**Pulse Amplitude Modulated (PAM) Fluorometry**

**Purpose/Aim**

Pulse amplitude modulation, or PAM, fluorometry operates on a basic signal modulation in which light is delivered in a series of signal pulses. The method can be used in photobiology and can obtain spectrofluorometric measurements of the kinetics of fluorescence by measuring the increases and decay of the light harvesting antennae of the thylakoid membranes (Maxwell and Johnson, 2000). Biologists use PAM fluorometry to provide a fast assessment of the overall photosynthetic state through comparison of photosynthetic rates based on fluorescence. Specifically, the measurement of fluorescence is often used as an indicator of the early effects of stress on autotroph photosystems caused by varying environmental conditions.

**Background**

Chlorophyll fluorescence is an indicator of photosynthetic energy conversion in higher plants, algae and bacteria (Consalvey et al., 2005). PAM fluorometers are designed specifically to measure chlorophyll fluorescence yield at a high degree of sensitivity. PAM measurements also are capable of differentiating between fluorescence from photochemical quenching as opposed to heat dissipation or non-photochemical quenching (NPQ) (Schreiber, 2004).

Hans Kautsky was the first to associate fluorescence with photosynthesis in 1931 through the illumination of dark adapted leaves and its correlation with CO₂ assimilation (Kautsky and Hirsch, 1931). Since its discovery in the 1960s, analysis of chlorophyll has been used as a fundamental method in understanding the reaction mechanics of photosynthesis as well as assessing plant stress physiology, ecophysiology and phytopathology (Enriquez and Borowitzka, 2010; Maxwell and Johnson, 2000). Tasks that, until the use of chlorophyll were severely limited (Schreiber, 2004).

All PAM fluorometers work under the principle in that they produce a series of modulated pulses of measuring light (useconds in duration) that induces fluorescence excitation. Another set of saturating light pulses is sent out that briefly suppresses the photochemical yield to zero, thus inducing a maximum fluorescence yield from which photosynthesis can be measured. PAM fluorometers expose samples to three different types of light. In order to distinguish between fluorescence and the ambient light, the fluorescent excitation must be modulated.

1. **Measuring Light** – a weak measuring light (0.15 mmol photons m⁻² s⁻¹) that induces fluorescence but not photosynthesis. The fluorescence emitted from this light is termed minimum fluorescence or F₀ and is used to measure the proportion of open reaction centres.
2. **Saturating Pulse** – a short pulse of light (>10000 mmol photons m⁻² s⁻¹) that closes all reaction centres resulting in maximum fluorescence (Fₘ).
3. **Actinic light** – ranging up to 2000 mmol photons m⁻² s⁻¹ and is used to induce and manipulate photosynthesis.

The fluorescence measuring features are very short, within the range of μ seconds and can be manipulated repetitively and at alternate frequencies. The fluorescence is then detected by a photodiode detector.

Another method of measuring photosynthesis that utilises a fundamentally different approach is the fast repetition rate fluorometer (FRRF) (Smith et al., 2004). The FRRF delivers a rapid series of flashes of light over 150-400 µs to obtain Fₘ, causing a single turnover of most reaction centres. By contrast, the PAM delivers a longer saturating pulse lasting 0.8 s which induces multiple turnovers of the reaction centres. As a result the FRRF produces a lower Fₘ and hence Fₘ/Fₐ than the PAM fluorometer.

**The PAM unit**

PAM units generally consists of three primary components:

**The PAM-CONTROL universal control unit**

This is generally the largest component of the instrument and houses the electronics that run the PAM. It can be used as a stand-alone means of measuring fluorescence or in conjunction with a PC.

**Fluorescence emitter-detector unit**

The emitter-detector unit contains an array of LED diodes with red (650-660nm) for fluorescence excitation, actinic illumination and saturation pulses. It also contains diodes in the far-red region of the spectrum for preferential excitation of PSI. The emitter detector unit also contains a highly sensitive photomultiplier that measures fluorescence emissions at wavelengths greater than 710nm.

**WinControl Windows software for operation with PC**
Optional control of PAM via PC.

Details concerning setup can be found in handling operation manuals:


Figure 1. a. Water-PAM Control unit and emitter-detector unit (with 15mm quartz cuvette), connected to PC; b. Diving-PAM connected to emitter detector unit. From www.walz.com.

