Dual-Channel PHOTOSYNTHESIS YIELD ANALYZER ToxY-PAM

Handbook of Operation

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Instructions for use (short version) for ToxY-PAM

A. Stand-alone operation

1) Switch device on using the ON-key
2) Place cuvette with investigated sample in Channel 1 (S) and cuvette with reference sample in Channel 2 (R).
3) Start a measurement using the START-key.
4) After c 10 s the resulting Inhibition % is displayed.
5) Upon signal saturation (values > 4000) the result is not valid. The individual fluorescence signals (F1, F2 and Fm1, Fm2) can be called on display using the arrow keys (press the ∧-key twice).
6) Generally, optimal results are obtained when F1 and F2 show values of 1000-1500. The chlorophyll concentration has to be adjusted accordingly.

B. PC-operation

1) Connect ToxY-PAM with the PC using the provided RS-232 cable.
2) The ToxyWin software should be installed on the PC (see Handbook).
3) Upon start of Toxy-exe, the instrument automatically is switched on.
4) Place cuvette with investigated sample in Channel 1 (S) and cuvette with reference sample in Channel 2 (R).
5) The fluorescence signal (F1) is displayed on the monitor screen (green box in upper left corner). By clicking the Channel 2-box also F2 can be displayed. The two fluorescence signals should not exceed 1500. Otherwise

signal saturation may occur during measurements. Chlorophyll content accordingly has to be adjusted.

6) A single measurement is started using the Start-key. Using Start-keys on the instrument and on the screen (in Measure-box, using left mouse key) is equivalent.

7) A series of repetitive measurements can be carried out using the Clock-function (Start-key in the Clock-box).

8) The data automatically are written into the Report-file. Before leaving the program the file has to be saved (Save Report-icon).

C. Instrument related requirements for optimal results

1) In most applications, optimal results are obtained using the Standard Settings, which can be installed via Reset Settings (Menu-point 10 of ToxY-PAM or corresponding icon under Settings on the screen).

2) The Zero Offset values (Menu-point 4 and under Settings) should correspond to the preset values (see sticker on the side of instrument) or should be carefully redetermined.

3) The value of Ch2-Adjustment (Menu-point 6 and under Settings) should correspond to the preset value (see sticker on the side of instrument) or should be carefully redetermined.

4) The value of Inh.-Correction (Menu-point 16 and under Settings) should correspond to the preset value (see sticker on bottom side of instrument) or should be carefully redetermined.

5) Battery voltage should not be less than 11 V. At lower values there will be a warning (BAT).

D. Sample related requirements for optimal results

1) The biological material should display high photosynthetic activity. Under Standard Settings Yield-values of 0.5-0.6 should be measured.

2) Algae suspensions should not display clumping or rapid settling, which would give rise to errors (sample noise).

3) The investigated water should not contain particulate matter, as this would cause noise and also induce a lowering of effective light intensity in Channel 1. Particulate matter should be removed by filtration.

4) If the investigated water contains fluorescent compounds, Zero Offset determination has to be carried out with this water in Channel 1. Otherwise inhibition would be simulated (Y1 lowered relative to Y2).

5) The investigated water should not contain compounds which cause significant absorption of the blue measuring light. Otherwise negative values of apparent inhibition will result (Y1 increased relative to Y2).

6) With algae suspensions, the osmolarity of the investigated water should not differ significantly from that of the normal suspension medium. Otherwise selective changes of activity may occur in Channel 1, which would lead to corresponding changes of Inhibition %.

7) The cuvettes regularly should be washed with alcohol in order to remove all material sticking to the walls, including potential inhibitors.

8) The outer walls of the cuvettes must be clean when placed into the ToxY-PAM.

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 sufficient 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. Ensure that no liquids or other foreign bodies can find their way inside the device.
10. The device should only be repaired by qualified personnel.

1.2 Special safety instructions

The ToxY-PAM 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 in order to avoid potential harm to the user and damage to the instrument.

2 General Information

The ToxY-PAM Dual-Channel-Yield-Analyzer is a specialized chlorophyll fluorometer for the assessment of small amounts of toxic substances in water samples using standardized photosynthetically active samples like dilute suspensions of unicellular algae, protoplasts, isolated chloroplasts, thylakoids and thylakoid membrane fractions. The particular choice of the photosynthetically active material depends on the type of toxic substances which are going to be detected and quantified. In the case of atrazin/diuron type herbicides, which act directly on photosystem II (PS II) reaction centers, thylakoids and PS II particles are well suited, with the advantage that this material can be stored in the frozen or freeze-dried state. On the other hand, when toxic substances are of interest which act on dark-enzymic steps of the photosynthetic process, such as Calvin cycle and photophosphorylation, freshly prepared chloroplasts, protoplasts or intact algae cells should be used. Only very small amounts of the biotest material is required, since chlorophyll concentrations in the order of 0.5 µg Chl/ml and test volumes of 1 ml are sufficient.

The toxicity test is based on highly sensitive measurements of the **effective quantum yield of photosystem II (PS II), Y**, via assessment of chlorophyll fluorescence yield and the so-called saturation pulse method (Schreiber et al. 1986; Genty et al. 1989). Any substance or treatment which causes a limitation of photosynthetic electron flow (from water-splitting in PS II to CO_2-fixation in Calvin cycle) will cause a corresponding lowering of the effective quantum yield, Y. The ToxY-PAM carries out Y-determinations with extraordinary accuracy, thus allowing to detect very small changes of Y induced by toxic substances. The standard deviation in Y-determination is in the order of 0.2 % which

relates to equivalent changes in the concentration of the standard PS II inhibitor Diuron in the order of 0.02 µg/l.

In order to detect very low concentrations of toxic substances which affect the quantum yield of PS II, the ToxY-PAM employs relatively strong measuring light. When applied at sufficiently high frequency, the measuring light will have a distinct actinic effect and thus can be used to establish an optimum rate of electron flow. Two fluorescence levels are measured with high accuracy, the basic level, **F**, shortly before application of a saturating light pulse and the maximal level, **Fm**, reached during the saturation pulse. The effective PS II quantum yield, Y, is calculated according to the expression:

Y = (Fm-F)/Fm (Genty et al. 1989)

A central feature of the ToxY-PAM is the use of two identical fluorescence measuring channels **(dual-channel principle)**. Channel 1 (Sample channel) contains the photosynthetically active sample suspended in the investigated water, whereas channel 2 (Reference channel) contains an identical sample suspended in control water. The two samples experience exactly the same conditions. In particular they are exposed to the same illumination in the course of repetitive measurements which are important for signal averaging and for decreasing the measuring error. Therefore, measurements are not disturbed by the unavoidable decrease of PS II quantum yield which is associated with preillumination of the samples.

The ToxY-PAM Dual-Channel-Yield Analyzer is an extremely compact device based on modern microprocessor technology and optoelectronics. It is battery powered, features a two-line LC-display and can be used as a stand-alone instrument. Alternatively, in standard laboratory applications, it normally is connected via RS 232 interface to a PC and operated via a PC-keyboard, with the data

being displayed on the PC-monitor screen. For PC-operation the dedicated Windows software **ToxyWin** has been developed. The Inhibition % parameter is calculated from the Y1- and Y2-values. On the basis of a stored calibration factor the **Diuron-equivalents** are calculated and displayed (in µg Diuron/liter). The data are automatically stored and written into a **Report-file**. A **Clock-function** is available for a set number of repetitive measurements at a set time interval. The data can be automatically averaged and standard deviations calculated.

3 Basic Operation of the ToxY-PAM

The ToxY-PAM is very easy to operate. It features a small tactile keyboard with **eight function keys (ON, OFF, MODE, MEM, ∧, ∨, START, SET)**. In order to get started, only the ON-key must be pressed and the system is ready for measuring. In the standard version of the instrument, the two channels feature pulse modulated blue measuring light which excites chlorophyll fluorescence in the photosynthetically active samples (thylakoids, algae etc.) contained in 13 mm ∅ quartz glass vials (cuvettes) provided with the instrument. The fluorescence measurement is quite sensitive and chlorophyll concentration should be rather low (≤1 µg/ml) in order to avoid saturation of the amplifier system (starting at signal amplitudes above 3500 unit). Always the activities of two samples are compared, with one serving as **investigated sample (Ch1, S)** and the other one serving **as reference sample (Ch2, R)**. For reliable results it is essential that the two samples are pretreated in exactly the same way (see also chapter 6). The actual measurement of the effective quantum yields of the two samples is extremely simple:

The two samples are placed into the measuring chambers and covered with the provided hoods, in order to protect them from ambient light, and then the **START**-key is pressed in order to start a measurement. Within c 10 seconds the results are displayed, e.g.

```
Inhib.(%)     1.2
D-equ(ug/l)  0.13
```

In this example, the quantum yield of the sample in Channel 1 (Ch1) is 1.2 % lower than that of the reference sample in Channel 2 (Ch2). This is equivalent to the presence of 0.13 µg diuron/l in the sample in Ch1, assuming a standard diuron calibration factor of 0.114, which means that 1 % inhibition is caused by 0.114 µg diuron/l.

Using the arrow key ∧ one can move in the MODE-menu and obtain more information on the last measurement, e.g.

```
F1:1129 Y1: 540
F2:1539 Y2: 547
```

In the given example, the fluorescence signals briefly before Y-determination were F1 = 1129 mV in Ch1 and F2 = 1539 mV in Ch2. And the determined effective quantum yields were Y1 = 0.540 in Ch1 and Y2 = 0.547 in Ch2.

Going to the next MODE-menu position via ∧ there is also the information on the maximal fluorescence signals, Fm, reached during the saturation pulses, e.g.

```
F1:1129 Fm:2457
F2:1539 Fm:3398
```

There are 17 more such **MODE-menu points**, all of which are briefly described in chapter 6. Most of them relate to Instrument Parameter Settings which normally are kept fixed and, hence, are of no concern for basic operation.

In practice, the ToxY-PAM favorably is connected via an **RS 232 interface cable to a PC**, such that it can be operated via the PC-keyboard and the Instrument Parameter Settings as well as the measured data are displayed on the PC-monitor screen. The dedicated Windows-software **ToxyWin** was developed for system operation, data acquisition and analysis (see chapter 5).

4 Description of the eight Keyboard Functions

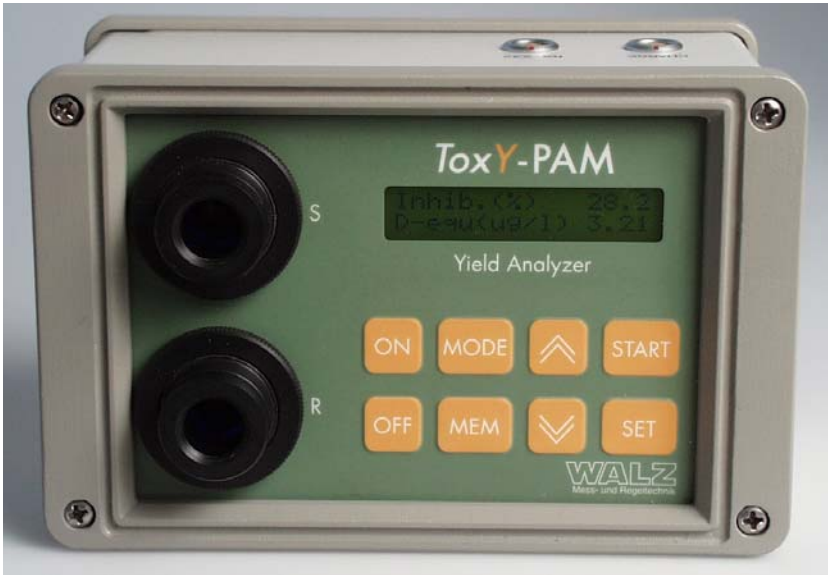


Fig. 1: Top view of the ToxY-PAM Dual Channel Yield Analyzer

4.1 Single key operations

ON To switch ToxY-PAM on (short pressing of the key). To reset the instrument.

