Cookbook checklist before making NPQ and other quenching measurements.

Quenching measurement parameters, such as NPQ, are the least understood, and most often misused parameters that are available with advanced chlorophyll fluorometers. This application note is designed to improve the understanding of proper quenching protocol usage.

There are a few quenching protocols to choose from, Kramer lake model, Hendrickson lake model, puddle model, and quenching relaxation protocols. For an in depth discussion of the differences, and advantages of each, please request the Opti-Sciences Quenching application note at www.optisci.com.

To get reliable measurements, one should follow tested guidelines.

1. Dark-adapt properly knowing the plant’s light history. It takes only a few minutes for the xanthophyll cycle and the ΔpH of the thylakoid lumen to return to a dark-adapted state. State transitions, however, take between fifteen to twenty minutes. These times can vary somewhat in field plants and can take slightly longer (Baker 2004). In addition, field plants and other plants that have been exposed to photoinhibition conditions for a number of hours, will retain a certain amount of NPQ for up to 30 to 60 hours (Lichtenthaler 2004). This means that even if dark adaptation is overnight, there will almost always be some residual NPQ built into summer field measurements of Fv/Fm, and other displayed quenching parameters. For this reason, it is important to only compare samples with a similar light history. When doing quenching measurements on field plants, it is common for researchers to choose pre-dawn dark adaptation values. Shorter times are not normally used to study quenching. (For more information, see dark adaptation application note.)

2. Samples that are compared, must have the same Fv/Fm values. Quenching measurements of different samples with different Fv/Fm values should not be compared (Baker 2008). Fv/Fm is used as the measuring standard for non-photochemical quenching measurements, and if the measuring standard is different, the quenching values are meaningless. Comparing values from samples with different Fv/Fm values is like measuring items with a ruler that has dimensions that change.

3. Modulation light intensity setting Fv/Fm is (F_M-F_O)/F_M. F_O, or minimum fluorescence is a dark-adapted value made by exposing the leaf antennae to a very low intensity modulated measuring light, that is not set high enough to drive photosynthesis. The modulation light intensity must be set correctly for best accuracy and repeatability. If it is set too high, it will drive photosynthesis and provide an F_O value that is too high. The modulated light allows the measurement of pre-photosynthetic antennae fluorescence. Maximum fluorescence is measured when exposing a leaf to a saturation flash with light intense enough to close all PSII reaction centers.
4. **Leaves must be at steady state photosynthesis for most quenching measurements.** This takes between fifteen and twenty minutes at a new light level (Maxwell and Johnson 2000) to reach steady state. For example, if there are ten saturation pulses spaced 120 seconds apart, the leaf will be exposed to the actinic light for twenty minutes after dark adaptation. Since an internal fluorometer artificial light source is normally used, the test allows one to compare below canopy leaves as long as the Fv/Fm values are the same. According to Klughammer (2008), the only non-photochemical parameter that does not have to be taken at steady state photosynthesis is Y(NO) from Hendrickson.

5. **Use a fluorometer with a stable actinic light output.** Depending on the brand and type of fluorometer, the intensity output of the actinic light can change over time. When an actinic light is on, it can heat the fluorometer and cause a lowering of the light output. The intensity of the actinic LED light source output changes as the heat from the lamp changes the lamp temperature. More advanced systems have ways to ensure a steady actinic light level.

6. **Y(II) values vary with light level and with temperature.** The higher the light level, the lower the Y(II) value. When measuring Y(II) in the field, it is extremely important to measure leaf irradiation or light level at the leaf, and leaf temperature. Comparing Y(II) values taken at different light levels, and different temperature levels, introduces a significant error, unless it is the change at different light levels, and heat levels, that is of interest. This is commonly done with a PAR Clip.

7. **Shade leaves vs. Sun leaves.** The Y(II) ratio will be higher on Sun leaves than on shade leaves (Lichtenthaler 2004).

8. **Only plants with the same Fv/FM should be compared** due to light history. (Lichtenthaler 2004) (Baker 2008)

9. **Leaf orientation is not important because an artificial actinic light source is used.**

10. **It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants** (Reuter and Robinson 1997). Other sampling plans are also used.

