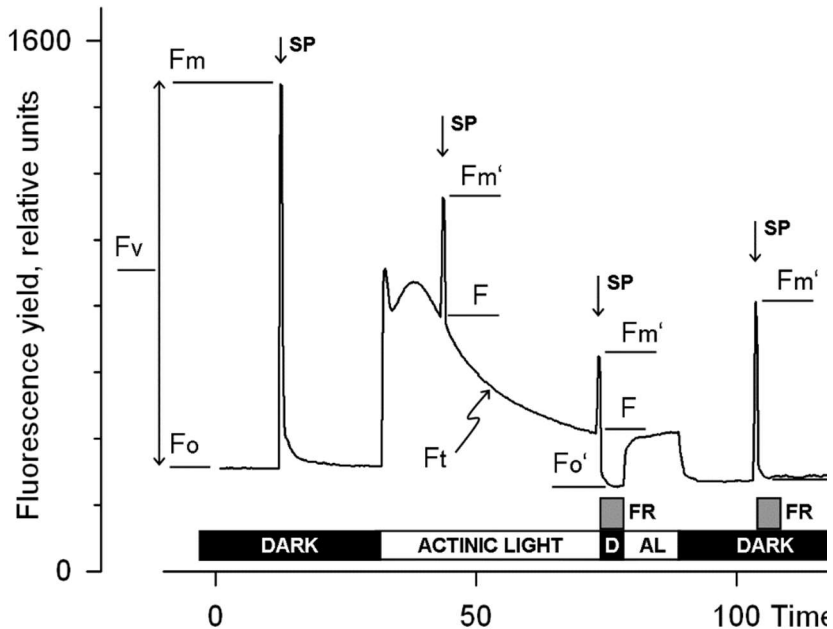
Fig. 9:   **Measurements for Saturation Pulse Analysis. AL, Actinic
Light; D, dark; SP, Saturation Pulse; FR, Far-red illumination.**

5.2   Measurements with Illuminated Samples

Fo'   Minimum fluorescence level of illuminated sample which is
lowered with respect to Fo by non-photochemical quenching.
When the measuring routine for Fo' is active, the Fo' level is
determined during a dark interval following the Saturation Pulse.
In the dark interval, far-red light is applied to selectively drive
PS I and to quickly remove electrons accumulated in the
intersystem electron transport chain, thus reopening PS II

reaction centers (see Fig. 9, time point 75 s). Alternatively, the Fo' can be estimated according to Oxborough and Baker (1997):

$$Fo' = \frac{1}{\dfrac{1}{Fo} - \dfrac{1}{Fm} + \dfrac{1}{Fm'}}$$

Fm'   Maximum fluorescence level of illuminated sample as induced by a Saturation Pulse which temporarily closes all PS II reactions centers. Fm' is decreased with respect to Fm by non-photochemical quenching.

F   The F corresponds to the momentary fluorescence level (Ft) of an illuminated sample measured shortly before application of a Saturation Pulse.

5.3   Fluorescence Ratio Parameters

To quantify photochemical use and non-photochemical losses of absorbed light energy, fluorescence ratio expressions have been derived which are based on the relative fluorescence yields introduced above. Table 1 compiles the fluorescence ratio parameters available in PhytoWin_3   . Subsequently, these parameters will be briefly explained.

Fv/Fm and Y(II)   Maximum and effective photochemical quantum yields of PS II

The Fv/Fm and Y(II) estimate the fraction of absorbed quanta used for PS II photochemistry. Fv/Fm corresponds to the maximum Y(II). For measurements of Fv/Fm, it is important that samples are acclimated to darkness or dim light so that all reactions centers are in the open state and non-photochemical dissipation of excitation energy is minimal.

Table 1:   Fluorescence Ratio Parameters.

Source	Equation
Maximum photochemical quantum yield of PS II (Kitajima and Butler, 1975)	$\dfrac{F_V}{F_M} = \dfrac{F_M - F_0}{F_M}$
Effective photochemical quantum yield of PS II (Genty *et al.*, 1989)	$Y(II) = \dfrac{F'_M - F}{F'_M}$
Quantum yield of light-induced (ΔpH- and zeaxanthin-dependent) non-photochemical fluorescence quenching (Genty *et al*. 1996)*	$Y(NPQ) = \dfrac{F}{F'_M} - \dfrac{F}{F_M}$
Quantum yield of non-regulated heat dissipation and fluorescence emission: this type of energy loss does not involve the action of a trans-thylakoid ΔpH and zeaxanthin (Genty *et al*. 1996)*	$Y(NO) = \dfrac{F}{F_M}$
Stern-Volmer type non-photochemical fluorescence quenching (Bilger and Björkman, 1990)	$NPQ = \dfrac{F_M}{F'_M} - 1$
Coefficient of photochemical fluorescence quenching (Schreiber *et al.* 1986 as formulated by van Kooten and Snel, 1990)	$q_P = \dfrac{F'_M - F}{F'_M - F'_0}$
Coefficient of photochemical fluorescence quenching assuming interconnected PS II antennae (Kramer *et al.* 2004)	$q_L = q_P \cdot \dfrac{F'_0}{F}$
Coefficient of non-photochemical fluorescence quenching (Schreiber *et al.* 1986 as formulated by van Kooten and Snel, 1990)	$q_N = 1 - \dfrac{F'_M - F'_0}{F_M - F_0}$

* Kramer *et al.* (2004) have derived more complex equations for Y(NO) and Y(NPQ). Klughammer and Schreiber (2008) have demonstrated that the equations by Kramer *et al.* (2004) can be transformed into the simple equations of (Genty *et al*. 1996) which are used by Phyto-Win-3 software.

In algae and cyanobacteria, the dark-acclimated state often is not showing maximal PS II quantum yield, as the PS II acceptor pool may be reduced in the dark by stromal reductants and consequently the so-called state 2 is formed exhibiting low PS II quantum yield. In this case, preillumination with moderate far-red light should precede determinations of Fo and Fm. The far-red preilluminated quasi-dark state normally serves as reference state with maximal PS II quantum yield for assessment of the functional absorption cross-section of PS II, Sigma(II).

The Y(II) value estimates the photochemical use of excitation energy in the light. It is lowered with respect to Fv/Fm by partial closure of PS II centers and various types of nonphotochemical energy losses induced by illumination. To derive from the Y(II) information on the overall state of photosynthesis, knowledge of the absorbed PAR is essential, as a sample e.g. may be severely damaged in Calvin cycle activity and still show a high value of Y(II) in weakly absorbed light. This aspect is particularly important in the study of algae and cyanobacteria, which display large wavelength-dependent differences in light absorption.

q_P **and** q_L Coefficients of photochemical fluorescence quenching.

Both parameters estimate the fraction of open PS II reaction centers. The q_P is based on the concept of separated PS II antenna units (puddle model), whereas the q_L assumes interconnected PS II antenna units (lake model) which appears to be the more realistic situation in leaves (*cf.* Kramer *et al.*, 2004). Determinations of q_P an q_L do not require fluorescence measurements with the dark-acclimated sample except when Fo' is not measured but calculated according to Oxborough and Baker (1997).

q_N and NPQ Parameters of non-photochemical quenching

Both parameters are associated with non-photochemical quenching of excitation energy, mainly involving a low thylakoid lumen pH- and a zeaxanthin-dependent quenching mechanism. In contrast to Y(II), qP and qL, calculations of the qN and the NPQ parameters always require fluorescence measurements with the sample both in the dark-acclimated and in the light-exposed states (see Table 1, page 37). Calculation of NPQ (or SV_N; Gilmore and Yamamoto, 1991) corresponds to the Stern-Volmer equation for fluorescence quenching which predicts proportionality between fluorescence quenching (NPQ) and the concentration of fluorescence-quenching centers in the photosynthetic antennae (e.g. zeaxanthin).

Y(NO), Y(NPQ) and Y(II) Complementary PS II quantum yields

Genty *et al.* (1996) first presented expressions based on basic fluorescence parameters that describe the partitioning of absorbed excitation energy in PS II between three fundamental pathways, which were further investigated by Klughammer and Schreiber (2004) and expressed in terms of the complementary quantum yields of

Y(NO)   sum of non-regulated losses of excitation energy including heat dissipation and fluorescence emission,

Y(NPQ) regulated energy losses of excitation energy *via* heat dissipation involving ΔpH- and zeaxanthin-dependent mechanisms, and

Y(II) use of excitation energy for charge separation.

The yields of photochemical energy conversion and non-photochemical losses sum up to 1:

Y(II) + Y(NPQ) + Y(NO) = 1

This concept of "complementary PS II quantum yields" is useful to analyze the partitioning of absorbed light energy in photosynthetic organisms. For

instance, under high light-conditions, a much higher Y(NPQ) than Y(NO) indicates that excess excitation energy is safely dissipated at the antenna level, that is, photosynthetic energy fluxes are well-regulated. In variance, high values of Y(NO) would signify that excess excitation energy is reaching the reaction centers, resulting in strong reduction of PS II acceptors and photodamage, e.g. via formation of reactive oxygen species.

6   Features of PhytoWin_3 Software

Operation of the PHYTO-PAM-II Phytoplankton Analyzer is based on the PhytoWin_3 software in conjunction with a Pentium PC. Installation of the software was described in chapter 2.2.2 and some first, basic measurements using this software were already described in chapter 4. Here the numerous functions supported by this software are described systematically in more detail.

The user surface of the PhytoWin_3 in the **MEASURE mode** is divided into 3 sections: 1. main menu, 2. one of nine output windows and 3. system operation elements
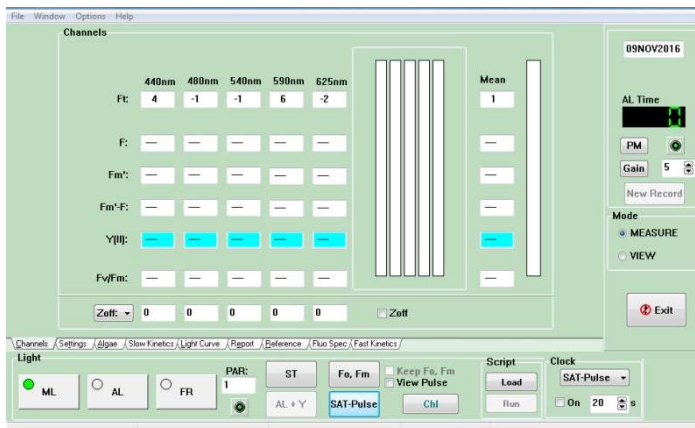
Fig. 10: PhytoWin_3 software upon start

1) **Main Menu:** At the top, the items of the main menu are listed (File, Window, Options and Help). Each item features a pull-down sub-menu.

2) **Window:** The major central part of the screen features the Channels window, which represents one out of nine windows offering various options for data output and user interfaces.

\Channels /Settings /Algae /Slow Kinetics /Light Curve /Report /Reference /Fluo Spec /Fast Kinetics/

- **Channels:** Original, unbiased fluorescence information at 5 different excitation wavelengths.

- **Settings:** Controls for instrument settings, like measuring pulse frequency, actinic intensity, saturation pulse width and intensity, clock interval, number of averages, etc.

- **Algae:** Deconvoluted fluorescence information for cyanobacteria, green algae, diatoms/dinoflagellates and phycoerythrin-containing organisms (e.g. cryptophytes) based on previously recorded reference excitation spectra;

- **Slow Kinetics:** Graphic display of saturation pulse analysis and online recordings of Ft.

- **Light Curve:** Measurement and graphic display of light response curves; effective quantum yield and relative electron transport rate (ETR) as a function of PAR

- **Report:** File in which all measured data and instrument settings are stored, which can be edited by the user and exported to other programs

- **Reference:** Recording and display of reference excitation spectra of cyanobacteria, green algae, diatoms/dinoflagellates and phycoerythrin-containing organisms

- **Fluo Spec:** Display of measuring light characteristics and unnormalized reference excitation spectra

- **Fast Kinetics:** Control and graphical display of fast kinetics analysis providing information on the functional absorption cross-section of PS II (σ_{II}).

3) **Elements for system operation and display**: Below and at the right-hand side of the central output window elements for system operation and display of instrument status are located. These elements are always accessible, independently of the selected window.

For all possible operations **"Tooltips"** are provided which are displayed whenever the cursor is moved into the vicinity of the corresponding switch or button. They give a brief explanation to guide the user through the program.

6.1   Elements for system operation and display of instrument status

The elements for system operation and display of device status are always available in MEASURE mode and independent of the displayed window.

Fig. 11   User surface of PhytoWin_3 elements for system operation and display of instrument status

Fig. 11 shows the user surface of the PhytoWin_3 in the **MEASURE-mode** of operation with the **Channels window** being selected. Elements for system operation and display of instrument status are marked red and will be briefly described starting from the lower left corner and ending at the upper right corner:
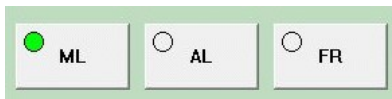
On/Off switches of Measuring Light (ML), Actinic Light (AL), Far Red Light (FR) respectively.

Display of current value of **incident photosynthetically active radiation** (quantum flux density) within the cuvette in units of μmol quanta $m^{-2}s^{-1}$. The displayed values derive from an internal

PAR-list unless the Spherical Micro Quantum Sensor for PAR list calibration is connected (see chapter 3.1). If the light sensor is connected the PAR field turns green.

This **indicator lamp** may light up alternatively green or red. When it lights up green, the system is **ready for measurement**. This means that a stable reading is reached, and the signal/noise ratio is high. The indicator lamp lights up red after an abrupt signal change (e.g. after switching on measuring or actinic light or after changing photomultiplier gain) and whenever the signal is disturbed by excessive background light.