Software

The PAM-Control unit is controlled efficiently via PC through WinControl (Walz GmbH, Effeltrich, Germany), widows software developed for the on-line operation of PAM-Control of fluorometers (Fig.2.). A PC can be connected to a PAM-Control unit via a RS 232 interface cable there forth allowing accessibility externally by PC. Settings changes made by PC will also be changed in the PAM-Control unit and vice versa. Data is automatically recorded by WinControl and allows calculation of fluorescence ratio parameters calculated from saturation pulse analysis as well as the real time creation, recording and display of F-E curves. All data is written into a report file similar to that of the stand-alone PAM unit. An extensive separate user’s manual is provided for the WinControl Windows-software (Heinz Walz GmbH, 2000). The most recent versions of WinControl software are available through the Walz homepage.

Figure 2. Wincontrol interface – main settings window. Image from www.walz.com.

Status of standard method
For standard methodology consult the PAM-Control Universal Control Unit Handbook of Operation for the respective PAM unit being utilised.

- Microscopy-PAM Handbook of Operations.
- Water-PAM Handbook of Operations
- Diving-PAM Handbook of Operations

A working knowledge of photosystems and photosynthetic parameters is recommended and can be ascertained through Chlorophyll fluorescence – a practical guide by Maxwell and Johnson (2000).

**Taxa**

PAM fluorometry is a rapid means for measuring photosynthesis in a variety of autotrophic taxa ranging from seaweeds, seagrasses and other macroalgae to marine phytoplankton, and ice algae and cyanobacteria. PAM modules have been developed as a non-intrusive means to measure fluorescence in organisms ranging from single cells to entire leaves. For example:

- **Diving-PAM** – A unique instrument designed for studying in situ photosynthesis of underwater plants, including sea grasses, macroalgae and zooxanthellae in corals.
- **Water-PAM** – Is used to analyse suspension samples of phytoplankton and algae that require a high degree of sensitivity.
- **Phyto-PAM** – Determines chlorophyll in surface waters and can also differentiate between differently pigmented groups of algae (green, algae, diatoms and cyanobacteria).

A full list of PAM units is available at Walz Mess- und Regeltechnik.

**Implementation**

PAM units themselves are the most expensive investment made when conducting PAM fluorometric analysis. The cost of performing the analysis in generally inexpensive in terms of expendables. Not a large amount of specific training is required for general use, however, an understanding of photosynthetic parameters is recommended. Introductory and specialised PAM workshops are held on occasion at various institutes. For further information on operation consult:

- Microscopy-PAM Handbook of Operations.
- Water-PAM Handbook of Operations
- Diving-PAM Handbook of Operations

Fluorometry has two main advantages over conventional spectrophotometry. These are specificity and sensitivity (Drees, and Wu, 2005). Specificity is increased in fluorometry through the selection of the optimal wavelength for both fluorescence and absorbance as opposed to solely absorbance. Fluorometry is also approximately 1000 times more sensitive than spectrophotometric methods (Drees and Wu, 2005). More specifically, PAM fluorometry is advantageous as it is a rapid and non-invasive method method that can act as an early indicator of environmental stress. PAM instruments are also portable and can even be used submerged.

The primary disadvantage to fluorometry, however, is that it is highly sensitive to environmental changes such as those of pH and temperature as well as the loss of electrons through collisions rather than fluorescence. Contamination of samples with solvents or UV radiation can all induce photochemical changes (Drees, and Wu, 2005). Due to this sensitivity it is imperative that care with analytical technique is taken.

**Data Collection**

The sample is typically dark adapted, a dark adaptation period of 30 minutes has been universally recognised as appropriate, although this is dependent and may also depend on the irradiiances that samples have previously been exposed to (Consalvey et al., 2005). Sample is then placed at the emitter-detector unit. Saturation pulses and rapid light curves (RLCs) are generally the most common tasks completed by the PAM unit. Data is logged in the PAM unit. This unit is capable of storing up to around 5000 lines of data, or 50 RLCs.

PAM rapidly produces a large amount of data that is automatically written into the ‘Report file’ of WinControl and in the memory of PAM-CONTROL. All data that is collected during the standalone operation of PAM-CONTROL can be readily transferred to PC via WinControl.

Data collected includes:

- Fluorescence yield \( (F_0) \) - measured briefly before the last saturating light pulse.
- Maximal fluorescence yield \( (F_m) \) measured during the last saturating light pulse.
- Temperature in degree Celsius, display of which requires optional temperature sensor.
- Momentary fluorescence yield displaying small fluctuations.
- Maximum quantum yield \( (Fv/Fm) \) of a dark adapted sample
- Electron transport rate (ETR)
Light intensity in terms of PAR (quantum flux density of photosynthetically active radiation)

Commonly used parameters include:

- Fv/Fm = inversely proportional to stress
- Rapid light curves = used for the assessment of photosynthetic activity under different light conditions
- Quenching analysis = used to understand the amount of light that is dissipated as heat to prevent damage
- Fast kinetics = used to understand some of the details regarding the fundamental structure of the photosystems and reaction centres

Consalvey et al. (2005) and Maxwell and Johnson (2000) provide a comprehensive account of photosynthetic parameters and their uses in assessing photosynthetic capacity.