OFF To switch ToxY-PAM off; will occur automatically, if no key operation for 4 min (power saving for field use), unless disabled via menu point 7.

MODE To return to MODE-menu after using the MEM-key.

MEM To enter the MEMORY-level of stored data with the last stored data set being displayed

∧, ∨	To select one of 20 points of the MODE-menu or one of maximally 4000 data sets when MEMORY is activated. To change a particular parameter setting in the MODE-menu after operating the SET-key. For advancement by several steps these keys can be kept pressed.
START	To trigger a measuring cycle involving two consecutive YIELD-determinations in Ch1 and Ch2
SET	To start and stop selected function.

4.2 Double key operations

Besides the single key operations, there is a number of double key operations which can serve as short-cuts for selecting/carrying out certain items/commands in the MODE-menu. For this purpose, the first key must be kept firmly pressed before briefly pressing the second key.

MODE + START	To return to standard display (menu position 1).
MODE + SET	To move from one functional block in the MODE-menu to the next (see list in section 7.1).
MODE + ∧	To move to MODE-menu point 11: Clock (started via SET).
MODE + ∨	To move to MODE-menu point 17: ML-Frequ.

5 Windows-software ToxyWin

The ToxY-PAM normally is operated in conjunction with a PC under laboratory conditions. For this purpose the dedicated Windows-software ToxyWin was developed, which is delivered together with the instrument on a program-disk. A PC with Pentium-processor is required. Software installation is carried out as follows:

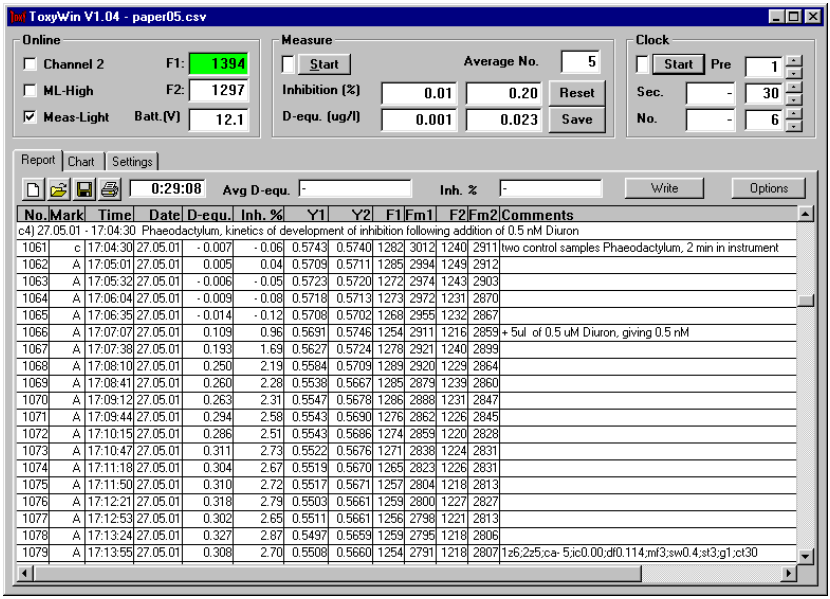
Installation of the ToxyWin-software

- Put disk into drive A of PC
- Call up „My Computer“ and select drive A
- Double click the file twsu104.exe (ToxyWin-Setup file for current program version 1.04)

  Now the toxy-directory will be installed on drive C of the PC.

5.1 User surface and Report-window

The instrument is connected via the provided RS 232 interface cable with the PC. When the program (Toxy.exe) is started, the instrument automatically switches on and communication between the PC and the instrument is established. Under laboratory conditions it is advisable to connect the instrument via the provided Battery Charger with the mains, such that battery voltage will remain high even at extended times of operation. After start of the program, the standard **ToxyWin Report-window** is displayed on the PC monitor screen.



No.	Mark	Time	Date	D-equ.	Inh. %	Y1	Y2	F1	Fm1	F2	Fm2	Comments
c4] 27.05.01 - 17:04:30 Phaeodactylum, kinetics of development of inhibition following addition of 0.5 nM Diuron												
1061	c	17:04:30	27.05.01	- 0.007	- 0.06	0.5743	0.5740	1282	3012	1240	2911	two control samples Phaeodactylum, 2 min in instrument
1062	A	17:05:01	27.05.01	0.005	0.04	0.5709	0.5711	1285	2994	1249	2912	
1063	A	17:05:32	27.05.01	- 0.006	- 0.05	0.5723	0.5720	1272	2974	1243	2903	
1064	A	17:06:04	27.05.01	- 0.009	- 0.08	0.5718	0.5713	1273	2972	1231	2870	
1065	A	17:06:35	27.05.01	- 0.014	- 0.12	0.5708	0.5702	1268	2955	1232	2867	
1066	A	17:07:07	27.05.01	0.109	0.96	0.5691	0.5746	1254	2911	1216	2859	+ 5ul of 0.5 uM Diuron, giving 0.5 nM
1067	A	17:07:38	27.05.01	0.193	1.69	0.5627	0.5724	1278	2921	1240	2899	
1068	A	17:08:10	27.05.01	0.250	2.19	0.5584	0.5709	1289	2920	1229	2864	
1069	A	17:08:41	27.05.01	0.260	2.28	0.5538	0.5667	1285	2879	1239	2860	
1070	A	17:09:12	27.05.01	0.263	2.31	0.5547	0.5678	1286	2888	1231	2847	
1071	A	17:09:44	27.05.01	0.294	2.58	0.5543	0.5690	1276	2862	1226	2845	
1072	A	17:10:15	27.05.01	0.286	2.51	0.5543	0.5686	1274	2859	1220	2828	
1073	A	17:10:47	27.05.01	0.311	2.73	0.5522	0.5676	1271	2838	1224	2831	
1074	A	17:11:18	27.05.01	0.304	2.67	0.5519	0.5670	1265	2823	1226	2831	
1075	A	17:11:50	27.05.01	0.310	2.72	0.5517	0.5671	1257	2804	1218	2813	
1076	A	17:12:21	27.05.01	0.318	2.79	0.5503	0.5661	1259	2800	1227	2827	
1077	A	17:12:53	27.05.01	0.302	2.65	0.5511	0.5661	1256	2798	1221	2813	
1078	A	17:13:24	27.05.01	0.327	2.87	0.5497	0.5659	1259	2795	1218	2806	
1079	A	17:13:55	27.05.01	0.308	2.70	0.5508	0.5660	1254	2791	1218	2807	1z6;2z5;ca- 5;ic0.00;df0.114;ml3;sw0.4;st3;g1;ct30

Fig. 2: ToxyWin user surface showing Report-window

Fig. 2 shows the ToxyWin user surface with the Report-window and some typical results. The user surface is divided into two main parts. At the top the **Online-, Measure- and Clock-boxes** are located which feature the essential controls for measurements as well as the display of the online measured data. Below either the standard **Report-** or alternatively the **Chart-** or **Settings-window** can be displayed.

In order to become familiar with the meaning of the various parameters and functions, the user should fill the two 13 mm ∅ cuvettes with dilute suspensions of photosynthetically active material (e.g. unicellular algae or thylakoids) and place them into the two measuring chambers. Only few chlorophyll is required to obtain a large fluorescence signal, which is displayed in the **Online-box** (featuring online measured fluorescence yield and related parameters). The displayed fluorescence yield **(F1)** is that of the

sample (S) in Channel 1. By clicking the **Channel 2 check-box**, online measurement of Channel 1 is disabled and instead Channel 2 is activated (now online measurement of **F2**, the fluorescence yield of the reference sample, R). The green box always is the active one. Please dilute the samples such that the fluorescence signals are in the order of 1000-1500. Then a measurement can be started by clicking the **Start-button** in the **Measure-box**. During the measurement, which lasts ca. 10 sec, the red indicator lamp at the left side of the Start-button lights up. One can see that first the fluorescence yield in Channel 1 (Ch1) and then that in Channel 2 (Ch2) is assessed. There is a beep when the measurement is started and two more beeps associated with the saturation pulses applied in Ch1 and Ch2. The measured data are written into the Report-file.

5.1.1 Report-file

The Report-file documented in Fig. 2 shows an experiment with *Phaeodactylum tricornutum* using the Clock-function. The information contained in the Report-file can also be viewed graphically in the Chart-window, with the Chart-Nr. corresponding to the Clock-series number (see 5.2 below). Starting from the left the following information is written into the Report:

No. current number of measurement; this number corresponds to the Memory-number (one of 4000 in cyclic memory) of the corresponding recording stored within the instrument.

Mark identification of type of sample or measurement; samples can be marked by letters A-Z; the first measurement of a Clock-series is marked with c. The current number of the Clock-series is documented in a special comment line inserted above the line where the

Clock-series is started.

Time time of the measurement

Date date of the measurement

D-equ. Diuron-equivalents, corresponding to the concentration (in $\mu g\, l^{-1}$) of the standard photosystem II inhibitor diuron which would be required to induce the same inhibition of the sample in Ch1 with respect to the reference sample in Ch2; calculation of this value is based on the measured Inhibition % and on the stored calibration factor (Diuron-factor, standard value of $0.114\ \mu g\, l^{-1}$); negative values of D-equivalents are shown when the sample in Ch1 is more active than the sample in Ch2 and, hence, negative values of Inhibition % are obtained (see below).

Inh.% Inhibition %, corresponding to the apparent relative inhibition of the sample in Ch1 with respect to the reference sample in Ch2, as judged from the measured values of effective quantum yields, Y1 and Y2. Normally, when Ch1 contains an investigated sample which is inhibited with respect to the reference sample in Ch2, Inhibition % is calculated from the equation:

for Y1 < Y2 Inhibition % = 100(Y2-Y1)/Y2

When almost identical samples are used, due to random noise it may happen that the sample in Ch1 shows a somewhat higher quantum yield than the sample in Ch2. Then negative values of Inhibition % will result. In this case, a slightly modified equation is applied:

for Y1>Y2 Inhibition % = 100(Y2-Y1)/Y1

When Inhibition % is calculated in this way, its absolute

value will be identical when the two samples are exchanged between Ch1 and Ch2 ('Cuvette-swap method', see 6.1.2).

Y1, Y2 corresponding to the effective quantum yields of the samples in Ch1 and Ch2, respectively, which are calculated from the fluorescence parameters F1, F2, Fm1 and Fm2 according to the equation:

Y = (Fm-F)/Fm (Genty-equation)

F1, F2 corresponding to the fluorescence yields measured shortly before application of a saturation pulse in Ch1 and Ch2, respectively.

Fm1, Fm2 corresponding to the maximal fluorescence yields measured during a saturation pulse in Ch1 and Ch2, respectively.

Comments at the end of each **Report-line** there is room for entering a comment applying to this particular measurement, e.g. concerning the type of photosynthetically active material, the type of investigated water, the selected instrument settings or the addition of an inhibitor. For writing a comment into a particular line, this has to be clicked with the left mouse-key. The program offers a routine for automatically writing a list of the momentary instrument settings into the Comment-line (see **Save Settings to Report** below).

5.1.2 Status-boxes: Online, Measure and Clock

The top part of the screen features the three status-boxes **Online**, **Measure** and **Clock**, the various elements of which shall be briefly described:

Online-box

Channel 2 checkbox for activating/deactivating online measurement of fluorescence yield F2 in Channel 2

ML-High checkbox for activating/deactivating a 20-fold increase of measuring light frequency which results in a ca. 20-fold increase of effective measuring light intensity; with ML-High being active, the measuring light displays a strong actinic effect, thus lowering the effective quantum yields, Y1 and Y2, substantially.