ST = 50 µs single turnover saturation flash

AL + Y = actinic illumination with terminal application of a saturation pulse for determination of effective quantum yield (Yield = (Fm-F)/Fm). This function is active only when the illumination time is defined (Act. Light Width on "Settings" window, see 6.4). Minimal Act. Light Width is 3 s.

Fo, Fm = determination of **dark fluorescence yield** of a dark acclimated sample. Fo, Fm-determination is essential for correct calculation of the quenching parameters. Fo, Fm values are stored in the Report as measurement No. 1.
Fo,Fm values can be retained by checking the "Keep Fo,Fm" checkbox before opening a new record.

SAT-Pulse = Yield-determination by a single saturation pulse. This measurement involves assessment of the fluorescence yield briefly before the saturation pulse**, F**, and of the maximal fluorescence yield, **Fm'**. While the Ft-values are continuously changing and not stored, the F- and Fm'-values are saved in the Report-file. The quantum yield of

photochemical energy conversion in PSII (**Yield**) is calculated by the equation:

$$Yield = (Fm'-F)/Fm'$$

The Yield-determination should be started only (via SAT-Pulse) when any signal disturbance, has settled down, i.e. when the indicator LED gives green light (see above).

**☐ Keep Fo, Fm**   mark this checkbox to keep a measured Fo, Fm when a new report or record is opened.

☐ View Pulse   **View Pulse:** Check box to display the **fluorescence rise kinetics** of the 5 signals during a saturation pulse. This function is important to ascertain that for a given sample the settings of SAT-Pulse (intensity and width) are appropriate to reach a plateau of maximal fluorescence yield during the pulse. This is a prerequisite for proper determination of effective quantum yield.
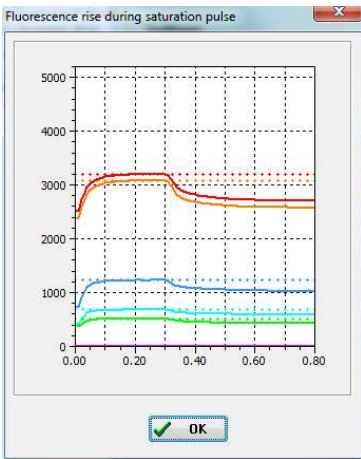
Chl   Start-button for **Chlorophyll a content determination** based on fluorescence yield. Please note: the measured apparent chlorophyll fluorescence is dependent on measuring light frequency and needs to be done at the measuring light frequency chosen for chlorophyll calibration during reference recording.

Script

Load

Run

Script files are used for automated execution of experimental procedures. The Load button opens the script file window for upload of an existing script file or creation of a new script. To execute script files, click the Run button. More information about script files is given in chapter 6.13.

Clock

SAT-Pulse ∨

SAT-Pulse
F
0-I1 rise
ST
FR
AL
AL + Y
Light Curve
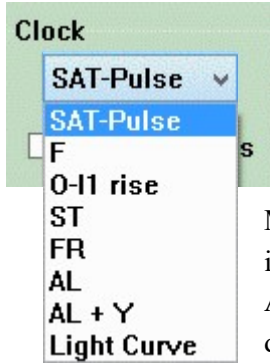
Repetition Clock: When switched on (check box), the selected command (**Clock-item**) is repeated indefinitely until manually switched off again. The **Clock interval** (time between two consecutive measurements) can be set (default value 20 s). Minimal Clock interval is 3 s maximal clock interval is 3600s. Please note that the Clock items AL and AL+Y can be activated only when AL Width has been defined under Settings (see 6.4).
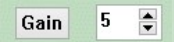
⊘ Exit   To leave the PhytoWin_3 program
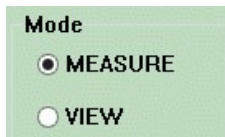
Mode
◉ MEASURE
◯ VIEW

In the MEASURE mode new data can be measured and analyzed online. The VIEW-mode is for offline inspection of previously stored data (see 6.11).

New Record   Manual start of a New Record, characterized by the time and date when New Record was clicked. The records are subdirectories of a Report file. A new record is started automatically upon start of the program, starting a new slow kinetic, starting a new light curve or when returning into the MEASURE-mode from the VIEW-mode (see 6.11). It is recommended to start New Record manually with every new sample or new experiment, and to write some relevant information to the record comment text.

Photomultiplier gain, which after start is by default at the low setting of 5. When the Gain-button is clicked, automatically the Gain-setting is adjusted to a value which is suitable for measurements with a given sample (**Auto-Gain** function). The gain steps are optimized to the extended dynamic range of the PHYTO-PAM-II photomultiplier and comprises 30 gain settings. The extended dynamic range distributes to the higher sensitivity of PHYTO-PAM-II.

For gain adjustment the maximal signal amounts to about 700 units (Ft-value on Channels window). The gain can also be set manually with the arrows or by double-clicking the gain field.

When the gain is too high the signal may run into "Overload" e.g. during saturation pulses. A low gain may result in a too low signal limiting the accuracy of fluorescence measurement. In both cases a corresponding warning appears:

overload

Attention:

Suboptimal data due to low
signal level. If possible,
increase Gain.

✓ OK
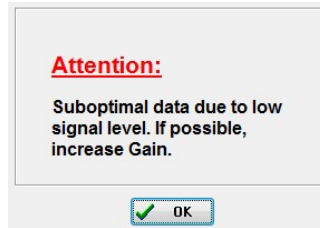
PM ◉   Photomultiplier activation button. The indicator lamp
lights up green indicating an active photomultiplier or red
if photomultiplier is switched off.

AL Time   Remaining illumination time during an actinic illumination
period. The actinic illumination period is defined on the
Settings window (Act. Light Width). In conjunction with
Light Curves (see 6.6), the width of each actinic step is defined under Light
Curves/Edit.
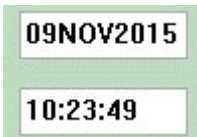
09NOV2015   Date (and in VIEW mode also time) of the start of the
current record.

10:23:49

6.2   Channels window

The channels window represents one out of nine windows which can be selected for different modes of signal display and analysis, as well as for definition of instrument settings.

The channels window displays the original, non-deconvoluted fluorescence information of the 5 excitation wavelengths.
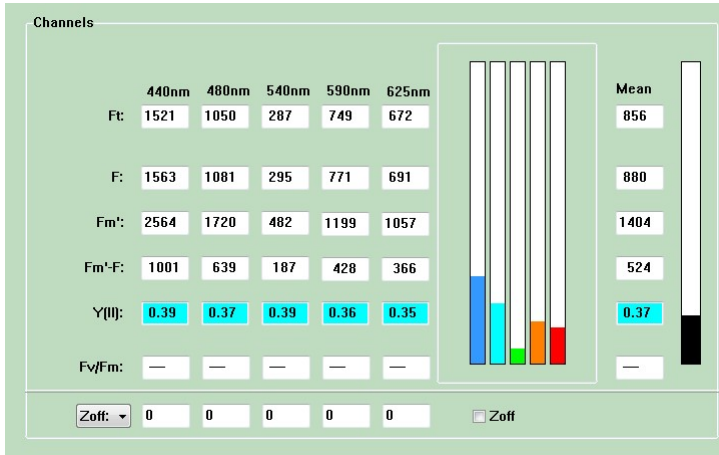
Fig. 12 Channels window for display of original fluorescence signals measured with 5 different excitation wavelengths

6.2.1 Ft, F, Fm', Fm'-F, Y(II), Fv/Fm and Mean Value display

The **current signals** of the five excitation channels are displayed in the **Ft**-line and by the five indicator-bars. Besides the individual Ft-signals, also the **Mean** (average of the 5 signals) is depicted.

After saturation pulse measurement (SAT-Pulse button) **F, Fm', Fm'-F** and **Y(II)** values are displayed for the five excitation channels. These values resemble the last stored values in the report window (see chapter 6.7). Also, the dark-adapted yield values of all five excitation channels are displayed as

Fv/Fm. If no measurement is taken the corresponding value fields are crossed out ⬚.

6.2.2 Zero Offset and noise N(t)

At low chlorophyll content, when high Gain is required, the Ft-signals are not only due to Chl fluorescence but also to an unavoidable background signal that originates from various system components. Furthermore, in natural surface waters fluorescing compounds like humic acids may be dissolved, which will contribute to the signal. The contributions of such background signals can be suppressed by the **Zero Offset (Zoff)** function.
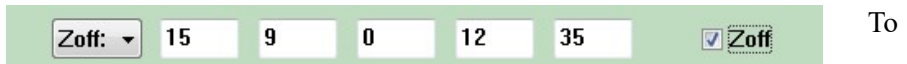
To determine Zoff, the cuvette is filled either with pure water or with the filtrate of a natural water sample. By clicking the Zoff button the five background signals are measured and substracted from the original Ft-signals, such that these are suppressed to zero. It is recommended to determine Zoff at the same Gain as used for the actual measurements. Please note that the Zoff values also are adjusted automatically to a changed Gain-setting based on the known photomultiplier gain characteristic.

NOTE:

Activation of Zoff function initiates the immediate measurement and subtraction of background signals. These Zoff values remain valid as long as the Zoff function stays activated. Measured Zoff values are noted in the report value, but not stored for later applications.

Previous determined Zoff values can be taken into account by manually editing the Zoff values (checkbox needs to be activated)

As an alternative to the display of the Zoff values, also the momentary noise, N(F), on the individual fluorescence channels can be shown. For display of N(F), please click on N(F) in the selection box. When the noise caused. by an external disturbance has settled down, the indicator LED (below PAR-box) gives green light for carrying out a measurement (e.g. application of SAT-pulse). Please note that N(F) will include any time dependent signal change, i.e. also time dependent fluorescence changes induced by actinic light.

It is possible to transfer a zero offset (Zoff) measurement from one record to another e.g. when the zero offset determination was done after the experiment with the sterile filtrated the sample.

To transfer a zero offset please follow the following procedure.

- Open the report containing the zero offset (Zoff) measurement in the view mode.

- Activate the settings check box and mark the Zoff

- Use the right mouse button to copy the Zoff values

- Open the measurement record without Zoff and apply the zero offset by using the right mouse button command Paste Zoff and recalc.

Please note that a zero offset can just be applied to records without an existing zero offset measurement. Please also note that applying a zero offset alters the rawdata within the record.

6.3   Algae window

The Algae window shows the deconvoluted fluorescence information for cyanobacteria, green algae, diatoms/dinoflagellates and PE-Type organisms (phycoerythrin-containing organisms e.g. cryptophytes). The deconvolution is based on Reference Excitation Spectra (see chapter 6.8) that were previously measured.
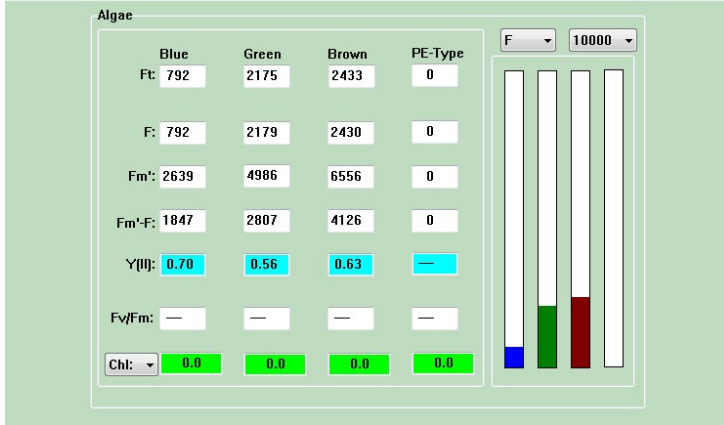
Fig. 13 Algae window displaying deconvoluted fluorescence information after application of a saturation pulse

In analogy to the Channels window, the deconvoluted fluorescence parameters Ft, F, Fm', Fm'-F, Y(II) and Fv/Fm are displayed. In addition, also the deconvoluted Chlorophyll concentrations of the four types of phytoplankton can be shown.

 In the same data boxes instead of Chl also the relative electron transport rate, **ETR** (see chapter 6.12), or the current noise, N(t) (see chapter 6.2.2), can be displayed.

 Besides fluorescence yield, the indicator bars can also show the Fm'-F induced by the last saturation pulse or the Chl concentration. A corresponding selection box is provided. Another selection box allows the user to define maximal signal range for display at different sensitivities.

The F-bar shows the current fluorescence yield (Ft) in the MEASURE mode. In the VIEW mode the F-value sampled in conjunction with the corresponding saturation pulse is displayed.

The Ft-values, as well as the corresponding indicator-bars, reflect the online measured deconvoluted contributions of the four types of phytoplankton to the overall fluorescence signal (i.e. the sum of the 5-channels signals). The sum of the four Ft-values displayed under Algae and the sum of the five Ft-values displayed under Channels are almost identical. The sum difference increases with increasing "Fit error". The F-values correspond to the fluorescence yields measured briefly before the saturation pulse and the Fm'-F values represent the increases induced by the pulse.

6.4   Settings window

In the settings window instrument settings are accessible for manual adjustment.
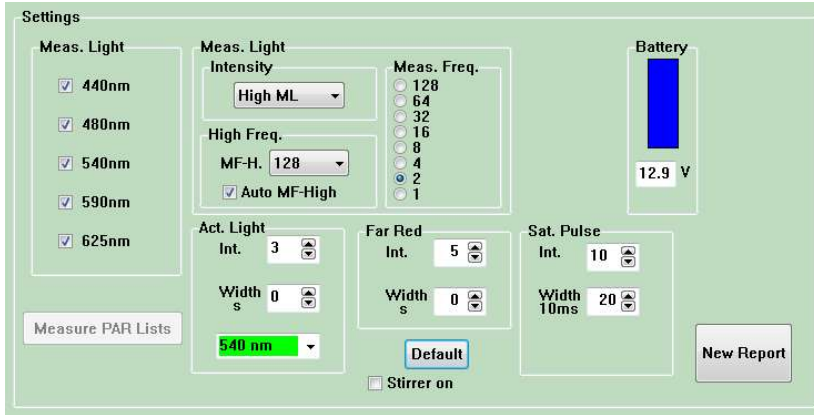
Fig. 14   Settings window PHYTO-PAM-II Modular Version

 This button opens a prompt for light list calibration. This routine includes calibration of the light sensor offset and determination of the overall characteristics of the LED array. Please read chapter 3.1.