Producing a Rapid Light Curve

A common and one of the more useful functions provided by PAM fluorometry is the rapid light curve (RLC). The PAM unit emits a red light from a light emitting diode (LED) with a peak emission at 650 nm. This acts as a measuring light. Chlorophyll a fluorescence is detected at wavelengths greater than 710 nm (Ralph and Gademan, 2005).

RLCs are measured using a preinstalled software routine where the actinic illumination is increased incrementally in eight steps plus an additional initial quasi-darkness measurement. Irradiance begins at a user defined irradiance (LC-INT from 1-5) for a prescribed duration, 10 seconds is common (Ralph and Gademan, 2005). LC-INT sets the initial irradiance and the subsequent irradiances that will comprise the parameters of the RLC.

The instrument's sensitivity can be altered by the user by adjusting the electronic signal gain. Increasing GAIN amplifies the signal coming from the sample, however, also increases the noise signal in proportion (Heinz Walz GmbH., 1998, 2000).

rETR should reach a plateau of at least two points and a decline over the last couple light increments indicative of photoinhibition although this is dependent on irradiance and adaptation level. RLCs on shade adapted samples would begin at LC-INT = 1 (low irradiance) whereas a light adapted sample would be exposed to a higher LC-INT to promote saturation and some degree of down regulation of photosystems.

Samples are shade adapted. For water PAM this is generally achieved by placing samples in a dark environment while diving PAM units utilise purpose-built clips. 20-30 minutes is generally accepted as appropriate dark adaptation time although this is dependent on taxa and prior irradiance exposure (Ralph and Gademan 2005). This ensures that PSII is indicative of the current acclimation state (Schreiber et al 1997).

RLCs take approximately 90 seconds to complete and record parameters F, Fm, FPSII and ETR that can be plotted against PAR irradiance. Data can be viewed through wincontrol software.

Data Processing and Analysis

PAM data should undergo some form of quality control before it is fitted to an exponential function. This generally involves the removal of obviously erroneous or erratic data points produced by noise in the sample. To prevent the use of incorrect values for absorbance and the fraction of that light absorbed by PSII, a new parameter, relative ETR (rETR) has been proposed (Beer et al., 2001) and is now widely accepted as common practice in PAM data processing. Relative electron transfer rate rETR values are calculated as the product of effective photochemical efficiency and the irradiance (Enriquez and Borowitzka, 2010).

P-E curves should generally exhibit a linear increase that eventual plateaus over at least two points before declining as photoinhibition sets in. It is not uncommon to remove the last point of photoinhibition. Curves that do not show these steady increases should be discarded.
Figure 3. P-E curve showing the response of photosynthetic parameters to light. Image from and explanation of parameters available in Consalvey et al. (2005).

To quantitatively compare RLCs a number of parameters must be defined such as alpha, Ek and rETRmax (Ralph et al., 2002). rETR and PAR data is fitted to one of a number of functions that describe resource limited growth. The most commonly used is the exponential function of Platt et al. (1980) (Fig.3).

$$rETR = rETR_{max} x (1-exp(-\alpha x PAR / rETR_{max}))$$

This function assumes that there is no photoinhibition. If the final rETR point declines, it should be removed from the analysis.

Products

Walz (Walz GambH, Effeltrich, Germany) was founded by Heinz Walz on July 1, 1972 and is one of the leading manufacturers in PAM fluorometer technology today.

The first PAM fluorometer (the PAM-101 chlorophyll fluorometer) was designed by Ulrich Schreiber and built by Walz in 1985 (Schreiber, 1986). These PAM units were used for terrestrial plants (PAM-101, 102, 103). The popular PAM 2000 was introduced in 1991.

The first PAM fluorometer units modified for use with marine plants were the Diving-PAM which was introduced in 1997 and the Water-PAM later introduced in 1999.

Later modifications gave rise to the Imaging-PAM series that are capable of a two dimensional analysis. These were developed by Oxborough and Baker (1997) and were commercialised by Walz (Walz GambH, Effeltrich, Germany) in 2003.

Follow this link for a full list of Walz PAM products.

Associated links

- Walz Mess- und Regeltechnik
- NOAA's Coral health and Monitoring Program

References


Background reading


Citation

Please cite this page as:

Page contributors: Jess Melbourne-Thomas , Thomas Coad

Last modified on: Apr 11, 2014 13:55