Meas-Light checkbox for switching on/off continuous measuring light; even when Meas-Light is off, it will be automatically switched on for the duration of a measurement; switching Meas-Light off may be advantageous when dealing with particularly light sensitive samples, the activity of which declines substantially when exposed to continuous measuring light.

F1 and F2 online measured fluorescence yields in Ch1 and Ch2, respectively; the momentary value of F1 is shown in a green box when the Channel 2 checkbox is deactivated, whereas the momentary value of F2 is shown in a green box when the Channel 2 checkbox is activated.

Batt.(V) online measured voltage of the internal battery; the battery should be recharged when voltage drops

battery should be recharged when voltage drops below 11 V; the battery charger routinely may stay connected to the instrument.

Measure-box

Start button to start a single measuring cycle which involves assessment of F1 and F2 as well as Fm1 and Fm2; during the course of a measurement, which takes ca. 10 sec, the red indicator lamp at the left side of the Start-button lights up.

Inhibition % relative inhibition of the sample in Ch1 with respect to the sample in Ch2 (see above); the left box shows the value calculated from the last measuring cycle which is identical to the value entered into the last Report-line; the right box shows the average of a given number of completed measuring cycles.

D-equ. (µg/l) equivalent concentration of diuron which is calculated from Inhibition % using the presently defined calibration factor (see above); the left box shows the value calculated from the last measuring cycle which is identical to the value entered into the last Report-line; the right box shows the average of a given number of completed measuring cycles.

Average No. number of current averages corresponding to the number of measuring cycles which resulted in the averaged values of Inhibition % and D-equ. (µg/l) shown in data-boxes below.

Reset button to reset the Average No. to zero, thus deleting the previously averaged values

Save button to save the information stored by the Average-function in the Report; when Save is pressed, not only the averaged values of Inhibition % and D-equ. (µg/l), but also the corresponding values of the averaged original data (F1, F2, Fm1, Fm2, Y1 and Y2) are written into the Report-file; furthermore, for each parameter also the standard deviation, SD, is calculated and written into the Report-file (see below).

Clock-box

Start/Stop button for start/stop of repetition clock which automatically triggers a defined number of consecutive measuring cycles at defined time intervals between measurements; when the clock is running, the red indicator lamp at the left side of the Start/Stop button lights up.

Sec left box: remaining time in seconds before triggering of next measuring cycle by the repetition clock;
right box: time interval between triggering of two consecutive measuring cycles by the clock, which can be adjusted with the help of the arrow-keys

No. left box: remaining number of measurements to be triggered by the running repetition clock;
right box: total number of measurements to be triggered by the repetition clock, which can be adjusted by the arrow keys. Please note that No. corresponds to the sum of the number of actual averages and the Pre-number (see below).

Pre number of pre-conditioning measuring cycles before start of averaging, which can be adjusted by the arrow keys; this function takes account of the fact that the samples become equalized by the identical preillumination experienced in the two measuring chambers; normally a Pre-number of 1 is sufficient (standard setting). Please note that the total number of measurements displayed under No. corresponds to the sum of Pre-number and number of actual averages.

5.1.3 Data management and offline averaging

The top part of the Report-window features a number of icons for management of the Report-file as well as special functions for offline averaging, data transfer etc.

Clear Report: Command to erase the present Report-file and to start a new Report-file. Before this command is carried out, there is a warning that all unsaved data will be destroyed

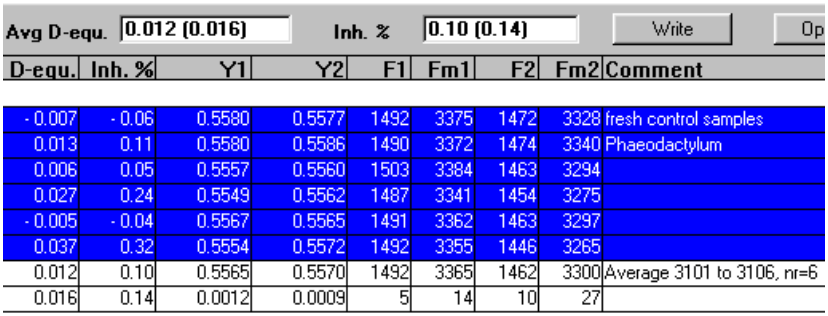
Load Report: Command to load a previously saved Report-file. This will overwrite the present Report-file. Before this command is carried out, there is a warning that all unsaved data will be destroyed.

Save Report: Command to save the present Report-file in form of a text-file (.txt) after the file is given a name by the user. This file is automatically written into the toxy-library which has been created upon installation of the ToxyWin software

Print Report: Command to print out present Report-file via a serial printer.

'Stop-watch'-function: The running time passed since Start of the last measurement is displayed. This time relates to the preillumination state of a sample. Maximal signal stability is reached under steady state conditions when the samples have been preilluminated at a constant dark-light rhythm, preferentially by making use of the Clock-function (see 5.1.2). The 'Stop-watch' is reset with every start of a measurement.

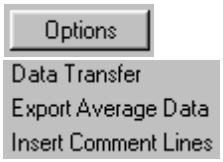
5.1.3.1 Offline Averaging routine



D-equ.	Inh. %	Y1	Y2	F1	Fm1	F2	Fm2	Comment
- 0.007	- 0.06	0.5580	0.5577	1492	3375	1472	3328	fresh control samples
0.013	0.11	0.5580	0.5586	1490	3372	1474	3340	Phaeodactylum
0.006	0.05	0.5557	0.5560	1503	3384	1463	3294	
0.027	0.24	0.5549	0.5562	1487	3341	1454	3275	
- 0.005	- 0.04	0.5567	0.5565	1491	3362	1463	3297	
0.037	0.32	0.5554	0.5572	1492	3355	1446	3265	
0.012	0.10	0.5565	0.5570	1492	3365	1462	3300	Average 3101 to 3106, nr=6
0.016	0.14	0.0012	0.0009	5	14	10	27	

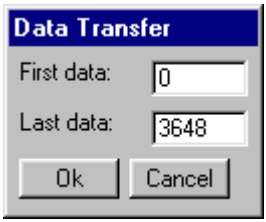
Data stored in the Report-file can be averaged at any time after the actual measurements. For this purpose the corresponding lines of the Report-file are marked by click-and-draw using the left mouse-key. Then the averages of Diuron-equivalents (**D-equ.**) and of **Inh.%** as well as the standard deviations (SD) are shown in the corresponding data boxes. The values in brackets are the **SD-values**. Using the **Write-button** two additional lines with the averages and the SD-values are written below the marked data-lines into the report-file. These lines also include the information on the Y- and F-values.

5.1.3.2 Options



Data Transfer: Routine for transfer of data from the instrument RAM-memory to a PC using the provided RS 232 interface cable. This function applies when data were collected by the ToxY-PAM without a PC being connected (stand-alone operation).

5.1.3.3 Data Transfer



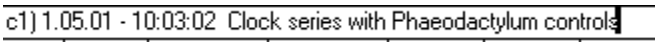
When **Data Transfer** is selected under **Options**, a dialogue-box is opened which allows to define the address of the first data set to be transferred. The last data set always corresponds to the last data set stored in RAM-memory (see chapter 8).

5.1.3.4 Export Average Data

To export specifically the averaged data from the Report-file to a spreadsheet program, like Excel. After selecting **Export Average Data** under **Options** and opening the spreadsheet program, the corresponding data lines can be inserted by the Insert-command. Please note that for export of other Report-file data these simply have to be marked and transferred to e.g. Excel by Copy/Insert commands using the right mouse key.

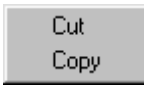
5.1.3.5 Insert Comment Line

To insert additional lines to a Report-file which was created by data transfer from the ToxY-PAM using the **Data Transfer** routine.



In particular, these lines are inserted to precede every data set recorded in conjunction with the **Clock-function** (see 5.1.2). The running number of the Clock-series with date and time is documented. In addition the user may add his own comments describing this particular experiment.

5.1.3.6 Cut and Copy



By clicking the right mouse key, the Cut and Copy commands can be called up. The Cut-command may serve to delete marked data sets from the Report-file. The **Copy**-command is useful for the export of marked data sets into spreadsheet programs, like excel. After Copy and opening of e.g. Excel, the selected data are entered via the **Insert**-command.

5.2 The Chart-window

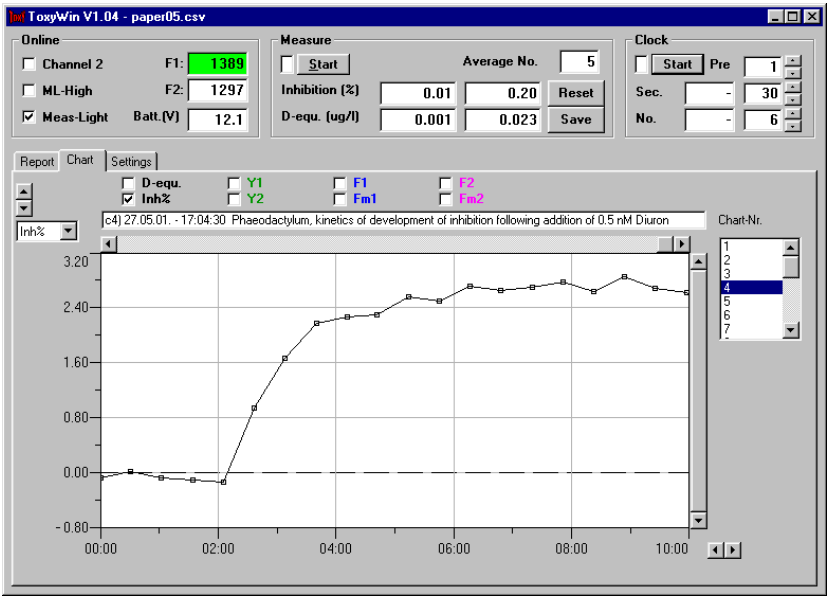
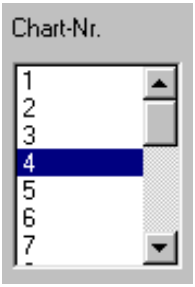


Fig. 3: Chart-window showing the kinetics of development of inhibition in *Phaeodactylum* following addition of Diuron to the sample in Ch1. Corresponding Report-file in Fig. 2.

When **Chart** is selected instead of Report, a graphics window is opened as shown in Fig. 3. In the given example, the same Inh.% data are plotted graphically as presented numerically in the Report-file of Fig.2. The Chart-data are defined by the **Chart-Nr.** (see below) and the comment in the **header line** of the window (see below). Each of the plotted data points corresponds to a measurement in a Clock-series. A total of 8 check-boxes are provided for display of the measured parameters (D-equ., Inh.%, Y1, Y2, F1, Fm1, F2 and Fm2). In principle, all parameters can be displayed on top of each other.

Selection of vertical scaling: The ordinate scale can be defined for each of the selected types of parameters (Inh.%, D-equ., Y and F). Hence, if several parameters are displayed on top of each other, the scale of the selected parameter type applies. The scaling range can be defined by the vertical arrow buttons. At the right hand side of the window a scoll bar is provided for moving the displayed curve on the screen up and down.

The **horizontal scaling** (time scale) can be defined by the horizontal arrow buttons. At the top of the window a scroll bar is provided for moving the displayed curve on the screen to the left and to the right.



The **Chart-Nr.** refers to the corresponding **Clock-series number** in the Report-file where with each start of a Clock-series a comment line is inserted, at the start of which the current Clock-series number is documented. The comment written into this line is identical to the text written in the **header line** of the corresponding Chart.