 Measuring Light Wavelengths: Five different measuring light colors contribute to the measuring light: 440 nm, 480 nm, 540 nm, 590 nm and 625 nm. Please note: For deconvolution of algae groups all five measuring light wavelengths need to be activated. If deconvolution is not required PHYTO-PAM-II instruments can also be used as single measuring wavelength PAM fluorometer by inactivating the unused measuring light checkboxes. This can have the advantage that the measurement light-mediated light input is significantly reduced, and the individual measuring light frequency can thus be increased without acquiring actinic character.

Measuring Light Intensity Settings: The measuring light intensity can be set to High ML or Low ML intensity by the upper pulldown menu. If the Auto MF-High checkbox is activated, the measuring light is automatically increased frequency during actinic illumination and saturation pulses to the frequency set in the lower pulldown menu (Freq. 8, 32 or 128 Hz)

Measuring Light Frequency: Meas. Freq. gives the pulse frequency of the measuring light in relative units. At the standard setting 2 the quantum flux of photosynthetically active radiation corresponds to ca. 1 µmol quanta $m^{-2}s^{-1}$, which in most cases is sufficiently low to allow assessment of the dark-adapted fluorescence yield, which in phytoplankton is not necessarily identical

to minimum fluorescence yield. The effective intensities of measuring light at different frequencies are shown in the PAR-box.

Actinic Wavelengths, Intensity and Width. A total of 20 intensity settings are provided. When Width is set to 0 (default-setting), the actinic illumination period is infinite and must be manually terminated using the AL-button in the elements for system operation bar at the bottom left. At Actinic Light setting 0 (zero) no separate Actinic Light is given, but if Auto MF-High is activated the high light measuring light conditions are applied. The actinic light color can be set to 440 nm, 480 nm, 540 nm, 590 nm, 625 nm or white via pulldown menu.

Far Red light is adjustable in 20 far red intensity levels with a width between 1 and 600 seconds. When width is set to 0 (default-setting), the far red illumination period is

indefinite and must be manually terminated using the FR-Button in the elements for system operation bar at the bottom left.

Stirrer on   This function is only available for the PHYTO-PAM-II / Modular version with a **Miniature Magnetic Stirrer PHYTO-MS** connected and the manual stirrer switch of the control unit in on position. Activation of the checkbox activates the stirrer. Magnetic stirrer speed is adjustable by the turning knob on the control unit front side.

Default   Clicking the Default-button will restore the **standard settings**. Settings changed by the user remain valid after the program is left (via Exit) and started again later.

Battery   Display of present voltage of Power-and-Control-Unit internal battery. When voltage drops below 11 V and the blue indicator-bar disappears, the battery is almost empty and needs to be charged via the provided battery charger. There will be a warning: "Attention low battery! Please connect battery charger."

13.2 V

Sat. Pulse
Int.   10
Width 20
10ms

Dial boxes for **Intensity and Width of saturation pulses**. A total of 20 intensity settings is provided. The pulse length (Width) can be varied between 40 ms and 500 ms. Default settings are 10 for Intensity and 200 ms for the Width. Whether or not Sat. Pulse Intensity and Width are appropriate, may be judged for a sample with the help of the View Pulse function.
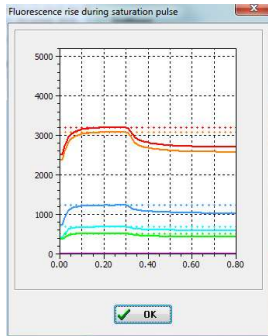
The **"View Pulse"** function allows to examine the fluorescence rise kinetics during a saturation pulse. In the example on the left a saturation pulse width of 0.3 s is displayed. In this particular saturation pulse, the fluorescence yield rises to a plateau but also does not declines during the saturation time widths of 0.3 s. For this sample the Saturation Pulse settings are appropriate.

Clicking the **New Report** button will create a new report file. The file will be created automatically by the program, written into the Data-directories of the applied Measuring Head. The file name is made up from the date and number of reports e.g. 20151117.rpt3 for the first report, 20151117_1.rpt for the second report started on the 17th November 2015. A report can include various record files characterized by the time and date of the moment "New Record" was started.

6.5   Slow Kinetics window

The Slow Kinetics window graphically displays all data measured by saturation pulse analysis. In addition, by activation via start the slow kinetic window provides online recording of current Ft values.
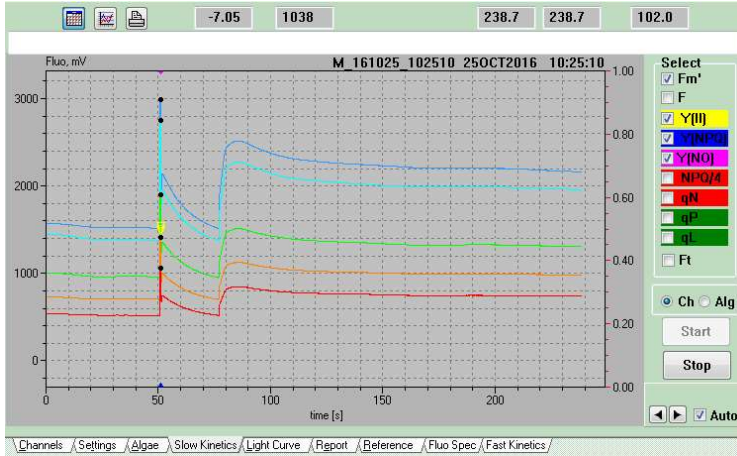
Fig. 15: Slow Kinetics window

Clicking the Start button initiates a new record and starts recording of current Ft values.

Clicking the Stop button terminates the slow kinetics recording.

Ten different fluorescence parameters can be selected for display. The display is controlled by checkboxes. Data points and background colors of the corresponding checkbox labels have identical colors.

Ch ○ ● Alg   It is possible to display either the **Channels** data or the deconvoluted **Algae** data by selection via the corresponding radio buttons.

Enables/disables Join to connect data points by line segments.

On/Off switch for grid display

Prints a black/white copy of the graph for documentation

Auto scale option to display the complete graph

89.98   3058   Courser position: left box displays x-axis value; right box displays y-axis value.

• Zoom in by clicking with the left mouse key on one corner of the rectangular area to be magnified, keeping the key pressed move to the opposite corner of the rectangle, and release key.

• Shift y-axis by moving the mouse cursor vertically with the shift key pressed.

◀ ▶   The arrow keys in the lower right-hand corner increase or decrease the x axis range. Employing the arrow keys deactivates the "Auto" function which otherwise automatically adjusts the x axis for full display the fluorescence trace.

After Start of a Slow Kinetics a comment line will appear above the graph. This comment line resembles the record comment of the report window (chapter 6.7).

6.6   Light Curve window

Light curves give information on the light adaptation state and photosynthetic capacity of a sample. With increasing quantum flux density, the effective quantum yield of PSII decreases, as PSII reaction centers progressively close and an increasing amount of energy is dissipated into heat.

Fig. 16   Light Curve window showing the effective quantum yield of PS II (Yield) and the relative electron transport rate (ETR) as a function of incident PAR. Display of original Channels data.

The Light Curve window shows plots of effective quantum yield (Yield) and relative electron transport rate (ETR) versus the incident photosynthetic active radiation (PAR). The PAR-scale is automatically adjusted to the selected range of light intensities, which are defined by the Edit-subroutine (see below). By double mouse-click on the Light Curves of Yield or ETR, either of these can be displayed full scale. With another double mouse click simultaneous display of both curves can be reinstalled.

It is possible to display either the Light Curves of the **Channels** data (Yield and ETR measured with five different excitation wavelengths) or the **Algae** data (Yield and ETR of four different pigment types of phytoplankton, deconvoluted using Reference Spectra for Blue, Green, Brown and PE-type Algae). For selection of Channels- or Algae-display the corresponding radio buttons are used. Check boxes are provided to select the **displayed** channels or types of phytoplankton. Deactivation of check boxes do not alter deconvolution (in contrast to the check boxes on the settings/reference window!).

If it is known that a sample does not contain a particular type of phytoplankton, the corresponding reference should be inactivated at the Reference window level. In this way, the deconvolution can be improved.

A light curve recording is started by clicking the **Start** button. A new record is started and the recording follows the light curve protocol unless stopped via **Stop**. Light curve protocols can be loaded and edited by the Edit function (see chapter 6.6.1). The first measurement is made in the absence of actinic illumination at the given measuring light frequency as Fo,Fm. This Fo,Fm is used for parameter calculations.

During a Light Curve recording the number of the **current illumination step** is indicated in the Step box. The remaining illumination time during a current step is shown as Next: or in the AL Time-box in the elements for system operation bar on the right hand side.

Light Curves can be auto scaled by clicking the **autoscale**-icon.

A **Print**-icon is provided for print-out of Light Curves via a serial printer (please define printer settings in the main menu).

| L Curve Details | ▸ | ✓ | Grid |
| L Curve Fit Parameters | | ✓ | Join |
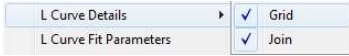
In the main menu **Options** some details in the way of Light Curve presentation can be defined. **Grid**: For display of grid. **Join**: To connect data points by line segments. For display of the **Light Curve Fit Parameters** a separate window is opened, which is outlined in sub-section 6.6.2.

6.6.1 Edit Light Curves

The **Edit** function allows the user to define the intensity settings and the time intervals of up to 20 steps in a light curve. Clicking the Edit button opens a Light Curve Edit window (Fig. 17).
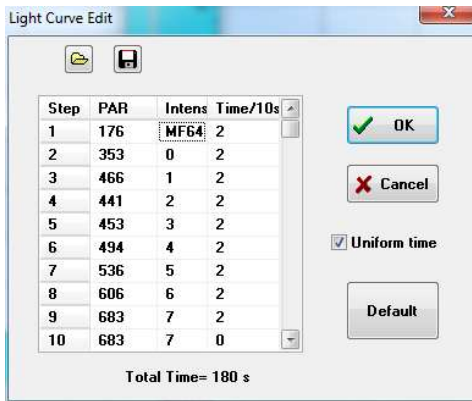
Fig. 17 Edit window for definition of Light Curve parameters

For changing the intensity setting or time (in units of 10 s) of a particular step, the corresponding fields first have to be marked by cursor/mouse click, then the setting can be typed in.

Steps

It is recommended to select progressively increasing PAR-values, with the highest value being at least two times higher than the PAR at which a sample was grown. Alternatively, it is also possible to edit up-down light curves with light intensity first increasing to saturation and then decreasing again. The observed hysteresis pattern provides information on light activation parameters.

A light curve recording does not necessarily have to involve 20 illumination steps. When at a step is given with a **0** (zero) for **Time/10s**, the **light curve is terminated** with the preceding step.

Intensity

One can choose between 20 intensity settings of actinic light and 8 frequency settings of the measuring light (MF1, MF2, MF4, MF8, MF16, MF32, MF64 and MF128), which at higher frequencies has a distinct actinic effect. The latter may be relevant for the very low light conditions light curves. MF128 is equivalent to actinic intensity-setting 0. **Please note that there is a significant difference between the spectral composition of the 5-wavelength measuring light and the chosen actinic light.**

PAR

The PAR indicated sums up incident PAR (PAR of the corresponding intensity as listed in the applied PAR list plus PAR resulting from the chosen measuring light frequency).

Time/10s

The Time-setting of each step can be set by typing units of 10 s (1 x 10 s up to 100000 x 10s) of each step.

Time/10s a **0** (zero) is entered, the **Light Curve is terminated** with the preceding step.

☑ Uniform time   The illumination period at each step is defined by Time/10s. When **"Uniform time"** is activated, the same time can be entered for all steps by typing a new value for one step and confirmation by mouse-click.

Light Curve recordings may be started repetitively with the help of the **Clock**-function (see 6.1). In this way, changes in the light adaptation state and the physiological health of a sample can be followed over longer periods of time.

☞ 💾   The **Light Curve Parameters** can be saved in **lcp-files** and reinstalled at any time by opening the corresponding file. This provides great flexibility in Light Curve recordings. The light curve files are stored within the report folder PhytoWin_3 folder sub-directory Data_US6, Data_ED6 or Data_CU6.

Default   Upon pressing the **Default** button, a standard list of intensity settings and a uniform illumination time of 20 s are installed.

✓ OK

✗ Cancel   After a previously defined list was changed, the changes can be confirmed by OK or cancelled by Cancel. In the latter case, the previously defined list of Light Curve parameters is maintained.

6.6.2 Light Curve Fit-parameters

Light curve data can be analyzed using the EP model (Eilers and Peeters (1988) Ecol Model 42: 199-215) or the Platt et al. model (Platt T, Gallegos CL, Harrison WG (1980) J Mar Res 38: 687-701).

 Rapid light curves plotting ETR/Yield versus PAR are often described by the following three cardinal points.

α (alpha), electrons/photons: Initial slope of RLC which is related to quantum efficiency of photosynthesis.

ETR_{max}, μmol electrons/(m^2 · s): Maximum electron transport rate.

IK, μmol photons/(m^2 · s): Transition PAR between light limited and light saturated conditions.