11. **The duration of the saturation pulse** should be between 0.5 seconds and 1.5 seconds for higher plants (Rosenqvist and van Kooten 2006), and 25 to 50 milliseconds for Phytoplankton and cyanobacteria (Schreiber 2005). Times outside these ranges increase the error in Y(II) and quenching measurements. Shorter durations prevent complete saturation of PSII regardless of the light intensity. Longer durations create a form of saturation pulse NPQ that rounds the tail end of the saturation pulse maximum value, and reduces the average maximum saturation pulse value. (Rosenqvist and van Kooten 2006). Some fluorometers allow adjustment of this parameter, and others are preset at the factory. The OS1p and OS5p use a rolling 25 ms average of the highest Fm and Fm' values. This ensures that the correct value is reported as long as the saturation pulse width is wide enough.
12. **Saturation pulse intensity**. Saturation pulse intensity is more of an issue with Y(II) than with Fv/Fm. While shade leaves will saturate at a few hundred \( \mu \)mols, sun leaves will usually saturate below 1,500\( \mu \)mols (Ralph 2005). A very intense saturation flash intensity does not damage light adapted samples, but may damage dark-adapted samples under cold stress conditions, if the saturation flash happens too frequently in the dark. Therefore, it is recommended that maximum intensity should be used for all quenching measurements. Saturation pulses used at night, should be at least ten minutes apart to one hour apart to prevent photo-damage from saturation pulses (Porcar-Castell A. 2008).

13. **The time between saturation pulses is important**. Rosenqvist and van Kooten (2006) state that between one to two minutes is required for complete relaxation of saturation pulse NPQ. If saturation pulses are not separated by this distance range, then an error caused by this type of saturation pulse NPQ will result. It will accumulate with each saturation pulse. When in doubt, space saturation pulses 120 seconds apart or more.

14. **Overlap of PSI fluorescence**. Part of the minimum fluorescence, the \( F_0 \) parameter, in Fv/Fm \( ((F_M - F_O)/F_M) \), contains PSI fluorescence as well as PSII fluorescence. With Fv/Fm, one is trying to measure the maximum variable fluorescence of PSII in a dark-adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces an error. In C\(_3\) plants, about 30% of \( F_0 \) fluorescence is due to PSI, and in C\(_4\) plants about 50% of \( F_0 \) fluorescence is due to PSI fluorescence. PSI produces about 6% of the fluorescence found in \( F_M \) in C\(_3\) plants, and about 12% in C\(_4\) plants. (Pfundle 1998). This not a problem when comparing quenching measurements for plant stress because, PSI fluorescence does not change with light level or plant stress.

15. **PAR** is photosynthetically active radiation. Radiation on the leaf is measured between the wavelengths of 400nm to 700 nm. PAR sensors, thermocouples and thermisters used for measuring light irradiation and temperature are calibrated to other instruments that are traceable to the National standards. Since Y(II) and quenching parameters change with light and temperature, as well as plant stress levels, there are advantages to using a shrouded leaf and PAR Clip when making quenching measurements.

16. **Leaf fluorescence heterogeneity**, presents itself as different measurements on different parts of the leaf. It has been found to occur under cold stress conditions, biotic stress, and under water stress conditions. By using multiple measurements and a sampling plan, heterogeneity can be overcome (Buschmann C. in correspondence by e-mail 2008). Imaging fluorescence can also be used.
17. Far-red pre-illumination. Some fluorometers have the ability to pre-illuminate dark-adapted leaves with far-red light. When this feature is used for five to ten seconds before an Fv/Fm measurement takes place. It activates PSI, and ensures that all electrons have been drained from PSII before the measurement of Fo. While this feature ensures that PSII is completely re-oxidized, it does not relax the xanthophyll cycle, state transitions, or photoinhibition. Time is still required in a darkened environment to relax all forms of NPQ and to obtain reliable quenching values.

18. Far-red illumination. This is usually used in the post actinic light mode to allow measurement of Fo’, a parameter that reflects quenched Fo. This value is used in Kramer lake model parameters, and puddle model qN. It is not used in Hendrickson simplified lake model parameters, or in NPQ.

The best experiments are ones that take these issues into account. PSI fluorescence is involved in all measurements. It does not vary with light level or plant stress (Schreiber 2004). With this in mind, comparing samples with similar light histories allows comparison of many types of plant stress.

The Plant Stress Guide provided by Opti-Sciences www.optisci.com, references papers that deal with specific types of plant stress and limitations of different chlorophyll fluorescence parameters for measuring plant stress.

Bibliography


Buschmann C. (2008) in correspondence by e-mail, Dr. Buschmann recommended taking multiple measurements per leaf to find potential infection locations as a substitute for fluorescence leaf imaging: Dr. Claus Buschmann (Priv.Doiz), Botanik 2, Universität Karlsruhe (TH), 76128 Karlsruhe (Germany).