5.3 The Settings-window

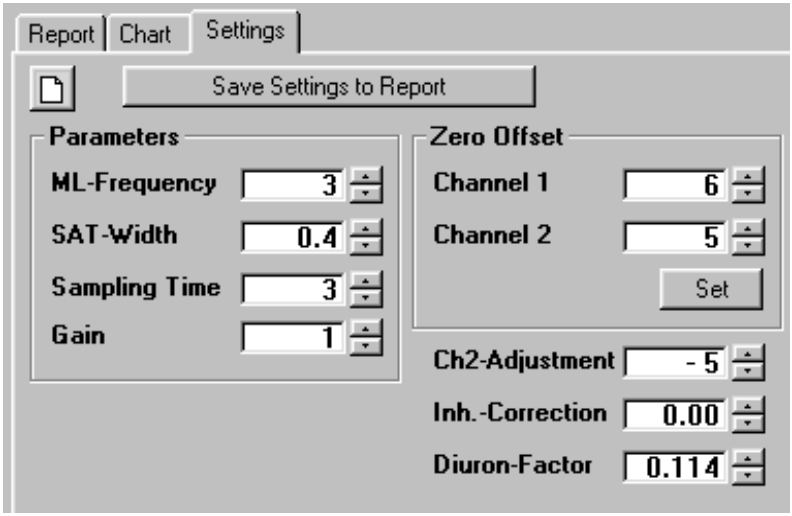


Fig. 4: Settings-window showing standard settings and instrument specific settings

When **Setting** is selected instead of Report or Chart a third window can be opened, as shown in Fig. 4 This window features the settings of a number of instrument parameters accessible to the user, the meaning of which shall be briefly outlined:

ML-Frequency **Repetition rate** (frequency) of the 5 µs pulses of the measuring light; this parameter defines the effective intensity of the measuring light, which increases linearly with the ML-Frequency; at the standard setting 3 of ML-Frequency the intensity amounts to ca. 3 µmol/m^{2}s quanta of photosynthetically active radiation (PAR). Measuring light intensity on purpose is relatively high in order to induce a significant flux of electrons, so that inhibition at any step in the electron transport chain will cause a drop in

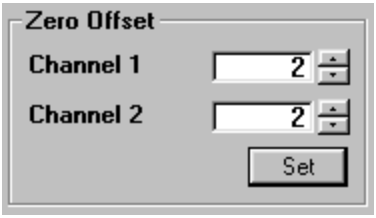
effective quantum yield.

SAT-Width **Width of Saturation Pulses** applied for assessment of Fm-values. At the standard setting of 0.4 sec normally a Fm-plateau is reached. In light sensitive samples, longer Saturation Pulses may lead to a gradual decrease of quantum yields with repetitive measurements.

Sampling Time Time interval over which the fluorescence yields are averaged in order to measure the values of **F1 and F2**; at a given ML-Frequency (see above) with increasing Sampling Time the accuracy of measurements is increased, while more time is required for a single measurement; at a given Sampling Time the accuracy increases with increasing ML-Frequency (see above), as it depends on the number of data points averaged.

Gain **Signal amplification factor**; please note that increasing the Gain from its standard setting 1 to higher values will not only increase the signal but the noise as well; therefore before increasing Gain it should be inquired whether increasing chlorophyll content would be a feasible alternative for increasing the signal, without affecting the signal/noise.

5.3.1 Zero Offset routine



The **Zero Offset routine** serves for suppression of small signals not originating from the photo-synthetically active biotest material. The two boxes show the currently effective Zero Offset values for Channel 1 and Channel 2. Each individual instrument shows characteristic **standard Zero Offset** values, which were determined at the factory with both cuvettes being filled with pure water. These values are documented on a sticker at the bottom side of each instrument. They can be reinstalled at any time with the help of the **arrow keys**. In order to determine Zero Offset the two cuvettes should be carefully cleaned (including an ethanol wash). The cuvette in Ch1 must be filled with the investigated water (without biotest material) and the cuvette in Ch2 with the reference water (without biotest material). The Set-button serves for starting a new Zero Offset determination. This function should be applied only if it is clear that the current settings are obsolete. The measured values will be automatically subtracted from the overall signal. Hence, they are reflected as negative F1 and F2 values in the Online-box when the measuring light is switched off (Meas-Light check box). Correct determination of Zero Offset is a prerequisite for accurate determination of fluorescence parameters and of the derived Inhibition % (see 6.1.2). Normally, the possible errors are relatively small, as the ToxY-PAM as such displays only small background signals. The influence of Zero Offset becomes more important when the investigated water contains fluorescent substances different from chlorophyll, as e.g. humic acids. Any uncompensated background signal which preferentially increases the overall signal in Ch1 with respect to Ch2 will cause an apparent increase of Inhibition %.

5.3.2 Ch2-Adjustment

Channel 2-Adjustment serves for increasing/decreasing the intensity of the measuring light of Ch2 using the **arrow keys**. This function allows to correct for small imbalances between the measuring light intensities of the two channels by adjustment of the measuring light in Ch2. The correct setting of Ch2-Adjustment is a prerequisite for reliable determination of Inhibition % (see 6.1.2). For each individual instrument the proper value of Ch2-Adjustment was determined at the factory. This value is documented on a sticker at the bottomside of each instrument. The setting should be changed only, if there is clear evidence that the relative intensities of the two channels have changed (see 6.1.2).

5.3.3 Inhibition-Correction

Inhibition-Correction can be applied in order to compensate for an apparent Inhibition % measured with two controls. This function should be applied only, if there is reason to believe that the apparent Inhibition % is not due to sub-optimal Ch2-Adjustment (see 6.1.2) and Zero Offset has been properly determined (see 6.1.3). Small values of apparent Inhibition % may result from the fact that the same increase of fluorescence (during saturation pulse) may be detected by the two channels with slightly different sensitivities. Such systematic error can be compensated: If, for example, the apparent Inhibition is 0.35, Inh.-Correction has to be set to -0.35. Please note that this correction does not affect the original fluorescence and Y-values. Therefore, the corrected value of Inh.% does not correspond to the value calculated from Y1 and Y2. For each individual instrument the proper value of Inh.-Correction was determined at the factory, which is documented on a sticker at the

bottomside of each instrument. Normally this value is 0.00, i.e.Inhibition-correction is too small to be of practical relevance. The setting should be changed only, if there is clear evidence that the properties of the two channels have changed (see 6.1.2).

5.3.4 Diuron-Factor

The **Diuron-Factor** serves as calibration factor for calculation of Diuron-equivalents (µg diuron/l) from measured values of Inhibition %. This factor corresponds to the concentration of the standard photosystem II inhibitor diuron (DCMU) which is required to induce under the given measuring conditions a value of 1 % inhibition of the effective quantum yield, Y. The preset value of 0.114 µg diuron/l was determined by Tischer and Strotmann (1977) for dilute suspensions of spinach thylakoids measuring photosynthetics electron transport rate via oxygen evolution. In practice, this factor should be determined by the user for any particular photosynthetically active biotest material at the chosen measuring conditions.

5.3.5 Reset Settings

Reset-icon serving to reset Instrument settings to standard values (see settings in Fig. 4). This command also applies to the settings and the status of the parameters in the Online-, Measure- and Clock-boxes (see Fig. 2). The only exceptions are the settings of Zero-Offset and Ch2-Adjustment, which are not affected by the Reset-command.

5.3.6 Save Settings to Report

This command serves for writing to the Report-file all current Instrument Settings and relevant parameters set in the Online-, Measure- and Clock-boxes. The momentary settings are documented in the **Comment-line** of the last data set. For this documentation abbreviations are used which are explained in the following list:

1z7 Zero Offset of Channel 1

2z9 Zero Offset of Channel 2

ca4 Ch2-Adjustment

ic0.00 Inh.-Correction

df0.114 Diuron-factor

mf3 Measuring Light Frequency

sw0.4 Saturation pulse width

st3 Sampling time

g1 Gain

ct30 Clock time (interval between measurements)

6 Important Points for Correct Determination of Inh. %

The main purpose of the ToxY-PAM is the quantification of toxic substances in water samples. The method is restricted to substances which directly or indirectly affect the complex process of photosynthesis and thus lower the quantum yield of energy conversion at the photosynthetic reaction centers. The ToxY-PAM is specialized in the determination of quantum yield of energy conversion at photosystem II (PS II) reaction centers, Y, using a highly sensitive and accurate chlorophyll fluorescence measuring technique. The YIELD-parameter ΔF/Fm (Genty-parameter) is determined with exceptional accuracy (c 0.1 %), such that in principle differences reflecting c 0.1 % inhibition of photosynthesis can be detected. This is e.g. equivalent to the presence of c 0.014 µg diuron l^{-1}. In practice, however, the detection limit of toxic substances may be higher. There are numerous parameters which can induce substantial changes in the effective quantum yield, Y. Some of these parameters may not be obvious to the user. While possible errors are minimized by the dual-channel measuring principle of the ToxY-PAM, they cannot be completely eliminated (see section 6.2).

6.1 Technical prerequisites

In order to judge the reliability of data obtained with the ToxY-PAM, the user must be convinced that technical errors are not responsible for apparent differences between Y1 and Y2. There are several possibities of technical error, all of which may be either excluded or quantified by appropriate tests:

- the determination of fluorescence yields (F1, F2 as well as Fm1, Fm2) could be inaccurate

- the effective light intensities in Ch1 and Ch2 could be different
- there could be signals not arising from the biological material
- the two cuvettes could be different to an extent that different light intensities in Ch1 and Ch2 arise

6.1.1 Paper-test for signal stability

The accuracy of the fluorescence measurement as such can be tested by substituting the biological samples in the two cuvettes by inert samples. Plugs of tissue-paper pushed into the two measuring chambers may serve for this purpose **(Paper-test)**. The signal levels can be appropriately adjusted to values also encountered with biological samples (up to c 3500 units). Then the clock for repetitive measurements can be started. It is normal that both signals decline somewhat with each measurement. This is due to the bleaching of the fluorescent components of the paper tissue. The actual noise should not be larger than 0.1 %, i.e. maximally 3 units at a signal of 3000 units.

F1	Fm1	F2	Fm2	Comment
Paper test for assessment of instrument accuracy				
3126	3125	3116	3116	Clock-time 30 s
3126	3125	3114	3114	
3124	3125	3114	3113	
3125	3124	3114	3113	
3125	3124	3114	3113	
3125	3124	3114	3114	Average 3130 to 3134, nr = 5
1	0	1	1	SD

Fig. 5: Paper test for assessment of accuracy of measurement

An example of a Paper-test with a standard instrument at standard instrument settings is given in Fig. 5. The Clock was applied to carry out 5 measurements at 30 s intervals. At the end, the

data were averaged and the Standard Deviation (SD) determined. It is apparent that the actual noise in both channels does not cause signal fluctuations exceeding 1 unit, corresponding to an apparent Inhibition of c 0.03 %. Hence, it may be concluded that the accuracy of the fluorescence measurements as such does not limit the performance of the method. If, as generally observed in practice, the SD in Y- and Inh.%-values is considerable larger than 0.03 % (in the best 0.1 %), this must be caused by other sources of error.

6.1.2 Ch2-Adjustment for balancing the two channels

Another important prerequisite for correct determination of Inhibition % is that the measuring light intensities of Ch1 and Ch2 are identical. This is essential, because the PS II quantum yield is not only decreased by inhibitors, but also by an increase in light intensity. In both cases, the amount of open reaction centers is decreased. Hence, any difference in light intensities will be expressed as Inhibition %. This effect is smallest at very low light intensities, when accumulation of reduced PS II acceptors is insignificant (close to maximal quantum yield), and at high light intensities, when quantum yields are low. Hence, the sensitivity to differences in measuring light intensities depends on **ML-Frequency** (see 5.3). Actually, at the lowest setting of ML-Frequency (mf1) the effect caused by differences up to 10 % is negligibly small. Therefore, any Inhibition % observed at ML-Frequency 1 is unlikely to be caused by differences in measuring light intensity, unless these were unusually large.