The EP function used by the software has been derived by Eilers and Peeters (1988) using a mechanistic model which considers the processes of photosynthesis and photoinhibition. Their final model function:

$$ETR = \frac{PAR}{a \cdot PAR^2 + b \cdot PAR + c}$$

In the above equation, the a, b, and c are free parameters which are varied by PhytoWin_3 until best fit between theory and data is achieved.

The cardinal points of a light curve are calculated according to subsequent equations:

$$\alpha = \frac{1}{c}$$

$$ETR_{max} = \frac{1}{b + 2 \cdot \sqrt{a \cdot c}}$$

$$I_K = \frac{c}{b + 2 \cdot \sqrt{a \cdot c}}$$

Fig. 18, illustrates the behavior of the Eilers and Peeters model function for 3 theoretical cases which show identical values of α but, at high light intensities, different degrees of photoinhibition.
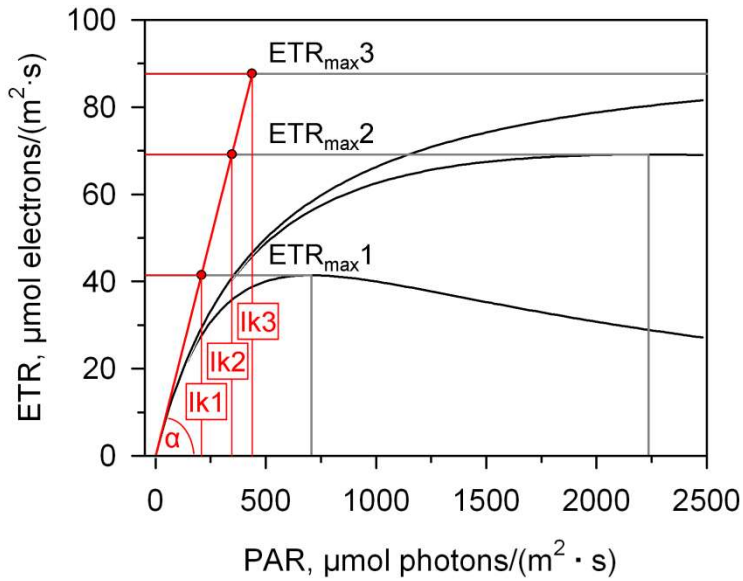
Curve	Parameter					
	a	b	c	α	ETRmax	Ik
1	$1 \cdot 10^{-5}$	$1 \cdot 10^{-2}$	5	0.2	41	207
2	$1 \cdot 10^{-6}$	$1 \cdot 10^{-2}$	5	0.2	69	345
3	$1 \cdot 10^{-7}$	$1 \cdot 10^{-2}$	5	0.2	88	438

Fig. 18:   Exemplary Light Curves.

Also, the Platt et al. (1980) model considers photoinhibition but their equation is not based on mechanistic considerations but on the empirical finding the many light response curves can be described by the product of 2 exponentials:

$$ETR = ETR_{mPot} \cdot (1 - e^{-\frac{\alpha \cdot PAR}{ETR_{mPot}}}) \cdot e^{-\frac{\beta \cdot PAR}{ETR_{mPot}}}$$

The free parameters in the latter equation are ETR_{mPot}, α and β, where the ETR_{mPot} is the asymptote value of the rising exponential in the equation above. For correct fitting it is important to give initial estimates for α and ETR_{mPot}.

The initial fitting values for α result directly from slope of the initial linear portion of the curve and ETR_{mPot} is given by the highest observed value of ETR.

The cardinal parameter β can be obtained with subsequent equation:

$$ETR_m = ETR_{mPot} \cdot (\frac{\alpha}{\alpha + \beta}) \cdot (\frac{\beta}{\alpha + \beta})^{\frac{\beta}{\alpha}}$$

$$I_K = \frac{ETR_m}{\alpha}$$

The model equation of Platt et al. (1980) considers photoinhibition by the "photoinhibition parameter" β. Platt et al. (1980) introduced the "Photoinhibition Index" (I_b) to quantify photoinhibition. The authors defined I_b as the PAR value required to photoinhibit ETR_{mPot} by the factor of 1/e according to:

$$I_b = ETR_{mPot}/\beta$$

Fit   The Fit button starts an iterative process during which the free parameters of the selected model equations (EP or Platt et al.) are varied until the best fit between theory and experiment is obtained. The fitted curves are displayed as continuous lines superimposed on the data points of the Yield- and ETR-Light Curves. Table 2 summarizes the units of these parameters.

Table 2: LC Fit Parameters.

		PAR
Fv/Fm x ETR fact or	Estimated alpha based on Fv/Fm.	
alpha	Initial slope of light curve.	$\dfrac{electrons}{quanta}$
ETRmax	Maximum electron transport rate	$\dfrac{\mu mol\ electrons}{m^2 \cdot s}$
I$_k$	Photon flux at which light-saturation of photosynthesis sets in.	$\dfrac{\mu mol\ quanta}{m^2 \cdot s}$

The original version of the model had to be modified to take account of the fact that some types of phytoplankton (particularly cyanobacteria) do not show maximal PS II quantum yield at PAR=0, as assumed by the model, but at significant levels of PAR (ca. 20-40 µmol quanta $m^{-2}s^{-1}$). Hence, there is an initial rise and peak of Yield, before the usual decline sets in at higher PAR values. This phenomenon is likely to reflect a state 2 - state 1 pigment change. The fitting routine applied by the PhytoWin_3 program ignores the data points in the rising part of the Yield Light Curve, including the peak value.

A speed button is provided for opening a window listing the **Light Curve Fit Parameters** for Ch1-Ch5 and for the deconvoluted types of phytoplankton (Blue, Green, Brown and PE-Type). The same window can be opened via the Main menu. The parameter α (**alpha**): reflects the maximal slope of the ETR Light Curve that, as outlined above, in phytoplankton samples is not necessarily observed close to PAR=0. The numerical value of α is equivalent to the maximal Yield multiplied by a PS II absorptivity term. If relative ETR is determined, a value of 0.42 is assumed for this term (ETR Factor multiplied by PS II distribution factor (PPS2/PPPS.), in numbers: 0.84 • 0.5) For measurement of absolute ETR the ETR Factor needs to be determined beforehand. The value can be entered under Options/ETR Parameters (see 6.12 and 6.10). **ETR_{max}** :represents maximal electron transport rate (relative units). I_k corresponds to the particular PAR-value at the crossing point of the lines defined by the initial slope (going through origin) and ETR_{max} (parallel to PAR-axis). It is calculated from the expression α/ETR_{max}. **I_k** is characteristic for onset of light saturation.

Fig. 19   Light Curve Fit Parameters

The quality of Light Curves for Blue, Green, Brown and PE-type, just like the deconvoluted values for fluorescence yield of Blue, Green, Brown and PE-type (see section 6.3), strongly depends on proper choice of Reference Spectra (see 6.7). Actually, in many cases the noise introduced by the fitting may prevent a satisfactory assessment of Light Curves for all four types of phytoplankton. This will be particularly true for a component at low content, when another type is dominating. The less reference spectra are used for fitting, the lower will be the fitting noise. Hence, if e.g. it is known from microscopic inspection that no or only few cyanobacteria are present, the Blue reference spectrum should be inactivated, to improve the results the other algae groups.

Exports fit results to csv-files (export folder of the corresponding data directory)

6.6.3 Comments on Light curves

While in green algae, just like in green leaves, maximal PS II quantum yield (Fv/Fm) is observed after dark adaptation (or adaptation to low measuring light frequency), this is not the case with other types of phytoplankton. Particularly in cyanobacteria dark adaptation leads to a state of low PS II

excitation and low PS II quantum yield (so-called state 2). In this case, maximal PS II quantum yield is induced at moderate light intensities.

Despite similarity of Rapid Light Curves with classical light response curves (P-I curves), there are also some basic differences, which should be kept in mind when evaluating Light Curves. In particular, the illumination time at each PAR-value generally is much shorter for Light Curves than for P-I-curves. At the default setting of 20 s not sufficient time is given for the sample to reach a light equilibrated state. Hence, such **Rapid Light Curves** (RLC) are expression of the momentary light adaptation state of a sample. For one and the same sample, there are as many different RLCs as there are different states of light adaptation. The user may convince himself about this fact by recording several consecutive RLCs starting with a dark-adapted sample. With increasing number of RLC-recordings, the ETR_{max} will increase: (unless there is photoinhibition). On the other hand, there should be only one P-I-curve for a given sample when illumination times are sufficiently long to allow full light adaptation at each intensity setting. In practice, however, it is not always possible to keep a collected sample physiologically healthy over longer periods of time (e.g. limitation by insufficient CO_2 supply must be avoided during such long-term light curves).

6.7   Report window

The "Report" documents and saves automatically all experimental data. A "Report" is organized in terms of separate "Records" with information on type of experiment, number of saturation pulse analyses, fast kinetics as well as the option to add comments. A report and its associated records are automatically saved to the report folder of the corresponding PHYTO-PAM-II measuring head. (e.g. C:\PhytoPam_DSP\ Data_CU6\Report).

In MEASURE mode, only the last record can be analyzed. In VIEW mode all Report/Record files are available for analysis.

Report ——— Record e.g. SAT-pulse measurements
         ——— Record e.g. light curve measurement
         ——— Record e.g. slow kinetics recordings
         ——— Record e.g. fast kinetics recordings
         ——— Record e.g. ...

Report:

📑 20151203_1.RPT3 📝

New reports can be started by clicking the New Report button in the settings window or by clicking the 📑 button in the report window.

report comments are entered in a text window which can be opened by clicking on the report comment icon 📝.

Record:

A new record can be manually defined with the help of the **New Record** button on the right-hand side of the elements for operation bar.

A light curve or slow kinetics recording automatically starts a new record. Also, upon program start and when returning to the MEASURE-mode from the VIEW-mode, automatically a new record is started.

It is recommended to enter a record Title/Comment at the start of each new record. This comment can be written in the head line available in the record view of the report window. This headline is also available above the graphs in the slow kinetics and light curve window. Additional information can be written in the text window becoming available by the 🗎 button.

🗎 20151221_1.RPT3 📊												🗎		
Please write a record title/comment in this headline.														
Date	Time	No.	Freq	Gain	Temp.	PAR	Y1	Y2	Y3	Y4	Y5		View	
22.12.15	11:48:10	Type:											○ Rpt ● Rec	
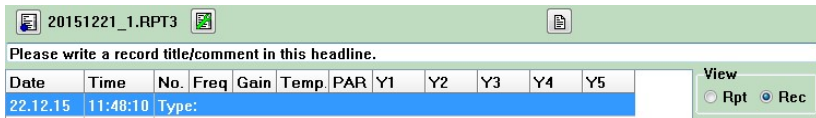
View ● Rpt ○ Rec   These radio buttons switch between report overview giving information about the records (Time, Type, numbers of saturation pulse analyses, numbers of fast kinetic measurements, number of chlorophyll concentration measurements, and the record comment/title). A closer view on the data can be obtained by selecting Rec (record view). Please note in MEASURE mode only the current record can be viewed. In the VIEW mode all records of the report are available for detailed information and analysis in the Rec view.

Select Column

☐ Fm'
☐ Fo'
☐ "Fo'
☑ Y(II)
☐ Y(NPQ)
☐ Y(NO)
☐ NPQ
☐ qN
☐ qP
☐ qL
☑ ETR

The record view (View Rec) provides detailed information on an individual experiment for the last record or in VIEW mode for all records. Minimum number of column headlines in the data field is 6 (Date, Time, No.,Temp, ML, PAR). Columns for Fo´ fluorescence and fluorescence ratio parameters can be added by selecting the item on the side bar by ticking checkmarks in "Select Column". The values of Fo and Fm are listed in the F and Fm' columns, respectively. They are distinguished from other data by column subtitles Fo and Fm.

☑ Settings

Activation of this checkbox will state settings in the record file. The abbreviations are G = Gain, MF = Measure Light Frequency, AI = actinic light intensity, AW = actinic light widths, FI = far red intensity, FW= far red widths, SI = saturation pulse intensity and SW = saturation pulse widths. Also setting changes within an experiment will be registered in the record file.

Open Report
Export Report
Print Report
Printer Setup

Exit

report-files can be analyzed at any later time in the VIEW-mode (see 6.11), which allows the user to try out different reference spectra for minimizing the fitting error etc. The **Open Report** and **Export Report** commands apply to the VIEW-mode only. In the VIEW-mode the report-file can be exported into other programs, like Excel or Sigma Plot for further analysis and different forms of data presentation. The exported report comprises values ticked with checkmarks in "Select Column" and will be saved as .csv file. If not the complete report but selected record files are supposed to be exported. The Export Record button ⯮ is used for export. The report-file can be printed out, given that a suitable serial printer is connected and has been defined by the Printer Setup routine.

Export Record button exports the selected record file as .csv file to the Export Folder of the corresponding measuring head. All values ticked with

checkmarks in "Select Column" will be exported.
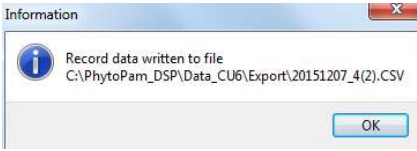
6.8   Reference window deconvolution and Chlorophyll determination calibration values

The primary signals measured by the PHYTO-PAM-II are five different fluorescence signals obtained by excitation of the sample with five measuring light beams at five different wavelengths (440, 480, 540, 590 and 625 nm). For the sake of **deconvolution**, these five wavelengths were chosen to give optimal differences in the excitation of the various antenna pigments that transfer excitation energy to the Chl a in PS II. Each of the four main pigment types of phytoplankton (cyanobacteria, green algae, diatoms/dinoflagellates and Phycoerythrin containing organisms) is characterized by a typical set of F-values at the five excitation wavelengths, which are called the **"Reference Spectra"** or more shortly **"References".** While they carry the information of five-point fluorescence excitation spectra, these "spectra" also are shaped by the intensities of the differently colored excitation beams that on purpose are <u>not</u> equal. The spectral composition of PHYTO-PAM-II instruments are standardized by spectral calibration using the fluorescence standard FluoRed and determined via the MEASURE PAR LIST routine when the instrument was put to operation. Thus, contrary to former versions of the PHYTO-PAM, PHYTO-PAM-II instruments provide standardized and calibrated light characteristics.