For matching the two measuring light intensities, the ToxY-PAM features the so-called Ch2-Adjustment function (MODE-menu point 6 and Settings-window under ToxyWin). Positive or negative values can be entered to increase or decrease the intensity of the measuring light LED-array in Ch2. One positive unit results in

approximately 1 % increase of Ch2-light intensity. An increase of Ch2-light intensity results in a decrease of the measured Inhibition %, as the effective quantum yield in Ch2 is decreased with respect to that in Ch1. The measuring light intensities of the two channels are adjusted at the factory for each individual instrument by measuring the intensities at the measuring sites within Ch1 and Ch2 with the help of a special spherical microquantum sensor (type US-SQS/T, Walz) (Optical method). In addition, by trimming of Ch2-Adjustment it is assured that with two identical samples in Ch1 and Ch2 the measured Inhibition % indeed is close to zero. This adjustment is carried out at ML-Frequency setting 5, where the Inhibition % caused by intensity differences is maximal. The obtained value of Ch2-Adjustment is documented on a sticker at the bottom side of each instrument. The value determined at the factory should remain valid for some time after instrument delivery. It would have to be corrected, if the LEDs in the two channels would display differences in ageing. In any case, the user should check once in a while whether with identical samples the measured Inhibition % is close to zero.

Ch2-Adjustment should be changed only if it is ascertained that the two samples indeed are identical and Inhibition % still reproducibly deviates substantially from zero (by more than 0.25-0.50 %). Furthermore, it is important that the Zero Offset is properly set (see 6.1.3) and that both cuvettes are free of traces of inhibitors. Therefore, before changing Ch2-Adjustment the cuvettes should be carefully washed with ethanol.

Reliable Ch2-Adjustment requires some time and experience. As explained above, for maximal sensitivity in the assessment of small differences in measuring light intensities the MF-Frequency setting 5 should be chosen. If such adjustment becomes necessary, it is recommended to use the following approach which further accentuates differences in the effective measuring light intensities of

the two channels. This method is based on the fact that at a given inhibition of a sample in Ch1 with respect to a sample in Ch2 upon exchanging the two cuvettes the same value of Inh.% should be displayed with negative sign, if Ch2-Adjustment is correct (**'cuvette-swap method'**).

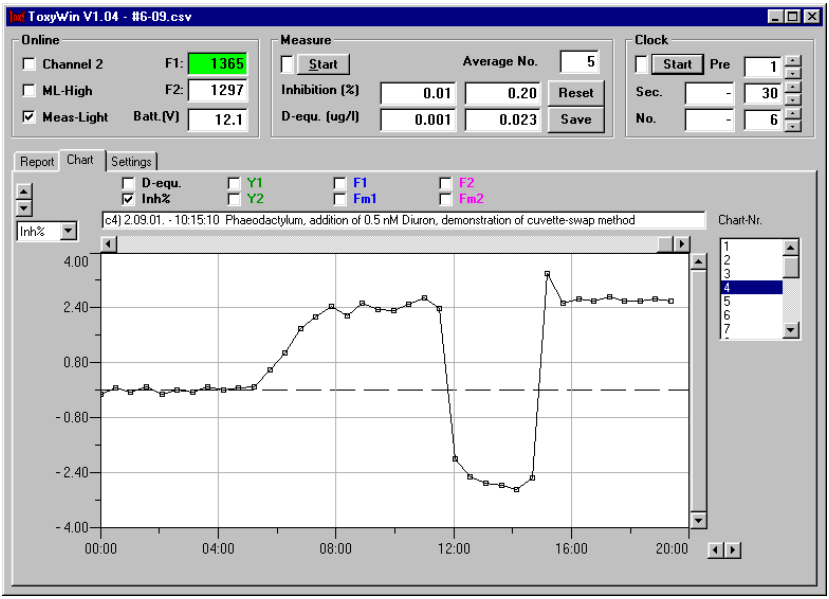


Fig. 6: Demonstration of cuvette-swap method after addition of 0.5 nM Diuron to Phaeodactylum sample in Ch1

Fig. 6 shows a Chart-registration of an experiment in which use of the cuvette-swap method is made. Inh.% is measured with two samples of Phaeodactylum, with a final concentration of 0.5 nM Diuron being added to Ch1 in a Clock-series directly after measurement 11. The cuvettes were swapped briefly after measurement 23 and back again after measurement 29. In the given example, the observed positive and negative values of Inh.% are almost identical, thus showing that Ch2-Adjustment is correct.

For the purpose of the cuvette-swap method a slightly different definition of Inh.% is applied in the case of Y1>Y2 than in the case of Y1<Y2 (see 5.1.1). In practice, the user should proceed as follows: Inhibit the sample in Ch1 with Diuron (or any other stable inhibitor) by at least 2-3 % and mark the cuvettes with and without inhibitor. Start the Clock using a Clock-Interval of 60 sec. After the Inh.% value has stabilized, carefully exchange the two cuvettes, without disturbing the samples, while the Clock is still running. If Ch2-Adjustment is correct, the same value of Inh.% will be measured, however with negative sign. If the measured value is not negative enough, the setting of Ch2-Adjustment has to be increased. Swapping the cuvettes should be carried out in dim light, such that the preillumination state is not significantly changed in either cuvette.

Even when the measuring light intensities of the two channels are identical, very small values of Inhibition % (up to 0.5 %) may be measured with two controls, which may arise from slightly different sensitivities of the two channels. This means that the same saturation pulse induced increase of fluorescence yield leads to slightly different signal increases in the two channels (unavoidable non-linearities in the signal vs intensity dependence). As this effect is independent of ML-Frequency, it can be assessed at ML-Frequency 1, where any imbalance caused by improper Ch2-Adjustment can be ignored (see above). Provided that the Zero Offset values are correct (see below) and the two samples are identical, any Inhibition % consistently measured at MF-Frequency 1 may be compensated by **Inh.-Correction** (see 5.3.3).

6.1.3 Proper setting of Zero Offset values

When the two cuvettes are filled with clean water, each channel displays a small background signal which normally is suppressed by

the Zero Offset function. The preset values of Zero Offset were determined at the factory using samples of clean tap water contained in the standard 13 mm ∅ cuvettes in the two channels. It may be necessary to change Zero Offset if the investigated water shows more fluorescence than tap water, e.g. due to dissolved fluorescent substances. For example, the background signal of deionized water stored in common polyethylene bottles is somewhat higher than that of normal tap water. Hence it is advisable to determine Zero Offset with samples of the investigated water (Ch1) and the control water (Ch2). Use of absolutely clean cuvettes is essential. Even traces of chlorophyll sticking to the walls may cause substantial errors. Therefore, a brief ethanol wash followed by rinsing with tap water is advisable.

The error caused by incorrect Zero Offset values normally is small. For example, when the value is 3 units off, at an Fm-level of 3000 the error in Y amounts to 0.1 % only. Much larger errors are possible, if fluorescent compounds are dissolved in the investigated water.

6.1.4 Use of optical grade cuvettes

Even if the intensity of Ch2 was well adjusted with respect to Ch1 by measuring the PAR-values with the US-SQS/T or by the 'Cuvette-swap method', small differences in the effective light intensities may arise from differences in the optical properties of the two cuvettes. Such differences are highly unlikely in the case of optical grade cuvettes, as provided with the instrument. They may however be encountered in the case of standard disposible glas vials. As the wall thickness of such vials is heterogeneous, the effective light intensity within each individual cuvette may differ. Furthermore, there may be even a small influence of the particular position of one and the same cuvette within the measuring chamber.

Unfortunately, the effective light intensity cannot be simply judged by the fluorescence amplitude, as in the disposible vials also the bottoms (through which fluorescence is measured) are heterogeneous. In practice, use of disposible glass vials instead of the provided optical grade 13 mm cuvettes can be recommended only, when errors in the order of 0.5 % Inhibition can be tolerated. When larger numbers of samples are prepared for incubation with the biotest material, the samples may be first stored in disposible glass vials. But for the actual measurements they should be transferred to the 13 mm cuvettes. Care should be taken that the cuvette walls are clean, such that no measuring light is absorbed or scattered away. Small differences in sample volume do not affect the outcome of the measurement.

6.2 Handling of samples and measuring conditions

A prerequisite for correct determination of Inhibition % is that the two samples are treated in exactly the same way. This can be best assured if the two samples are prepared only briefly before the actual measurement by mixing aliquots of the photosynthetically active material with the investigated water and reference water samples, respectively. Preparation of the samples should be carried out under controlled light conditions. It is essential that preillumination of the two samples before placing them into the measuring chambers is as much identical as possible. The user may convince himself about the importance of this point by briefly exposing the sample in Channel 1 (Ch1) to somewhat stronger light. This will result in apparent inhibition (start the Clock for repetitive measurements), the extent of which will slowly decline as the sample re-adapts to the light conditions within the instrument. Differences in preillumination will decline with the time for which samples are within the instrument. Hence, the reliability with which Inh.% is measured, will normally

increases with the time after installing the two cuvettes. The equalization of the samples is improved by the light conditions within the two chambers, provided it has been assured that the effective measuring light intensities are equal (see section 6.1.2).

6.2.1 Choice of ML-Frequency

The effective intensity of the measuring light on purpose is relatively high in order to induce a substantial rate of electron transport, such that inhibition of this transport will result in maximal lowering of the quantum yield. The effective intensity can be modified via the ML-Frequency (MODE-menu point 17; Settings-window under ToxyWin). For the choice of ML-Frequency the following aspects have to be considered:

1. The accuracy of Y-determination increases with increasing values of Y and as Y decreases with light intensity, the ML-Frequency should not be too high. Control Y-values below 0.4 should be avoided.
2. The apparent Inhibition % at a given inhibitor concentration depends on light intensity, showing an optimum curve, which depends on the type of biological sample. In the case of *Phaeodactylum* cells grown in daylight, ML-Frequency 5 was found optimal.

6.2.2 Biological test material

For making optimal use of the ToxY-PAM, the choice of the biological test material and its appropriate handling are of utmost importance. Advantages of freeze dried thylakoids are:

1. Once they are prepared, they can be kept for years at -20 °C, thus providing a very convenient test material with constant properties.
2. Inhibitors can penetrate readily to potential inhibition sites on the thylakoid membranes.
3. Thylakoids lack the complex induction kinetics observed upon dark-light changes in whole cells, which may affect the PS II quantum yields measured by the ToxY-PAM.

On the other hand, advantages of algae are:

1. Algae are not only sensitive to PS II inhibitors, but also to numerous other substances which affect the overall process of photosynthesis. Because of the interaction of photosynthesis with other metabolic processes, the range of potentially inhibiting substances is even further extended.
2. There is much less investment and technical know-how required for maintaining an algae culture than for isolating and lyophylizing thylakoids.

Different algae display different sensitivities to inhibitors (see e.g. Juneau et al. 2001; reference in chapter 13) which may reflect differences in physiology or inhibitor penetration. While this is a complicating factor, the ToxY-PAM is ideally suited to investigate dose-response curves and inhibitor uptake kinetics. The knowledge gained by such basic research can be used for the practical application of the ToxY-PAM in pollution control using particularly sensitive types of algae for various types of potential pollutants.