Depending on conditions the fluorescence excitation spectra for the fluorescence yield, F, and the saturation pulse induced increase, Fm-Fo, can show significant differences. Therefore, the instrument measures both **F and Fm-Fo** reference spectra and stores all relevant instrument specific information within the reference spectra. The instrument specific information allows normalization of the references with respect to the spectral composition of the PHYTO-PAM-II instrument. Therefore PHYTO-PAM-II reference spectra are universal and can be exchanged between instruments and users. Please find references on our Reference exchange Database: https://www.walz.com/products/chl_p700/phyto-pam/share_references.php

Additionally, to the fluorescence excitation characteristics, PHYTO-PAM-II references provide information about chlorophyll content biased on fluorescence yield. Therefor each reference spectrum includes chlorophyll calibration data which can be set during reference generation (see 6.8.1).
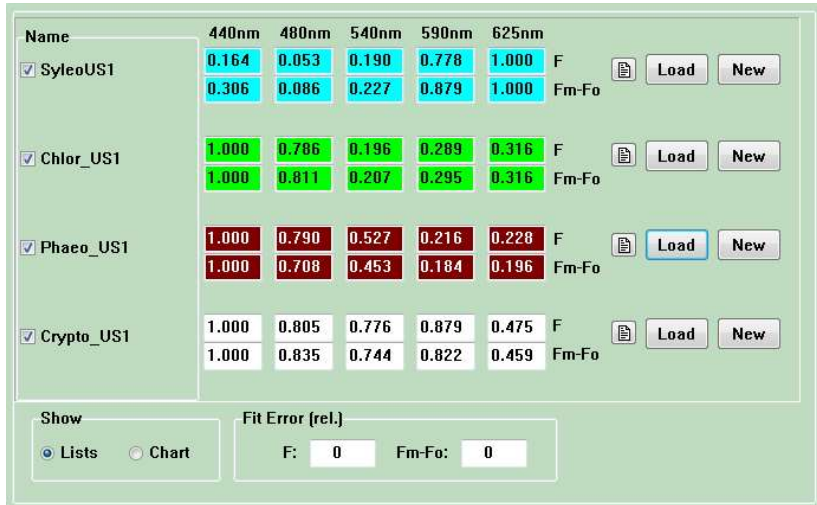
Fig. 20: Reference window Lists, numerical display of the five-point fluorescence excitation spectra

In the Reference window the references can be viewed either in the Lists view or the Chart view:

In the lists view the Reference window shows 2x5 Reference lines with the data boxes containing the numerical values of the normalized 5-channels fluorescence signals F and Fm-Fo (in different colors for each algae group: blue, green, brown and white).

The corresponding data points and connecting lines displayed in the chart view are blue, green, brown and black.
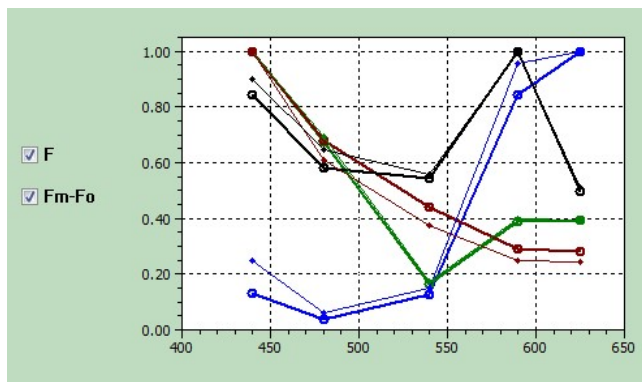
Fig. 21   Simultaneous display of F and Fm-Fo Reference Spectra in chart view

At the beginning of each reference line is a check box to activate or deactivate a particular reference. Only when activated, the spectrum of this reference is used for deconvolution. If it is known, that a given sample does not contain substantial amounts of a special type of phytoplankton, deconvolution of the other types of phytoplankton will be improved by deactivating the corresponding Reference.

Deconvolution consists in the fitting of the measured 5-channel signals (F and Fm-Fo separately) by the sum of fluorescence excitation characteristics given in the blue, green, brown and PE-type reference file. The relative contribution of each component to the 5-channel signals is determined and the sum of the squares of the deviations of the measured data points from the fitted values is minimized (least squares method).

Fit Error (rel.)

F: 10   Fm-Fo: 11

The quality of fitting with a given set of reference spectra can be judged by the **Fit Error**, which is shown for the fluorescence yield, Ft, and for the saturation pulse induced fluorescence increase, Fm-Fo. The Fit Error depends on the selection of references (via check-boxes) and on the choice of the particular reference spectra files. If it is known that a sample does not contain a particular type of phytoplankton (e.g. by microscopic inspection), it is better to inactivate the corresponding reference.

New

A **New Reference Spectra** can be measured via the **New**-button on the Reference window. Reference Spectra are measured with samples of pure cultures of phytoplankton and comprise the wavelength dependent fluorescence characteristics as well as the algae specific chlorophyll calibration. Instructions how to generate reference spectra are given in chapter 6.8.1.

Together with the reference a **Comment file** can be saved, in which the type of sample and the conditions of reference measurement are documented.

Load

Previously saved **References** can be installed in the corresponding reference line by clicking the **Load** button and selecting the file name in the Data-directory of the applied measuring head. Also references measured by other instruments can be copied to the Data-directory of the applied measuring head. Loaded references are normalized in respect to the spectral composition of the PHYTO-PAM-II instrument.

6.8.1 How to generate Reference Spectra

The quality of deconvolution bases on the quality of the applied reference spectra. Reference spectra are generated from pure cultures samples of algae. Data can be recalculated with new reference spectra at any time. Also, the reference spectra implemented chlorophyll concentration calibration can be corrected manually at any time after reference spectra generation.

- Make sure your cuvette is clean. If necessary, apply the Zoff-determination using filtrate of sample to exclude background fluorescence.

- Prepare 4 ml of pure sample.

- Set the measuring light frequency. Keep in mind that chlorophyll determination should be done at the same measuring light frequency as chlorophyll content calibration. Please set the measuring light frequency during reference spectrum generation to the frequency which will be used in following experiments.

- Apply auto-gain to define gain settings at which the measurement will be carried out.

- Click New to start reference spectrum generation.
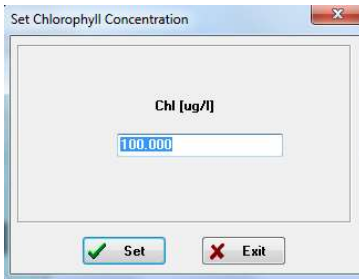
A prompt to set chlorophyll concentration appears. The chlorophyll concentration is predefined to 100 µg/l. The exact chlorophyll concentration used as calibration value for chlorophyll determinations can be edited manually e.g. after wet lab determination of the chlorophyll content of this reference sample.

- Finalize the reference spectrum by saving.

To edit the chlorophyll calibration at a later time, please click on the reference text button on the reference side. This first opens the reference text file followed by the Set Chlorophyll Concentration prompt.

6.9   Fluo Spec window

PHYTO-PAM-II References are universal. The Fluo Spec window shows raw data of the reference spectra and some of the spectral information used for normalization.
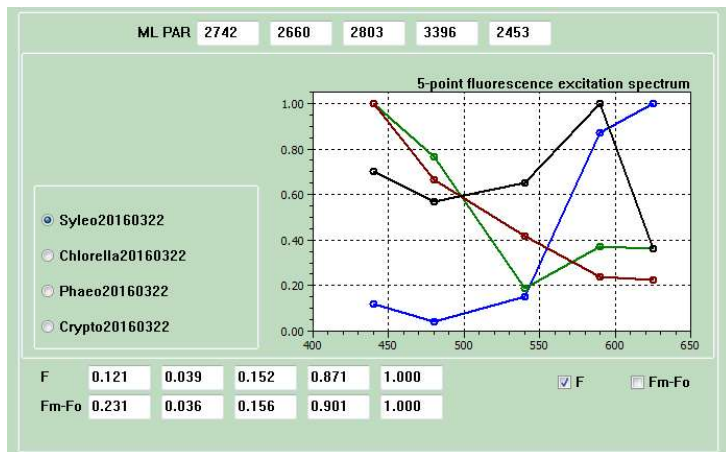
Fig. 22: Fluo Spec window displaying raw data of universal reference spectra

 The ML PAR displays the measuring light values of ActP 440 nm, 480 nm, 540 nm, 590 nm and 625 nm according to the internal light lists of the instrument which measured the reference spectrum.
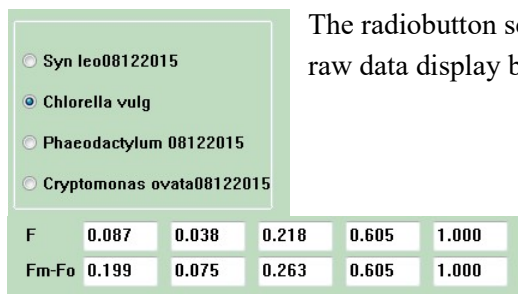
 The radiobutton selects the reference for F, Fm-Fo raw data display below.

6.10   Fast Kinetics - window

The Fast Kinetics window provides analysis of the wavelength depended O-I_1 fluorescence rise kinetics upon onset of pulses of strong actinic light. The obtained data can be fitted in the O-I_1 Fit window (chapter 6.10.1) for evaluation of the functional optical cross-section of PS II, Sigma(II). Important prerequisites for analysis of the fast kinetics are

    a) Low chlorophyll content (< 300 µg/L for 440 nm excitation) to avoid light gradients.

    b) Oxidized PS II acceptor-side (QA and PQ-pool) achieved by far-red background or far-red pre-illumination.

    c) Accurate determination of the O- and I_1-levels. Specifically, the O-level is assessed briefly before onset of actinic illumination and the I_1-level is the fluorescence level reached by a single turnover saturating flash (ST) at the end of the illumination period (depicted as dot).
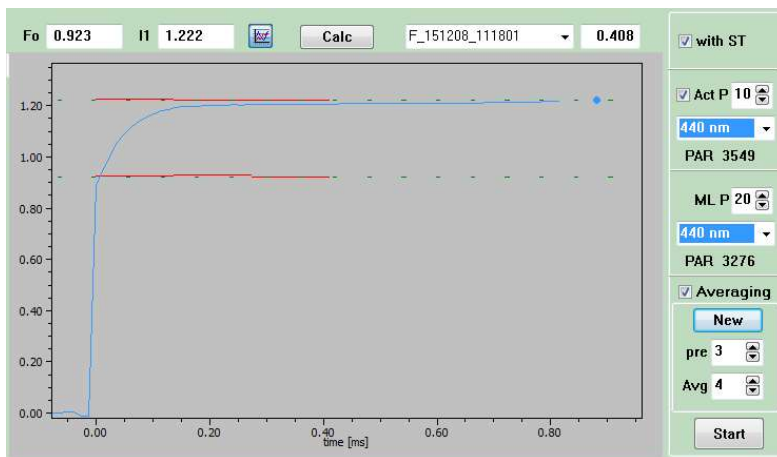
The Start button in the bottom right corner executes the fast kinetics light flashes and records the resulting fluorescence kinetics.

When ST is checked, all actinic LEDs contribute to a single turnover flash which then is particularly strong. Selection of a color for measuring light automatically sets actinic light to the same color. On the other hand, changing the actinic light color does not affect measuring light color so that all combinations of measuring and actinic colors are available. Via pulldown menu the intensity of ML and Act pulse can be defined. The corresponding PAR value is indicated.

Point averaging leads to reduction in noise on the cost of time resolution. Hence, point averaging is equivalent to signal damping. The number of averages can be defined by the pulldown menu Avg. All measured fast kinetics will be averaged and saved in one fast kinetic file. The pulldown menu pre-defines a series of fast kinetics before averaging is started e.g. to exclude "Fast Kinetic" recordings at non-steady-state conditions. The New button starts a new series of fast kinetic averaging.

The display of the fast kinetics graph can be changed. Zoom in by clicking with the left mouse key on one corner of the rectangular area to be magnified, keeping the key depressed move to the opposite corner of the rectangle, and release key. Zoom out by clicking the autoscale button above the chart.

Displays the values for Fo and I_1 of the fast kinetic recording
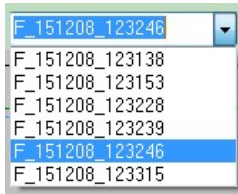
F_151208_123246 ▼ F_151208_123138 F_151208_123153 F_151208_123228 F_151208_123239 F_151208_123246 F_151208_123315	Pulldown menu to select a fast kinetics file for graphical display
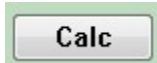
Calc	The Calc button provides access to the O-I$_1$ Fit window

6.10.1 O-I$_1$ Fit window

The O-I$_1$ Fit window serves for analysis of the O-I$_1$ rise kinetics by a special fitting routine. The routine was developed for determinations of the functional optical cross-section of PS II, Sigma(II), which is wavelength- and sample-specific. The routine is based on the reversible radical pair model of PS II (Schatz et al., 1988, Biophys J 54: 397-405; Lavergne and Trissl, 1995, Biophys J 68: 2474-2492) and takes into account reoxidation of the primary PS II acceptor QA as well as energy transfer between PS II units (connectivity).