For standard measurements with the ToxY-PAM the diatom *Phaeodactylum* can be recommended which displays good growth at a wide range of salinities. Actually, very satisfactory results can be obtained with a simple *Phaeodactylum* culture in standard growth

medium using an Erlenmeyer flask covered with a cotton plug on a window sill. No fancy algae culturing facilities are required. However, it is important that the algae are gently shaken every day and overgrowing is avoided. Preliminary experiments show that most common inhibitors penetrate more readily into *Phaeodactylum* than into the green algae *Ankistrodesmus* or *Chlorella*, as judged from severalfold higher levels of Inhibition % measured with the ToxY-PAM.

The measured value of Inh.% at a given inhibitor concentration may not only vary with the type of biological test material and the light conditions within the instrument (see 6.2.1), but also with the light conditions during growth. For example, in the case of *Phaeodactylum* long time dark-adapted cells are distinctly less sensitive to Diuron than preilluminated cells. Hence, for reproducible results, it is important to standardize the growth conditions of the biological test material.

When using algae as test material, it is important to realize that algae show dynamic regulatory reactions in response to changing environmental factors which may affect the measured values of quantum yield. This is particularly true for changes in light intensity and light quality, which may induce regulatory reactions leading to Y-changes, which often display oscillatory patterns. When these oscillations are slightly phase shifted in the two samples, they may cause significant fluctuations in Inh.%, the amplitude of which declines with an increasing number of repetitive measurements (use Clock-function). In order to avoid this problem, it is advised not only to pay attention to equal light pretreatment, but also to keep the algae stock before preparation of the two samples in a light environment similar to that encountered in the instrument.

6.2.3 Possible sample-related errors

There are a number of other sample-related aspects, which should be considered in order to avoid errors and to obtain optimal results:

1. The investigated water should be free of any particulate, potentially fluorescing material, except for the added photosynthetically active material. This can be ascertained by filtering the water using a membrane (0.2 μm pores). If this is done, the same filtered water should be used for Zero Offset determination and the Inhibition % test.

2. Presence of colored substances in the investigated water may give rise to substantial systematic errors by lowering the effective measuring light intensity in Ch1 relative to that in Ch2. This leads to an increase of Y1 with respect to Y2 and consequently negative values of Inh.%. As the standard version of the ToxY-PAM features blue measuring light (peak at 470 nm), this 'color problem' is particularly serious when dealing with yellow and brown water samples (e.g. from soil extracts). In this case, it is recommended to apply a special version of the ToxY-PAM with red measuring light, which is absorbed c 8 times less by yellow-brown substances. In principle, the 'color effect' can be corrected for by appropriate lowering of the measuring light in Ch2 (i.e. lowering of Ch2-Adjustment value). However, determination of the correct Ch2-Adjustment value is problematic, if the colored substance also displays fluorescence (like humic acids).

3. As the photosynthetically active material is in aqueous suspension, its addition to the investigated water will lead to dilution of the latter and to a corresponding decrease of the concentration of the dissolved toxic substances. Hence, for optimal detection of Inhibition %, the dilution factor should be

low. On the other hand, it is also important that the chlorophyll concentration is sufficiently high to reach high fluorescence values. For optimal resolution Fm values should be in the order of 2500-3400 units which is equivalent to c 0.5 µg Chl ml^{-1}. The standard deviation in Inhibition % linearly decreases with signal amplitude. In practice, it is e.g. feasible to use a stock suspension of photosynthetically active material with c 2.5 µg Chl ml^{-1} and to dilute it to ca. 0.5 µg Chl ml^{-1} with the investigated water (mixing ratio 1:4). In this case toxic substances in the investigated water will be diluted by a factor of 0.8 and, hence, the measured Inhibition % (as well as Diuron-equivalents) have to be multiplied by a factor of 1.25 (assuming linearity between Inhibition % and concentration, which is given up to at least 10 %).

4. Algae suspensions should be homogenous. Any clumping will lead to increased sample noise. Sample noise will also increase with the extent that algae cells settle during measurements.

7 The MODE-Menu

The MODE-menu contains 20 items corresponding to a variety of measured values, instrument settings or special commands. Increasing or decreasing item numbers are selected by the ∧- or ∨-arrow keys, respectively. Changes are terminated via SET or MODE. Starting from position 1, at increasing numbers there are first MODE-points featuring the display of measured fluorescence parameters (like F1, F2, Y1, Y2), while at decreasing numbers the MODE-points for instrument settings prevail. Some of the MODE-menu positions can be directly reached via double key operations (see list in section 4.2 above).

Irrespective of the selected menu item, a measurement of Inhibition %- can be initiated at any time by pressing the START-key. Normally, the system then automatically returns to the menu position 1 where the measured data are displayed.

The operations related to the various points of the MODE-menu are either directly carried out via SET (e.g. 11: Clock) or initiated/terminated (e.g. 17: ML-Frequ.) by pressing SET. Settings are changed by arrow key operations (∧, ∨) and become immediately effective. The numbers following the double points show the present settings.

7.1 List of Menu points

The Menu points are organized in functional blocks. The starting point of each block can be reached successively by simultaneous pressing of MODE and SET.

The below list shows the default settings, which can be reset at any time by the command 10: Reset Settings. The first points of the functional blocks which can be quickly reached by the MODE+SET

command, are emphasized by boldface printing. The double-key commands by which some of the menu points can be quickly accessed are also listed.

Menu points:	Quick access via:
1. Standard display	MODE+START
2. F1, F2, Y1, Y2	
3. F1, F2, Fm1, Fm2	
4. Z-Offs. Ch1, Ch2 (Set)	
5. Channel 1 (SET)	
6. Ch2 Adjust (SET)	
7. Auto-OFF: On (SET)	
8. Battery 12.5V	
9. Ver.T0.92 101001	
10.Reset Settings (SET)	
11.Clock OFF (Set)	MODE+∧
12.Clock Time 30s (SET)	
13.Clock No. 6 (SET)	
14.Sampl.Time..3s (Set)	
15.SAT-Width 0.4s (Set)	
16.Inh.Corr. 0 (Set)	
17.ML-Frequ. 3 (SET)	MODE+∨
18.Meas-Light ON (SET)	
19.ML-High OFF (SET)	
20.Diuron-F. 0.114 (SET)	

7.2 Description of the MODE-menu points

The following list briefly describes the items contained in the MODE-menu.

```
Inhib.(%)     1.2
D-equ(ug/l) 0.13
```

Standard menu-position for display of the result of the last measuring cycle triggered by START. The inhibition of the investigated sample in Ch1 with respect to the reference sample in Ch2 as well as the Diuron-equivalents (µg l^{-1}) based on stored calibration factor (Diuron-Factor, menu-point 20) are displayed.

```
 F1:1129 Y1: 540
 F2:1539 Y2: 547
```

Display of fluorescence yields F1 and F2 (determined briefly before application of saturating light pulses) as well as the calculated effective quantum yields Y1 and Y2. Data valid for last measuring cycle triggered by START.

```
 F1:1129 Fm:2457
 F2:1539 Fm:3398
```

Display of both fluorescence parameters, F and Fm, for Ch1 and Ch2. The displayed data are valid for the last measuring cycle triggered via START.

```
  4.Z-Offs.(SET)
Ch1:   3 Ch2:  6
```

Zero-Offset command for determination of signals in absence of chlorophyll (background signals), the values of which are displayed and automatically substracted, such that signals become zero without samples. The offsets remain valid for all consequent measurements until being deliberately changed. The command is carried out via SET. Correct Zero-Offset values depend on the fluorescence properties of the investigated water in the absence of the photosynthetically active material (see section 6.1.3).

```
  5.1129    (SET)
 Channel     1
```

Command for switching between display of Channel 1 (default) and of Channel 2. The displayed value corresponds to the momentary fluorescence yield of Ch1 (default) or to the momentary fluorescence yield of

Ch2, depending on which of the two channels is active. This momentary fluorescence yield is also displayed in the top line of all of the following MODE-menu points 6-20.

```
 6.1129   (SET)
Ch2 Adjust  -1
```

Setting of Ch2-Adjustment. The setting can be modified via SET and the arrow keys. It should be changed only if it is ascertained that the preset values are not valid anymore, e.g. due to differences in ageing of the LEDs in the two channels (see section 6.1.2). In the top line the momentary fluorescence yield of the active channel (Ch1 by default) is displayed. Display of Ch2 can be installed via menu-point 5.

```
 7.1129   (SET)
Auto-OFF    ON
```

On/off switch to enable/disable the power saving automatics which turn off the ToxY-PAM after 4 min without key operation. It is advisable to disable the AUTO-OFF when the instrument is connected to an external power supply (via CHARGE-socket).

```
 8.1129
Battery   12.4
```

Display of battery voltage. Measurements may become erroneous, if the voltage drops below 8.0 V (Error message : CHECK BATTERY). The battery voltage is a non-linear function of the remaining battery capacity. When dropped below 11.2 V (without saturation pulse) the remaining capacity is approx. 20 % and recharging soon will become necessary. In this case there is a warning (BAT-sign blinking in the left corner of the upper display line).

```
 9.1129
Ver.T0.92 101001
```

Display of number and date of origin of present ToxY-PAM software version resident on EPROM within the instrument. This software can be updated by the user after downloading via internet (Walz Homepage) with the help of special cable delivered with the instrument (see chapter 10.3).

```
10.1129   (SET)
Reset Settings
```

Command to reset all instrument settings (which are accessible via the MODE-menu) to the standard settings preset at the factory.

```
11.1129   (SET)
Clock       OFF
```

On/Off switch of repetition clock which serves to trigger a fixed number of measuring cycles (set via menu-point 13) at fixed intervals between two consecutive measuring cycles (set via menu point 12).

```
12.1129   (SET)
Clock Time  30s
```

Setting of clock interval, which is the time between start of two consecutive measuring cycles triggered by the repetition clock (menu-point 11). The setting can be modified via SET and the arrow keys.

```
13.1129   (SET)
Clock No.   10
```

Setting of number of repetitive measurements triggered by the clock (menu-point 11). The setting can be modified via SET and the arrow keys.

```
14.1129   (SET)
Sampl.Time   3s
```

Setting of the Sampling Time for assessment of the F1 and F2 values. During this time the data points are averaged to decrease random noise. At the standard time of 3 s a high signal/noise ratio is obtained, which is not much further improved when longer times are applied.

```
15.1129   (SET)
SAT-Width  0.4s
```

Setting of the Saturation Pulse Width. Values higher than the standard 0.4 s may lead to a decrease of effective quantum yield when longer sequences of saturation pulses are given.

```
16.Y2: 547(SET)
Inh.Corr.    8
```

Setting of the Inhibition-Correction which may be applied in order to compensate an apparent Inhibition % which is neither due to differences in measuring light intensities of the two channels, nor to incorrect Zero Offset values nor to differences in the two samples.

```
17.1129   (SET)
ML-Frequ.    3
```

Setting of Measuring Light-Frequency in 12 steps. Measuring light intensity is a linear function of ML-Frequency. With increasing setting the actinic effect of the measuring light increases and the effective quantum yield, Y, decreases, which affects the accuracy with which Inhibition % is measured. On the other hand, at higher intensities the inhibitory effect of toxic substances may be enhanced. Hence, the optimal setting of ML-Frequency depends on the light saturation properties of the photosynthetically active material and the particular type of inhibitors involved. The standard setting 3 is well suited for assessment of diuron/atrazin type of inhibitors in conjunction with thylakoids or unicellular algae.

```
18.1129   (SET)
Meas-Light  ON
```

On/off switch of measuring light. Under standard conditions the measuring light is on. When switched off, a negative signal indicates the ZERO-Offset value of the active channel.

```
19.1129   (SET)
ML-High     OFF
```

On/off switch of the Measuring Light – High function. In the standard OFF-position, the measuring light is applied in form of 5 Hz packages of 5 µs measuring light pulses (so-called burst-mode). Frequency of the measuring light packages is increased to 100 Hz when ML-High is switched on. This leads to a 20-fold increase of light intensity, thus resulting in a strong actinic effect and considerable lowering of effective quantum yield, Y. While ML-High normally is not suited for measurement of Inhibition %, it may be useful for defined preillumination of samples.

```
20.1129   (SET)
Diuron-F. 0.114
```

Setting of the Diuron-Factor which is the calibration factor for the calculation of Diuron-equivalents from Inhibition %. The preset value of 0.114 µg diuron l^{-1} corresponds to the concentration of diuron which was found by Tischer & Strotmann (1977) to cause 1 % inhibition of

photosynthesis in dilute spinach thylakoid suspensions. In practice, in order to obtain 1 % Inhibition with the ToxY-PAM different diuron concentrations may be required, depending on the type and concentration of the photosynthetically active material as well as the selected Instrument Parameter Settings. Hence the Diuron-factor has to be determined by each user for the particular biotest system and experimental conditions.

8 The Memory-level

By pressing the **MEM-key** the user can move from the MODE-level to the **MEMORY-level** where up to 4000 data sets are stored.

```
A3648 08:24 1605
Inh%  0.3 D 0.03
```

On the display the data of the last measurement are shown, with Mark, current No., Time, Date, Inhibition % and Diuron-equivalents. When the **Set-key** is pressed once, also the original Y1- and Y2-values are shown, on which calculation of Inhibition % was based:

```
A3648 08:24 1605
 Y1: 574 Y2: 576
```

By pressing the **Set-key** once more, one can return to the display of Inhibition % and Diuron-equivalents.

With the help of the **arrow-keys** the user can move within the Memory and select any of the previously stored data sets for display. However, if a PC is available, it is more comfortable to first transfer the data to the PC (see 5.1.3) and then to view the data at the level of the Report-file (see 5.1).

9 Components of the ToxY-PAM

All basic components of the ToxY-PAM Dual-Channel Photosynthesis Yield Analyzer are contained in one compact, portable unit. Additional peripheral components can be connected to the three sockets at the rear side of the housing

9.1 Dual-channel optical units

The ToxY-PAM features two identical optical units for comparison of an investigated sample (Channel 1, top unit, S) with a reference sample (Channel 2, bottom unit, R). Each channel features the following components:

- 10 blue light emitting diodes (LEDs) for pulse modulated fluorescence excitation (for special applications, also instruments with differently colored LEDs are available); the LEDs are arranged in form of concentric rings around the two measuring chambers
- the LED light passes a special scattering filter in order to prevent heterogeneities in the light fields
- the measuring chambers house standard 13 mm ∅ optical grade quartz cuvettes (Hellma)
- 1 mm perspex tubes protect the LEDs against injury and O-rings in the chamber walls prevent moisture to penetrate to the optoelectronics
- O-ringed plugs with central 13 mm ∅ holes form the top of each unit and serve for centering the cuvettes; these plugs can be removed for cleaning the chambers
- 12 mm ∅ detector filters (RG645, Schott, 2 mm thick) form the bottom of each chamber

- spherical lenses (12 mm ∅, which are located between filters and photodetector, effectively collect fluorescence originating at the crossing points of the LED excitation beams
- PIN photodiodes with pulse preamplifiers serve for detection of the pulse modulated fluorescence signals

9.2 Electronic components

The extremely compact design of the ToxY-PAM is a consequence of recent progress in miniaturization of solid state integrated circuits. The central processing unit features a powerful CMOS microcontroller. The instrument software is stored in a programmable CMOS EPROM. This EPROM can be re-programmed by the user, when program updates become available (see 10.3).

9.2.1 Rechargeable battery

A relatively large rechargeable lead acid battery (12 V/2 Ah) is mounted in the bottom of the ToxY-PAM housing. For recharging, the Battery Charger MINI-PAM/L is provided which is connected to the CHARGE-socket at the rear side of the ToxY-PAM. The charger, which operates at input voltages between 100 and 240 V AC, features an overload protection. Full charging of an empty battery takes approx. 5 hours. Battery voltage is displayed under MODE-menu point 8. The warning 'BAT' is given in the upper left corner of the display when voltage drops below 11 V.

With a fully charged battery the displayed voltage is 12.5 - 12.9 V. In first approximation, battery voltage can be taken as a measure of remaining battery power. The functional relationship between capacity (Ah) and voltage of a new battery is depicted in Fig. 7. It is apparent that battery voltage first drops steeply to about

12.3 V and then slowly decreases to about 11.8 V, from whereon there is a steep further drop to values below 11 V.

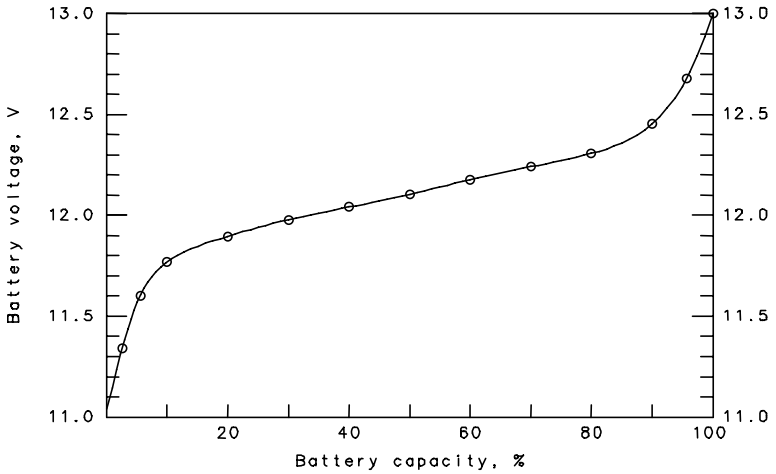


Fig. 7: Relationship between Battery Capacity and Battery Voltage

9.2.2 Description of the connectors

At the rear side of the ToxY-PAM the following electrical connectors are located:

a) CHARGE

Together with the ToxY-PAM the Battery Charger MINI-PAM/L is delivered which connects to the CHARGE-input at the rear side of the instrument. The charger can be used with line voltages of 100 to 240 V at 50-60 Hz. When used in the laboratory the charger can remain permanently connected.

b) RS 232

An RS 232 interface cable is provided to connect the ToxY-PAM to IBM or IBM-compatible PCs for operation under ToxyWin software.

10 Maintenance

10.1 Internal battery and its replacement

The internal battery is essentially 'maintenance free'. However, even when the instrument is switched off, there is some discharge, which is stimulated by elevated temperatures. If it is forseeable that the instrument will not be used for some months, the battery should be charged beforehand. Excessive discharge of the battery should be avoided, as this may cause irreversible damage. Such damage involves lowering of the capacity and increase of internal resistance, with the consequence that recharging becomes necessary after relatively short times of operation and that there is an excessive lowering of voltage during saturation pulses. In this case, battery replacement is recommended.

If replacement of the battery becomes necessary, this is readily accessible after removing the 4 screws at the bottom of the ToxY-PAM. The battery is attached to the bottom piece by double-sided adhesive tape. After disconnecting the cables, the battery can be detached by means of a screw-driver used as a lever. The replacement battery comes with adhesive tape. When connecting the cables, please note the proper contact polarities (red/positive and black/negative).

10.2 Fuse replacement

For access of the fuse, the four screws holding the lid of the instrument have to be removed, the lid lifted and placed upside down. Then the fuse is located at the upper right hand side. If the fuse should ever be burnt, it can be replaced by the one which was provided upon delivery of the instrument.

10.3 EPROM and update of instrument software

The ToxY-PAM features two types of software, the instrument software, which is resident on an EPROM within the instrument and the Windows-software, ToxyWin, which is delivered on a program disc and stored within the PC. The EPROM is located on the microcontroller board. When new instrument software versions become available, it can be reprogrammed by the user with the help of a special programming cable which is provided with the instrument.

For this purpose the top of the instrument must be lifted after removing the 4 screws. On the right hand side a 6-pin socket becomes accessible into which the corresponding connector of the programming cable PDA-100/K1 can be plugged. The other connector (25-pin) is plugged into the serial interface socket (normally used for connecting serial printer) of the PC on which the new instrument software is stored. Please note that the nose on the cable connector connecting to the EPROM has to point outwards. Update versions of the instrument software can be downloaded via internet from the WALZ-homepage http://www.walz.com. The new program version is automatically installed when the instrument is linked to the PC via the provided cable and the update file is double clicked.

11 Some Relevant Information on Chlorophyll Fluorescence Measurements with the ToxY-PAM

Chlorophyll fluorescence is a large signal and in principle its measurement is rather simple. Hans Kautsky already observed chlorophyll fluorescence changes by his bare eyes in 1931 and suggested that these are related to photosynthesis. In the following 50 years, with the progress of modern electronics and photooptics, highly sensitive and fast fluorometers were developed which contributed substantially to the elucidation of the basic mechanisms involved in the complex process of photosynthesis. Progress in this field of research has been greatly stimulated by the invention of the Pulse-Amplitude-Modulation (PAM) measuring principle (see section 11.2 below). PAM Chlorophyll Fluorometers have been successfully used all over the world, as can be judged from the large number of publications based on investigations carried out with numerous versions of these instruments.

The ToxY-PAM differs from the previously issued PAM fluorometers in that it is further miniaturized and specialized to perform one particular type of measurement with the greatest ease, accuracy and reliability, namely the inhibition of an investigated sample (e.g. algae or thylakoid suspension) relative to a reference sample. This measurement is based on the determination of the effective quantum yield of photosynthetic energy conversion, $Y=\Delta F/Fm'$, the so-called Genty-parameter. In the following section some background information on this and other fluorescence parameters is given, and special aspects on fluorescence measurements with the ToxY-PAM are outlined, in order to make optimal use of this instrument.

11.1 Chlorophyl fluorescence as an indicator of photosynthesis

Photosynthesis involves reactions at five different functional levels:

- processes at the pigment level
- primary light reactions
- thylakoid electron transport reactions
- dark-enzymic stroma reactions
- slow regulatory feedback processes

In principle, chlorophyll fluorescence can function as an indicator at all of these levels of the photosynthesis process. To what extent the fluorescence response is effected at these different levels depends on the choice of photosynthetically active material and the measuring conditions.

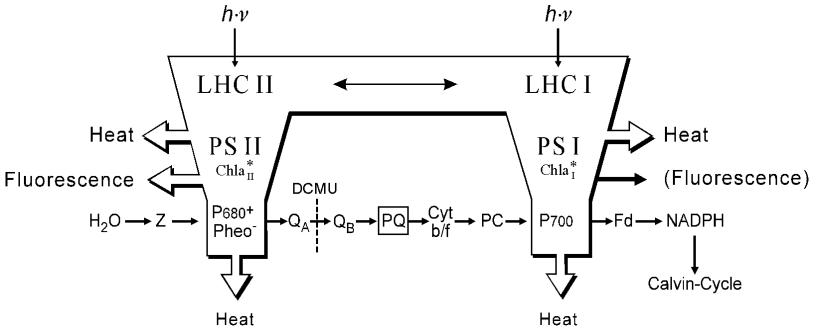


Fig. 8: Schematic view of primary energy conversion and primary electron transport in photosynthesis. LHC, light harvesting pigment-protein complex; P680 and P700, energy converting special chlorophyll molecules in the reaction centers of photosystem II (PS II) and photosystem I (PS I), respectively; Pheo, pheophytin; DCMU, PS II inhibitor (diuron); PQ, plastoquinone; PC, plastocyanin; Fd, ferredoxin

Chlorophyll is the major antenna pigment, funneling the absorbed light energy into the reactions centers, where photochemical conversion of the excitation energy takes place (see Fig. 8). The indicator function of chlorophyll fluorescence arises from the fact that fluorescence emission is complementary to the alternative pathways of de-excitation, which are photochemistry and heat dissipation. Generally speaking, fluorescence yield is highest when the yields of photochemistry and heat dissipation are lowest. Hence, changes in fluorescence yield reflect changes in photochemical efficiency and heat dissipation. In practice, the variable part of chlorophyll fluorescence originates mainly in photosystem II and excitation transfer to photosystem I may be considered an additional competitive pathway of de-excitation.