The immediate result of an O-I$_1$ Fit is information on the time constant of the fluorescence rise. With information on the incident PAR driving this rise (saved in the PAR Lists), the functional absorption cross-section of PS II is calculated by the program.
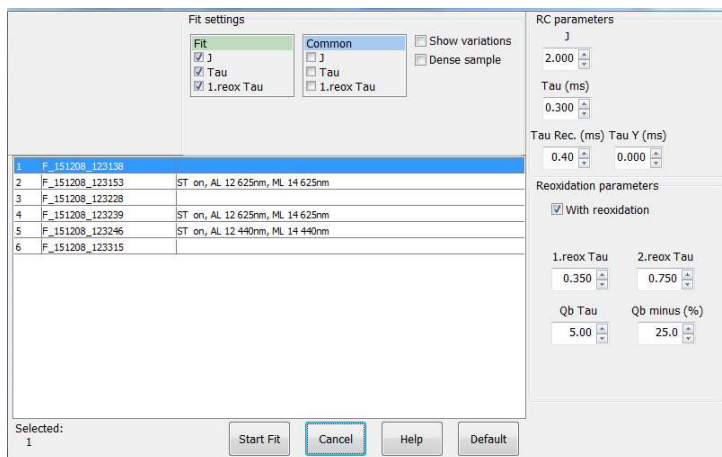
Knowledge of Sigma(II) permits transformation of incident PAR (in µmol quanta · m-2 · s-1) into the rate of quantum absorption per PS II complex, PAR(II) (in quanta · PS II-1 · s-1). Then, the rate of linear electron transport per PS II reaction center, ETR(II) (electrons · PS II-1 · s-1) can be estimated according to

ETR(II)=PAR(II) · Y(II)/(Fv/Fm)   where   Y(II)   and   Fv/Fm   are   the photochemical yields of PS II in the light-exposed and quasi-dark-acclimated state (i.e. with weak far-red background light), respectively.
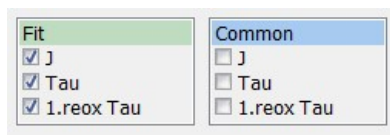
One kinetic file (Fit) or if more than one kinetic file is marked several fluorescence kinetics (Common) can be simultaneously analyzed by curve fitting. To this end, the kinetics of interest have to be marked in the data list of the O-I$_1$ fit window. Thereafter, the field "Common" is available. A parameter selected in the Common box will be identical for all marked curves. By assignment of "common" parameters the reliability of the fitting analysis can be improved, if it is unlikely that the enabled parameters vary significantly between measurements. For example, the rate of QA- reoxidation is not expected to be influenced by the color of light used for driving the O-I$_1$ rise.
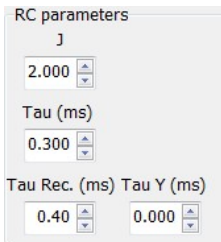
Hence, it makes sense to enable the rate constant "1.reox Tau" when fitting O-I_1 rise curves measured with various AL and MT colors

☐ Show variations

☐ Dense sample

Display in O-I_1 Fit table for all fitted parameters the +/- % signal variations required for +/- 10% changes in fit error. Note that small signal variations are indicative of a reliable fit and normally are written in green. When written in red, a parameter limit was reached during the fitting process. If you work with dense samples please activate the dense sample checkbox.

RC parameters

J

2.000

Tau (ms)

0.300

Tau Rec. (ms)   Tau Y (ms)

0.40   0.000

J = Initial value (at start of O-I_1 Fit) of the free parameter J.

Tau (ms) = time constant of QA- formation (QA reduction); initial value (at start of O-I_1 Fit) of free parameter Tau (ms).

Reoxidation parameters

☑ With reoxidation

1.reox Tau	2.reox Tau
0.350	0.750

Qb Tau	Qb minus (%)
5.00	25.0

Reoxidation of QA- formed during the O-I_1 rise is taken into account. "With reoxidation" should be disabled if QA- reoxidation cannot occur, e.g. in presence of the PS II inhibitor DCMU. 1. reox Tau (ms) = Time constant of QA- reoxidation by QB (first QA reoxidation time constant). The 1. reox Tau is varied during curve fitting.

Start Fit The button starts O-I_1 fitting. The results are compiled in the O-I_1 Fit data table. At the same time, original traces and fitted curves are shown in the fast kinetics window. If the experiment used a single turnover flash to elicit I_1 level fluorescence, the result is depicted as dot.

Cancel Cancels O-I_1 Fit and closes the calculation window

Default Sets all parameters to default values

6.10.2 Parameters and Output of Fo-I$_1$ Rise Analysis

Table 3:   Adopted from: Schreiber et al. (2012) Photosynth Res 113: 127-144

Parameters	Definition
Free model parameters	
J Unit: dimensionless	Sigmoidicity parameter. $J = p/(1-p)$, p is Joliot's connectivity parameter describing excitation energy flux from a PS II to neighboring PS II.
Tau Unit: s	Time constant of light-driven Q_A reduction.
Tau(reox) Unit: s	Time constant of Q_A^- reoxidation.
Data input	
Fo (or O) Unit: mV	Fluorescence in the dark-acclimated state. Derived from initial fluorescence level of $F_0 \rightarrow I_1$ rise.
I$_1$ Unit: mV	Maximum fluorescence elicited by a saturating single turnover flash. Derived from back-extrapolation of fluorescence decay after saturating single turnover flash.
PAR Unit: µmol quanta$\cdot$(m² $\cdot$ s)$^{-1}$	Photosynthetically active radiation.
Derived parameters	
k(II)=1/Tau Unit: s^{-1}	Rate constant of light-driven Q_A reduction
Sigma(II)$_\lambda$=k(II)$\cdot$(L$\cdot$PAR)$^{-1}$ Unit: nm²	Wavelength-dependent functional cross-section of PS II
PAR(II)=k(II)=Sigma(II)$_\lambda\cdot$L$\cdot$PAR Unit: quanta$\cdot$(PS II $\cdot$ s)$^{-1}$	Rate of quantum absorption of PS II
ETR(II)=PAR(II)$\cdot$Y(II)$\cdot$(F$_V$/F$_M$)$^{-1}$ Unit: electrons$\cdot$(PS II $\cdot$ s)$^{-1}$	Wavelength-dependent PS II-based electron transport rate
Constant	
L=6.022 $\cdot$ 10^{23} mol^{-1}	Avogadro constant

6.11   VIEW mode
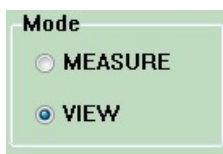
The PhytoWin software can be used in two modes, the **MEASURE mode** and the **VIEW mode**. While the MEASURE mode requires connection of the PC with the turned-on PHYTO-PAM-II, for the VIEW-mode only the PC is required. The MEASURE-mode is for data generation, the VIEW mode for Data inspection, recalculation or export.

Data generated in the MEASURE mode are automatically stored in report/record files. In the VIEW-mode these data can be viewed as originally recorded or modified e.g. by selection of varying reference files (see 6.8). In this way, all data can be analyzed on the basis of new information on the composition and properties of a particular sample.
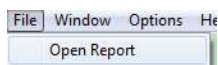
Reports can be opened via the Open Report command of the Main Menu (–File –Open Report) or via the Open Report button ▤underneath Select Record. The open report selection window (see below) displays all report folder on the left-hand side and windows for direct view of the report text (middle) and information about records-dates, -times, -types as well as the record comments (right hand side) for easy data assignment.
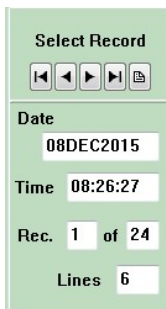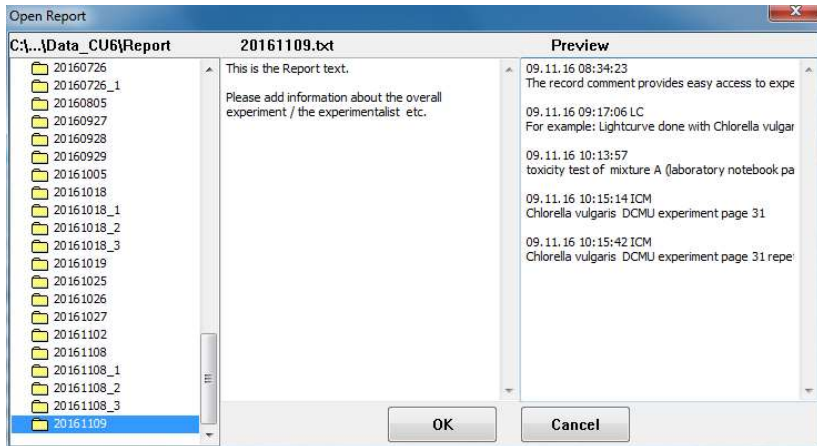
The opened report file is displayed on the report window. The report is organized into records, defined by Date and Time, referring to the moment at which a particular record was started in the MEASURE mode.

The arrows allow to jump to the first or the last record and to move forward and backwards by single steps in the records.
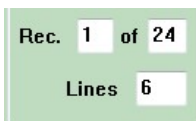
The records contained in a report-file are numbered. The number of the selected record (characterized by Date and Time) is shown, as well as the total number of records. Also, the number of "Lines" which relate either to a measurement involving the application of a saturation pulse or Chl determination is listed.

After selection of a record, the data of the corresponding measurement are selected to be viewed and analyzed in detail on the various display windows (**Channels, Algae, Slow Kinetics, Report, Light Curve, Reference, Fluo Spec or Fast Kinetics**). Upon start the first line of the corresponding record is displayed. The display can be altered by marking other lines in the record window. The user may visualize this aspect by selecting a record of interest and calling up the channels window. With each jump on the record display from one line to the next, there are corresponding changes in the data displayed on the Channels window. fast kinetics recordings can be selected via drop down menu in the fast kinetics window.

As in the MEASURE mode the selected references determine the deconvolution of the measured data. In the VIEW mode data can be recalculated recording to the applied references.
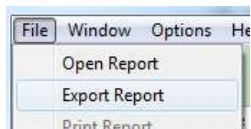
Data selected in the VIEW mode can be exported in form of a csv-file (comma separated values). In this form, the data can be further analyzed with any spread-sheet program. To export a complete Report file please use the Export report command in the Main Menu File, to export single record please use the **Export record** button in the report window.

If the instrument is properly connected, the user can switch anytime from the MEASURE- to the VIEW-mode and *vice versa*. Upon return to the MEASURE-mode a new record is opened.

6.12   Main Menu

The Main Menu bar contains pull down menus for File, Window, Options and Help.

File		Window		Options		Help
Open Report		Channels		L Curve Details ▶		✓ Tooltips
Export Report		Settings		L Curve Fit Parameters		Info
Print Report		Algae				Hotkeys
Printer Setup		Slow Kinetics		ETR Parameters		
Exit		Light Curve		AL-current/PAR List		
		Report				
		Reference				
		Fluo Spec				
		Fast Kinetics				
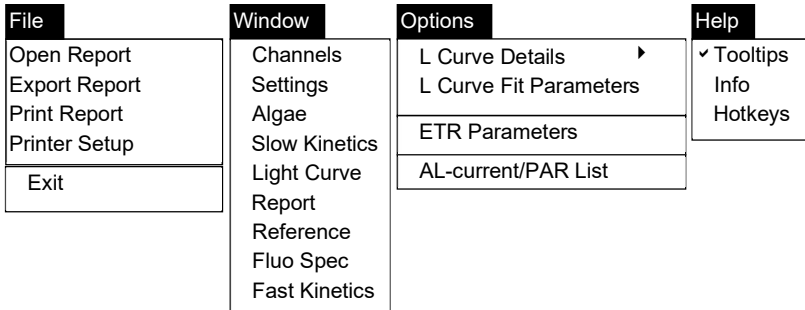
File:

- Open Report is only available in the VIEW mode

- Export Report is only available in the VIEW mode and exports the complete report to the export folder.

- Print Report function is available in the VIEW mode. Prints report data under given printer settings.

- Printer Setup to define printer settings

Window:

- Provides access to Channels-, Settings-, Algae-, Slow Kinetics-, Light Curve-, Report-, Reference-, Fluo Spec- or Fast Kinetics window.

Options:

- L Curve Details to select graphical display of light curves. Enables grid (Grid) or (Join) joining of data points with line segments.

- L Curve Parameter opens the light curve Fit Parameter window displaying the fit parameter α, ETRmax and IK of the Eilers and Peeters or Platt et al model fit. This window is also available via calculation button in the light curve window (more information see 6.6.2).

- ETR-Parameter opens the ETR-Parameter as outlined in chapter 6.12.1.

- Al current/PAR lists opens PAR list window where the actinic light current and resulting PAR values are listed. This window provides the function to save or load PAR lists.

Help:

- Activation of the tooltips checkbox gives access to short help messages. The tooltips appear moving the courser close to functional elements throughout PhytoWin_3 software.

- Info provides information of the used software version.