Measuring chlorophyll fluorescence is rather simple: The emission extends from 650 nm to 760 nm, and if shorter wavelength excitation light is used, separation of fluorescence from the measuring light is readily achieved with the help of optical filters. In the ToxY-PAM blue light is used for excitation and fluorescence is assessed at wavelengths above 650 nm. The challenge arises with the necessity to apply very strong light for the so-called 'quenching analysis'. For this purpose the PAM measuring principle has been developed.

From the viewpoint of fluorescence emission there are two fundamentally different types of competing de-excitation processes:

- photochemical energy conversion at the PS II centers
- non-photochemical loss of excitation energy at the antenna and reaction center levels

By both mechanisms, the maximal potential fluorescence yield is 'quenched' and, hence, 'photochemical' and 'non-photochemical fluorescence quenching' can be distinguished. For interpretation of

fluorescence changes, it is essential to know the relative contributions of these two different quenching mechanisms to the overall effect. If, for example, fluorescence yield declines, this may be caused by

- an increase of the photochemical rate at the cost of fluorescence and heat-dissipation
- or an increase of heat-dissipation at the cost of fluorescence and photochemistry

These two possibilities can be distinguished by the so-called 'saturation pulse method':

With a very strong pulse of white light the electron transport chain between the two photosystems can be quickly fully reduced, such that the acceptors of PS II become exhausted. Hence, during the saturation pulse photochemical fluorescence quenching becomes zero and any remaining quenching must be nonphotochemical. It is assumed that changes in nonphotochemical quenching are too slow to become effective within the short (0.2-0.8) duration of a saturation pulse.

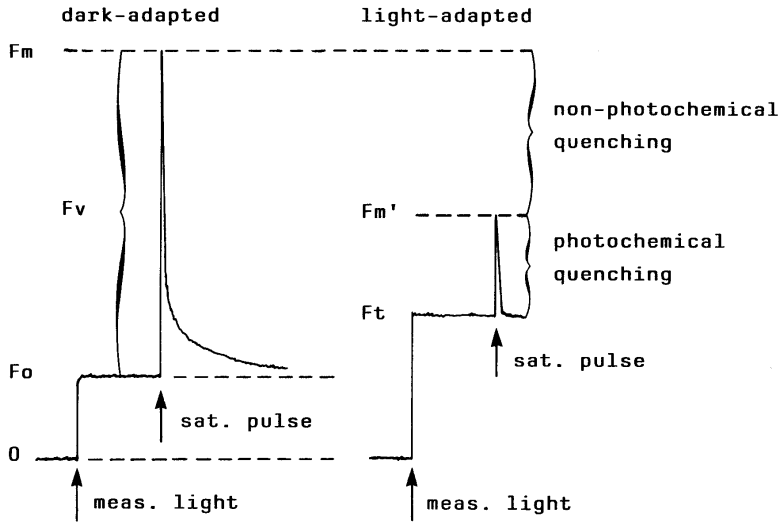


Fig. 9: Principle of saturation pulse quenching analysis

In recent years, evidence from a number of research groups has shown that the overall quantum yield of photochemical energy conversion can be assessed by the simple expression:

$$\mathbf{YIELD = (Fm\text{-}F)/Fm = \Delta F/Fm}$$

where F can correspond to Fo (dark-adapted) or Ft (illuminated at any time,t) and Fm, when measured in an illuminated sample, corresponds to Fm'.

This expression, which was introduced by Genty et al. (1989) is identical to the Y-parameter measured by the ToxY-PAM. The ToxY-PAM is specialized to compare the Y-value of an investigated sample with that of a reference sample and, hence, to determine the relative inhibition of the investigated sample with respect to the reference sample. With this fluorometer, determination of Inhibition % has become exceedingly simple: The two cuvettes with investigated and reference samples are placed into the measuring

chambers, covered with the provided hoods and the START-key is pressed. Everything else is proceeding automatically within 10 seconds:

- the fluorescence yield F1 of the investigated sample in Channel 1 (Ch1) is sampled
- a saturation pulse is applied and Fm1 is sampled
- the fluorescence yield F2 of the reference sample in Channel 2 (Ch2) is sampled
- another saturation pulse is applied and Fm2 is sampled
- the effective quantum yields, Y1 and Y2, of investigated and reference samples, respectively, are calculated from the equation Y=(Fm-F)/Fm
- the relative inhibition of the investigated sample with respect to the reference sample is calculated from the equation:

Inhibition % = 100(Y2-Y1)/Y2

- the equivalent concentration of the standard photosystem II inhibitor diuron is calculated on the basis of a stored calibration factor (Diuron-Factor preset at 0.114 µg Diuron l^{-1} being equivalent to 1 % inhibition)
- all measured and calculated fluorescence parameters are displayed on the LC-display of the instrument and on the PC-monitor screen under ToxyWin
- under ToxyWin all data are stored in a so-called Report-file and, if desired, averages of a number of measurements can be calculated and the standard deviations determined
- all data are also stored in the Memory of the instrument, from where they can be transferred on a PC under ToxyWin

Since the introduction of the PAM Fluorometer in 1985, there has been a boom in chlorophyll fluorescence research, at the basic as well as at the applied level. This is reflected in a large number of publications, which have given rise to considerable progress in the understanding of the chlorophyll fluorescence indicator function and of the photosynthesis process as well. At the end of this handbook (chapter 13) a list of literature on chlorophyll fluorescence and related topics is given. This literature may be useful to become informed in more detail on chlorophyll fluorescence and its relationship to photosynthesis.

11.2 The PAM measuring principle

With conventional chlorophyll fluorometers, the same light is used for driving photosynthesis and for exciting fluorescence. Separation of fluorescence from stray excitation light then is achieved by appropriate combinations of optical filters (e.g. excitation by blue light and protection of the detector by a red filter, which only passes the red fluorescence). Such conventional fluorometers measure the intensity of chlorophyll fluorescence. However, for determination of the effective quantum yield of photosynthetic energy conversion it is necessary to measure fluorescence yield at largely different light intensities, which are applied with the purpose of causing changes in fluorescence yield rather than of measuring it. In order to distinguish between fluorescence and other types of light reaching the photodetector, fluorescence excitation can be 'modulated': When a special 'measuring beam' is rapidly switched on/off, the fluorescence signal follows this on/off pattern and with the help of suitable electronic devices the resulting modulated signal can be separated. Standard devices for this purpose are lock-in amplifiers which tolerate background signals several hundred times larger than the

fluorescence signal. For the extreme requirements of chlorophyll fluorescence quenching analysis by the so-called saturation pulse method, a new modulation principle was developed which tolerates a ratio of $1:10^5$ or even higher between fluorescence and background signal. Fluorescence is excited by very brief but strong light pulses from light-emitting diodes. With the ToxY-PAM, individual measuring pulses are 5 µs long and applied at largely varying frequencies, such that largely different effective light intensities can be obtained at identical intensities of the individual measuring light pulses. A highly selective pulse amplification system ignores all signals except the fluorescence excited during the 5 µs measuring pulses.

12 Appendix

12.1 Technical specifications

Dual-Channel Photosynthesis Yield Analyzer ToxY-PAM (standard version)

Measuring light	source: 10 light emitting diodes per channel, emission maximum at 470 nm; 12 x 12 settings of effective intensities set via frequency and amplitude of 5 µs measuring light pulses; standard intensity 10 µmol $m^{-2}s^{-1}$ PAR; ML-High function causing frequency as well as integrated intensity increase by factor of 20
Signal detection:	two PIN-photodiodes protected by long-pass filters (λ>640 nm); selective window amplifiers (patented)
Saturation pulses:	for the duration of a saturation pulse (standard width 0.4 s) all LEDs are driven at maximal intensity resulting in ca. 2000 µmol quanta $m^{-2}s^{-1}$.
Measured and calculated parameters:	steady-state fluorescence yields, F1 and F2, maximal fluorescence yields, Fm1, Fm2, effective quantum yields, Y1 and Y2, Inhibition % and Diuron-equivalents (µg diuron l^{-1})
Peak detection:	optional function to determine Fm as peak value of moving average over 150 msec
Pre-Pulse:	optional function to apply short (standard length 5 ms) saturating flash 1.4 sec before assessment of steady-state fluorescence yields F1 and F2

Microcontroller:	CMOS 80C52
Display:	2 x 16 character alphanumerical LC-display with backlight; character size 4.5 mm
User interface:	2 x 4 tactile keypad
Power supply:	Internal rechargeable battery 12 V/2 Ah, providing power for ca. 24 hours of continuous off-line operation; automatic power/off when not used for 4 min; Battery Charger MINI-PAM/L
PC-terminal operation:	Via RS 232 interface using dedicated ToxyWin-software
Data output and transfer:	Transfer on PC via RS 232 using ToxyWin-software
Dimensions:	17 cm x 11.5 cm x 13 cm (L x W x H)
Weight:	1.8 kg
Permissible ambient temperature:	-5 to 45 °C

Windows-Software ToxyWin

for online PC-operation via RS 232-interface

Features:	Report-window Chart-window Settings-window Clock-function Online and offline Averaging functions Calculation of Standard Deviation Export to clip-board Data storage and printout

Battery Charger MINI-PAM/L

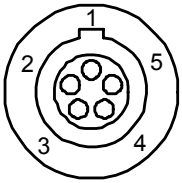
Power Supply:	100 to 240 V AC, 50/60 Hz
Output:	18 V/45 W
Dimensions:	13.5 cm x 6 cm x 3.6 cm (L x W x H)
Weight:	0.26 kg

Transport Case ToxY-PAM/T

Design:	Plastic case with custom foam packing
Dimensions:	42.5 cm x 34 cm x 13.5 cm (L x W x H)
Weight:	1.9 kg

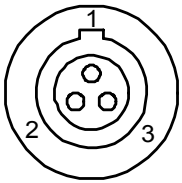
12.2 PIN-assignments

"RS 232"



1: Not used
2: Not used
3: TxD
4: RxD
5: GND

"CHARGE"



1: Charge input +18 V
2: GND
3: External input +12 V (max. 13.8 V). ATTENTION: Internal battery cannot be charged via this input.

13 Literature on chlorophyll fluorescence, herbicide action and related topics

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14 Rechargeable battery

The Dual-Channel-Yield-Analyzer ToxY-PAM 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!**

15 Warranty conditions

All products supplied by the Heinz Walz GmbH, Germany, are warranted by Heinz Walz GmbH, Germany to be free from defects in material and workmanship for one (1) year from the shipping date (date on invoice).

The warranty is subject to the following conditions:

1. This warranty applies if the defects are called to the attention of Heinz Walz GmbH, Germany, in writing within one year (1) of the shipping date of the product.
2. This warranty 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.
3. This warranty shall not apply to any product supplied by the Heinz Walz GmbH, Germany which has been subjected to misuse, abuse, abnormal use, negligence, alteration or accident.
4. This warranty does not apply to damage caused from improper packaging during shipment or any natural acts of God.
5. This warranty does not apply to underwater cables, batteries, fiberoptic cables, lamps, gas filters, thermocouples, fuses or calibrations.

To obtain warranty service, please follow the instructions below:

1. The Warranty Registration form must be completed and returned to Heinz Walz GmbH, Germany.
2. 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, custom duties,

and/or shipping costs incurred in returning equipment for warranty service are at customer expense.

3. All products being returned for warranty service must be carefully packed and sent freight prepaid.

4. 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.