- Activation of the Hotkeys giving quick access to:

a = Algae window, c = Channels window, e = Report window, l = Light Curve window, r = Reference window, t = Settings window

s = triggers a SAT pulse measurement

Hotkeys giving access to windows are underlined in the window name, e.g. t = Settings

6.12.1 Options: ETR Parameter
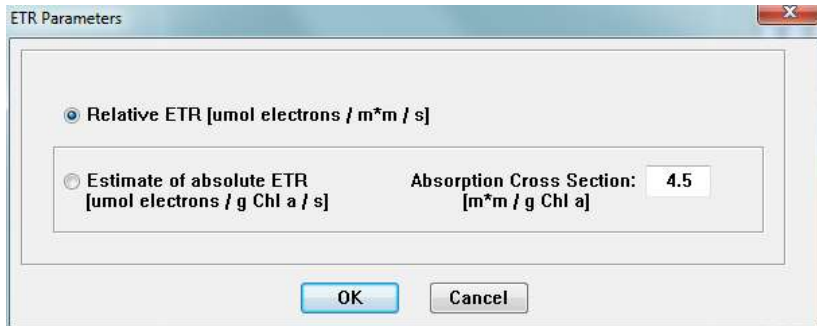
Relative ETR: When Relative ETR is selected, no attempt is made to estimate the absolute rate of photosynthetic electron transport, as no assumption is made on the absorption of the incident PAR. In the case of dilute phytoplankton suspensions, it is clear that only a minute fraction of the incident light is actually reaching the PS II reaction centers where primary charge separation takes place. Nevertheless, it is informative to compare values of relative ETR of various photosynthetic organisms at the same intensity of incident light. Many researchers are familiar with ETR measurements in higher plant leaves for which normally an absorptivity of 0.84 is assumed.

For the sake of comparison the same fictive absorptivity may be assumed for a phytoplankton suspension:

Relative ETR = Yield x PAR x P_{PS2}/P_{PPS} x ETR-Factor

P_{PS2}/P_{PPS}: Photons absorbed by PS II relative to photons absorbed by all photosynthetic pigments, default value for leaves is 0.5

ETR-Factor: ratio of photons absorbed by photosynthetic pigments to incident photons, default value 0.84

It is assumed that half of the quanta of the incident PAR are distributed to PS II, the quantum yield of charge separation of which is measured via fluorescence. Maximum relative ETR-values calculated in this way, range

from ca. 30 µmol electrons $m^{-2}s^{-1}$ with shade grown samples to ca. 150 µmol electrons $m^{-2}s^{-1}$ in the case of high light grown samples, irrespectively of whether phytoplankton suspensions or leaves (or any other photosynthetically active organisms, like corals, sea grasses or lichens) are studied.

Estimate of absolute ETR: In order to estimate absolute ETR-values, quantitative information on the absorption of the incident PAR is required. Such information can be obtained from measurements of the absorbance spectrum and the Actinic Light spectrum (see e.g. Gilbert et al 2000. J Plant Physiol 157: 307-314). Alternatively, the so-called PSII absorption cross-section can be determined from the light saturation curve of the fluorescence increase induced by a single turnover flash (Ley and Mauzerall 1982. Biochim Biophys Acta 680: 95-106; Dubinsky et al 1986. Plant Cell Physiol 27: 1335-1349). Under Options/ETR Parameters a value for Absorption Cross Section (in $m^2/gChl$) can be entered on which the estimate of absolute ETR is based. The default value of 4.5 $m^2/gChl$ was proposed by Nicklisch A and Köhler J (2001) Estimation of primary production with Phyto-PAM-fluorometry. Ann Report Inst Freshw Ecol Inland Fish Berlin 13: 47-60.

6.12.2 Al current/PAR list

PHYTO-PAM-II functions depend on accurate calibration of the LED array including the generation of a valid internal PAR list. This is done via automated routine of the MEASURE PAR LIST function on the settings side (see chapter 3.1). The Al current/PAR list menu in the menu bar opens the currently used PAR list in a separate window.
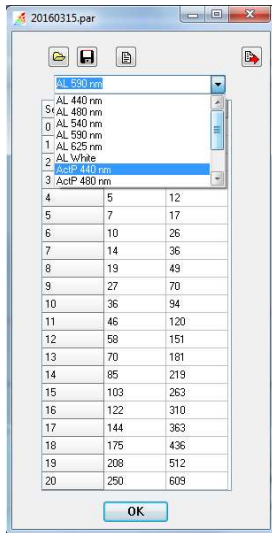
The pull-down menu determines which part of the overall PAR list is displayed. The PAR list contains a list for each actinic light color and ActP (actinic light pulses) of the fast kinetic mode. All PAR list consists of three columns: <Setting> is the setting number for the Actinic Light intensity, <Current> represents relative values for the LED current and <PAR> lists values of PAR in µmol photons/($m^2 \cdot s$).

Setting 0 resembles a characteristic of the corresponding measuring light color.
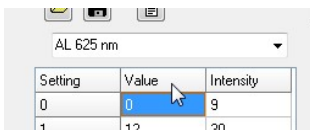
The LED currents are optimized for algal investigations which are most times adapted to lower light conditions. If the actinic light steps do not suit the requirements a double click on the column heading "Value" gives access to edit the current values (and therefore to the irradiance of each actinic light condition). The maximum current value is 500. **Changes of current values requires recalibration of the internal PAR List** via Measure Par List routine in the settings window – otherwise PAR values will be incorrect!

Button to open a PAR file

Button to save the PAR file

gives access to the PAR comment file to see or edit PAR list comment text.

Button to export PAR list as .csv file

6.13 Script files

Script files are used for automated execution of experimental procedures. Particularly, script files are advantageous when the same type of analysis needs to be repeated frequently and when complicated protocols must be exactly reproduced.

Generally, all manual operations in the Phyto-Win software can also be carried out under the control of a Script file. In addition, Script files offer commands for time management, combinations of sub-programs and conditional commands.

To access the script file window, click on the $\boxed{\text{Load}}$ button on the bottom bar of Phyto-Win. To execute script files, click on the $\boxed{\text{Run}}$ button below.
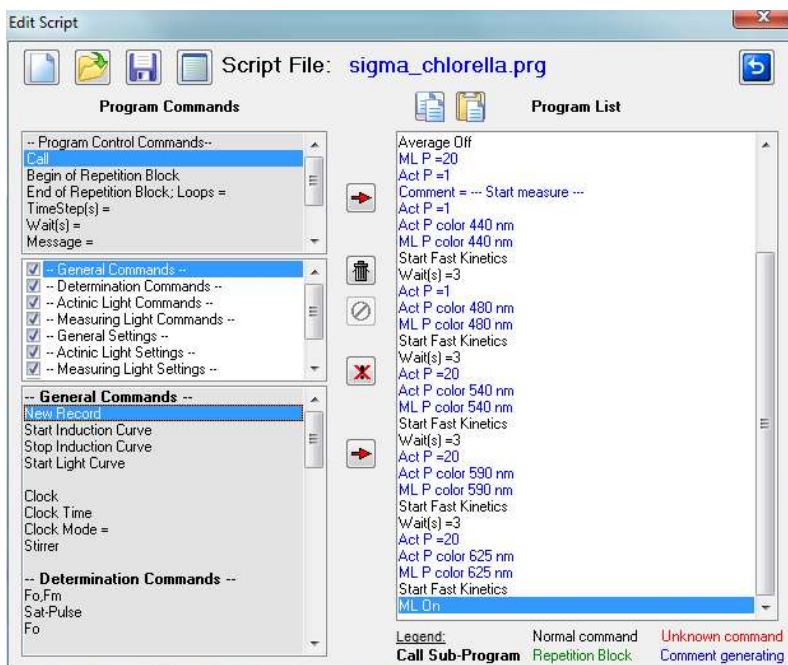
Fig. 23: Script file window

6.13.1 Data Management

Four commands are provided for script file management:

New Script File   Clears the script file window and prompts for a new script file name.

Open Script File Opens a script file with name format "filename.PRG". The default directory for script files is C:\PhytoPam_DSP\Script Files. No other directories can be selected.

Save Script File   Saves to C:\PhytoPam_DSP\Script Files.

Script File Comment Displays a text window for notes on the current script file. The content of the window is saved as text file with name "filename01.TXT" which is associated with the script file "filename01.PRG".

6.13.2 Editing Tools

Copy   The command stores one or several lines of the current script file in the clipboard. To execute the copy command, select one or several lines using the mouse cursor (Left-click once to pick one line. Hold down Shift key and select first and last line of a series of script file commands. Hold down Ctrl key to select several scattered lines.) Click Copy icon. The selected commands are now available for pasting within the current or into another script file using the Paste command.

Paste   To paste previously copied commands, select a line in the target script file and click the Paste icon. The pasted lines will be written below the selected line.

Insert   Generally, "Insert" icons transfer commands from the command box to the script file window. The upper insert icon transfers commands from the box "Program Control Commands", the lower icon transfers commands from the "General Commands" box. Double click on the command to be transferred is equivalent to the insert icons.

To insert a new command in the program list:

- Select the command to be inserted by mouse cursor in the command box - Select by mouse cursor in the script file window (right text window of the line below which the command shall be inserted

- Click Insert button.

Delete   Select one or several commands to be deleted (see above) and click icon. The command is equivalent to pressing "delete" on the keyboard.

Undo Delete   Reverses the last delete action.

Disable/Enable   Disables command lines of the current script file, or enables previously disabled command lines. Disabled lines are printed in grey. To execute the command, select line(s) in script file window and click icon.

BACK   Closes script file window. In case that a sub-program is displayed, the Back button returns to the script file which calls the sub-program.

6.13.3 List of Script File Commands

The list of script file commands are divided into three sections. The upper one contains commands controlling the progress of script files (Program Control Commands). The middle panel contains the title of nine groups of commands which are listed in the bottom panel. Unchecking titles in the middle panels hides the corresponding group of commands in the bottom panel.

Program Control Commands:

Parameter	Command, Comment
Call	Executes another Phyto-Win script file as a sub-program. A sub-program can be displayed by double-clicking an existing calling line. Use the Back button (↺) to return to main script file. Sub-programs may call further sub-programs.
Begin of Repetition Block	Marks the beginning of a series of commands (repetition block) which is to be repeated.
End of Repetition Block; Loops =	Marks the end of a repetition block. A repetition block may contain other repetition blocks. Repetitions can also be terminated depending on the levels of Ft or temperature (see section "Condition Commands" at the end of this list).
TimeStep (s) =	Defines the time interval between the beginnings of two consecutive events; if the TimeStep command is not preceded by an event, the time interval starts with script file execution. A series of time steps forms a time scale along which actions can be performed with defined time pattern.
Wait(s)	Defines the time interval after the last command has been completed and next command is carried out. Wait steps can be placed inside of a TimeStep interval provided that the total

	time required for command execution plus wait steps is shorter than the time step interval.
Message =	Halt script execution and display a message. Clicking OK on the message window or hitting the Enter key continues execution of the script file.
Comment =	Inserts a comment line in the script file to add notes to the script.
Paste to Record Comment Line	Inserts a comment to the Record Comment Line for labeling the experiment.
Spacer	Inserts an empty line in the script after the currently selected line (to visually subdivide scripts)
Exit	Terminates and exits script files.

General Commands:

Parameter	Command, Comment
New Record	Start a new record.
Load Par File=	Loads a PAR file. Please enter the filename exactly and without file extension. (E.g. for PAR file 20190628_lowlight.par enter: 20190628_lowlight)
Load Lcp File=	Loads a light curve parameter file (chapter 6.6.1). Please enter the filename exactly and without file extension. (E.g. for Routine1.lcp enter: Routine1. Please note that the actinic light color is not set within the lcp file but needs to be set separately (Actinic Light Settings)
Load Reference1=	Loads a reference file to the first reference position (marked blue). Please enter the filename exactly and without file extension.

Load Reference2=	Loads a reference file to the second reference position (marked green). Please enter the filename exactly and without file extension.
Load Reference3=	Loads a reference file to the third reference position (marked brown). Please enter the filename exactly and without file extension.
Load Reference4=	Loads a reference file to the forth reference position (marked white). Please enter the filename exactly and without file extension.
Export Record to csv	Exports current record as displayed (Channel or Algae view/ all marked parameters) to the Export folder
Export Report to csv	Exports the complete Report as displayed (Channel or Algae view/ all marked parameters) to the Export folder
Start Slow Kinetic	Start recording of a slow kinetic: equivalent to the "Start" button on slow kinetics window.
Stop Slow Kinetic	Stop recording of a slow kinetic: equivalent to the "Stop" button on slow kinetics window.
Start Light Curve	Start recording of a light curve: equivalent to the "Start" button on the light curve window.
Clock	Start or stop the repetitive trigger.
Clock Time	Sets or modifies clock interval.
Clock Mode=	Selects the action to be triggered by the clock.
Stirrer	modular version only: Switches stirrer on and off. Stirring speed can be adjusted on the front plate of the control unit.

Determination Commands:

Parameter	Command, Comment
Fo,Fm	Determines Fo and Fm level fluorescence and calculates Fv/Fm.
Sat-Pulse	Performs saturation pulse analysis
Fo	Determines Fo level fluorescence
Chl	Executes Chlorophyll content measurement

Actinic Light Commands:

Parameter	Command, Comment
AL	Switches actinic light on/off.
FR	Switches far red light on/off.
ST	Triggers a single turnover flash.

Measuring Light Commands:

Parameter	Command, Comment
ML	Switches measuring lights on/off

General Settings:

Parameter	Command, Comment
Auto Gain	Executes the Auto Gain function for the automatic gain adjustment
Set Gain	Sets or modifies gain (1 to 30). Gain setting (= #). Increment for decrease (- #) or increase (+ #)

Keep Fo,Fm	Off: Fo and Fm values at start of each light curve are determined. On: Uses the previous Fo and Fm values for all subsequent saturation pulse analyses. No Fo,Fm is determined at the start of the light curve

Actinic Light Settings:

Parameter	Command, Comment
AL Color=	Selects actinic light color.
AL Int	Intensity setting of actinic light. Intensity setting (= #). Increment for decrease (- #) or increase (+ #)
FR Int	Intensity setting of far red light. Intensity setting (= #). Increment for decrease (- #) or increase (+ #)
SP Int	Intensity setting of saturation pulse. Intensity setting (= #). Increment for decrease (- #) or increase (+ #)
AL Width	Illumination time interval for actinic light
FR Width	Illumination time interval for far red light
SP Width	Duration of the saturation pulse

Measuring Light Settings:

Parameter	Command, Comment
ML Freq	Sets frequency of measuring light

Fast Kinetics:

Parameter	Command, Comment
Start Fast kinetics	Starts recording of a fast kinetics. Equivalent to the "Start" button on the fast kinetics window

New Average	Starts recording of a fast kinetics average series
With ST	fast kinetics recording includes single turn over flash
With AL	fast kinetics recording includes actinic light illumination
Average precycles =	Set number of fast kinetics precycles
Curves to Average	Defines the numbers of fast kinetic recordings to average
Average	Fast Kinetic recordings are averaged
ActP	Set Intensity of ActP
ActP color	Set color of ActP
ML P	Set Intensity of measuring light pulse
ML color	Set color of measuring light

7   Technical Specifications

Subject to change without prior notice

7.1   PHYTO-PAM-II Compact Version

7.1.1 PHYTO-PAM-II/ED

General design: Metal housing for PHYTO-PAM-II Power-and-Control-Unit including all opto-electronic components as well as the measuring chamber for 15 mm Ø quartz cuvette WATER-K.

Chip-on-board multi-wavelength measuring light LED emitter: 440, 480, 540, 590, and 625 nm for pulse-modulated measuring light; 2 intensity settings; 8 settings of pulse frequency and 3 settings of auto MF-high pulse frequency

Chip-on-board multi-wavelength actinic LED array: 440, 480, 540, 590, 625 and 420-640 nm (white) for continuous actinic illumination, up to 1400 µmol m^{-2} s^{-1} PAR; fast kinetic flashes up to 7000 µmol m^{-2} s^{-1} PAR; saturation pulse up to 5000 µmol m^{-2} s^{-1} PAR,

Far-Red LED: peak wavelength 725nm

Signal detection: Photomultiplier detector based on Photosensor Module H-10720 (Hamamatsu)

Standard detector filter: long-pass filter > 650 nm

Sockets: charge socket for Battery Charger MINI-PAM/L, input socket for US-SQS/WB Spherical Micro Quantum Sensor, USB socket

Communication: USB 1.1, USB 2.0 and USB 3.0 compatible

User interface: Windows computer with PhytoWin_3 software

Power supply: Rechargeable sealed lead-acid battery 12 V/2 Ah; Battery Charger MINI-PAM/L (100 to 240 V AC)

Dimensions: 29 cm x 30 cm x 20.5 cm (l x w x h), aluminum housing with carrying handle and cuvette cover

Power consumption: Basic operation 1.3 W, ML +AL at maximum output 3.7 W. Saturation Pulse at maximum intensity, 6.2 W

Weight: 4.42 kg (including battery)

Operating temperature: -5 to +40 °C

7.1.2 Battery Charger MINI-PAM/L

Input: 90 to 264 V AC, 47 to 63 Hz

Output: 19 V DC, 3.7 A

Operating temperature: 0 to 40 °C

Dimensions: 15 cm x 6 cm x 3 cm (l x w x h)

Weight: 300 g

7.1.3 Spherical Micro Quantum Sensor US-SQS/WB

Design: 3.7 mm diffusing Plexiglass sphere coupled to integrated PAR-sensor via 2 mm fiber, compact amplifier unit and special holder for mounting on sample cuvette;

Connects to: PHYTO-PAM-II LIGHT SENSOR connector

Cable length: 3 m + 0.5 m

Size: Sensor: diameter 1 cm, length: 11 cm; 15.2 mm spacer ring for light sensor positioning; Hood: 3.4 cm diameter, 3.2 cm height; Amplifier: 5 x 5 x 5 cm (w x l x h),

Weight: 175 g

7.1.4 Transport Box PHYTO-T

Design: Aluminum box with custom foam packing for PHYTO-PAM-II and accessories

Dimensions: 60 cm x 40 cm x 34 cm (l x w x h)

Weight: 5 kg

7.1.5 Accessory Stirring Device WATER-S

Design: Miniature stirring motor in plastic housing with adapter to mount on top of the PHYTO-PAM-II cuvette; equipped with disposable perspex stirring paddle; self-contained unit featuring long-life 3 V Lithium Battery; potentiometer for adjustment of stirring rate

Dimensions: 80 mm x 50 mm x 30 mm (l x w x h)

Weight: 95 g (incl. battery)

7.2   PHYTO-PAM-II MODULAR Version

7.2.1 Power-and-Control-Unit PHYTO-II-C

Sockets: CHARGE socket for Battery Charger MINI-PAM/L, AUX socket for US-SQS/WB Spherical Micro Quantum Sensor, USB socket, socket for Miniature Magnetic Stirrer PHYTO-MS, DETECTOR socket for connecting PHYTO-II-D Photomultiplier-Detector Unit, LED ARRAY and FLUO ML sockets for connecting PHYTO-II-E Multi-Color-Emitter Unit

User interface: Windows computer with PhytoWin-3 software

Communication: USB 2.0 and USB 3.0 compatible

Power supply: Rechargeable sealed lead-acid battery 12 V/2 Ah; Battery Charger MINI-PAM/L (100 to 240 V AC)

Dimensions: 29 cm x 30 cm x 18 cm (l x w x h), aluminum housing with carrying handle

Power consumption: Basic operation, 1.5 W; with Measuring and Actinic Light at maximal currents, 4.5 W. Saturation Pulse at maximum intensity, 7 W

Weight: 4.2 kg (including battery)

Operating temperature: -5 to +40 °C

7.2.2 Multi-Color-Emitter Unit PHYTO-II-E

Chip-on-board multi-wavelength measuring light LED emitter: 440, 480, 540, 590 and 625 nm for pulse-modulated measuring light; 2 intensity settings; 8 settings of pulse frequency and 3 settings of Auto MF-high pulse frequency

Chip-on-board multi-wavelength actinic LED array: 440, 480, 540, 590, 625 and 420-640 nm (white) for continuous actinic illumination, up to 3000 μmol m-2 s-1 PAR; actinic pulses for Fast Kinetics, up to 11000 μmol m-2 s-1 PAR; saturation pulses up to 12000 μmol m-2 s-1 PAR,

Far-Red LED: peak wavelength 725nm

Dimension: 6.6 cm x 10.6 cm x 10 cm (l x w x h)

Weight: 490 g

7.2.3 Photomultiplier-Detector Unit PHYTO-II-D

Signal detection: Photomultiplier detector based on Photosensor Module H-10720 (Hamamatsu)

Standard detector filter: long-pass filter > 650 nm

Weight: 510 g

Dimension: 5.3 cm x 11 cm x 7.1 cm (l x w x h)

7.2.4 Battery Charger MINI-PAM/L

Input: 90 to 264 V AC, 47 to 63 Hz

Output: 19 V DC, 3.7 A

Operating temperature: 0 to 40 °C

Dimensions: 15 cm x 6 cm x 3 cm (l x w x h)

Weight: 300 g

7.2.5 Spherical Micro Quantum Sensor US-SQS/WB

Design: 3.7 mm diffusing Plexiglass sphere coupled to integrated PAR-sensor via 2 mm fiber, compact amplifier unit and special holder for mounting on sample cuvette;

Connects to: PHYTO-PAM-II AUX connector

Cable length: 3 m + 0.5 m

Size: Sensor: diameter 1 cm, length: 11 cm; 19 mm spacer ring for light sensor positioning; Hood: 3.4 cm diameter, 3.2 cm height; Amplifier: 5 x 5 x 5 cm (w x l x h),

Weight: 175 g

7.2.6 Transport Box PHYTO-T

Design: Aluminum box with custom foam packing for PHYTO-PAM-II and accessories

Dimensions: 60 cm x 40 cm x 34 cm (l x w x h)

Weight: 6 kg

7.2.7 Temperature Control Unit US-T

Power-and-Control Unit US-T/DR

Display: Three-line LCD display

Control range: 0 °C to 50 °C at 0.1 K steps

Operating voltage: 11 V - 14 V DC

Maximum Peltier current: 1 A

Size: 10.5 cm x 13 cm x 9 cm (l x w x h)

Weight: 570 g

Peltier Heat-Transfer Head US-T/DS

Achievable temperatures: 12 K below ambient temperature, 15 K above ambient temperature (Quartz cuvette placed in Optical Unit for Suspensions ED-101US/MD with 1.5 ml water and stirrer PHYTO-MS on)

Size: diameter 55 mm, 110 mm height

Cable length: 130 cm

Weight: 290 g (including cable)

AC Adapter

Input: 100 V - 240 V AC 1.5 A 50-60 Hz

Output: 12 V DC 5.5 A

Size: 130 mm x 56 mm x 30 mm (L x W x H)

Weight: 500 g (including cable)

7.2.8 Miniature Magnetic Stirrer PHYTO-MS

Design: Based on rotating magnetic field; connecting to Power-and-Control Unit PHYTO-II-C; with special adapter plug to be mounted in bottom port of Optical Unit ED-101US/MP

Weight: 16 g

7.3   System Control and Data Acquisition

Software: PhytoWin_3 System Control and Data Acquisition Program (Windows XP/Vista, Windows 7 , 8 and 10) for operation of measuring system via PC. Advanced multi-wavelength analysis of Chl fluorescence in suspensions down to 0.5 µg/l, including measurements of slow and fast induction kinetics (Kautsky effect, O-I1 fluorescence rise), deconvolution of major pigment types of phytoplankton (green algae, diatoms, cryptophytes, cyanobacteria). Higher sensitivity (e.g. 0.1 µg/l) can be obtained by signal averaging.

Saturation Pulse Analysis: <u>Measured:</u> Ft, Fo, Fm, F, Fo' (also calculated), Fm'. Fast polyphasic rise kinetics (time resolution up to 13 µs). PAR using Spherical Micro Quantum Sensor US-SQS/WB. <u>Calculated:</u> Fo' (also measured), Fv/Fm, Y(II), q_L, q_P, q_N, NPQ, Y(NPQ), Y(NO) and ETR,

Fitting Routines: Fitting routine for fast fluorescence rise from 0 to the I_1 level to determine functional absorption cross-section of PS II. Fitting routine for determination of the cardinal points α, I_k and ETR_{max} of light curves.

Computer Requirements: Processor, 0.8 GHz. RAM, 512 MB. Screen resolution, 1024 x 600 pixels. Interface, USB 2.0/3.0. Operating system: Microsoft Windows Windows 7, 32 and 64 bit, Windows 10

Technical specifications are subject to change without prior notice.

8   Rechargeable battery

The Phytoplankton Analyzer PHYTO-PAM-II is equipped with a rechargeable sealed-lead acid battery.

The life time is 1-3 years and it depends on the specific application. A 10 °C rise of the temperature will decrease battery life by approx. 25%. Near the end-of-life the standby capacity of the battery will be reduced. When this reduction becomes persistently, please replace the battery.

The battery cannot be overcharged, when the battery charger supplied with the instrument is used! Do not use any other battery charger!

Never store the instrument with a discharged or partially discharged battery! It is recommended to charge the battery every three months during the storage period.

- **For optimum performance always recharge the battery immediately after discharging!**
- **Never leave the battery in a discharged stage!**
- **Never short-circuit the battery terminals!**

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11 Manufacturer's Guarantee

Under this Manufacturer's Guarantee ("Guarantee"), subject to the Conditions and Instructions below, Heinz Walz GmbH, Germany ("Manufacturer"), guarantees (§443 BGB) to the end customer and user ("Customer") that all products supplied by it shall substantially conform in material respects to the Specifications for 24 months from the delivery date (date on invoice). In this Guarantee, "Specifications" means the product's features (as may be amended by Manufacturer from time to time), which are set out under the headings "specifications" and/or "technical specifications" within the product's respective brochure, data sheet, or respective tab on the Manufacturer's website for such product, and which may be included with the documents for the product when delivered. In case of an eligible guarantee claim, this Guarantee entitles the Customer to repair or replacement, at the Manufacturer's option, and this Guarantee does not include any other rights or remedies.

11.1   Conditions

This Guarantee shall not apply to:

-    Any defects or damage directly or indirectly caused by or resulting from the use of unauthorized replacement parts and/or service performed by unauthorized personnel.
-    Any product supplied by the Heinz Walz GmbH, Germany which has been subjected to misuse, abuse, abnormal use, negligence, alteration or accident.
-    Damage caused from improper packaging during shipment or any acts of God.
-    Batteries, cables, calibrations, fiberoptics, fuses, gas filters, lamps, thermocouples, and underwater cables.
-    Defects that could reasonably have been detected upon inspection of the product when received by the Customer and not promptly noticed within ten (10) days to Heinz Walz GmbH.

- Submersible parts of the DIVING-PAM or the underwater version of the MONITORING-PAM have been tested to be watertight down to the maximum operating depth indicated in the respective manual. Guarantee shall not apply for diving depths exceeding the maximum operating depth. Further, guarantee shall not apply for damage resulting from improper operation of devices, in particular, the failure to properly seal ports or sockets.

11.2   Instructions

- To obtain guarantee service, please follow the instructions below:
- The Walz Service Information Form available at http://www.walz.com/support/repair_service.html must be completed and returned to Heinz Walz GmbH, Germany.
- The product must be returned to Heinz Walz GmbH, Germany, within 30 days after Heinz Walz GmbH, Germany has received written notice of the defect. Postage, insurance, and/or shipping costs incurred in returning equipment for guarantee service are at customer expense. Duty and taxes are covered by Walz.
- All products being returned for guarantee service must be carefully packed and sent freight prepaid.
- Heinz Walz GmbH, Germany is not responsible or liable for missing components or damage to the unit caused by handling during shipping. All claims or damage should be directed to the shipping carrier.

11.3   Applicable law

This Guarantee is governed by German law. Place of jurisdiction is Bamberg, Germany.