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Photosynthesis of Submerged and Surface Leaves of the Dwarf Water Lily (Nymphoides aquatica) Using PAM Fluorometry

Tharawit Wuthirak¹ Raymond J. Ritchie²*

1. Department of Biology, Faculty of Science, Prince of Songkla University, Hat Yai, 90112, Thailand
2. Tropical Plant Biology Unit, Faculty of Technology and Environment, Prince of Songkla University, Phuket, 83120, Thailand

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1. Introduction

The Dwarf Water Lily (Nymphoides aquatica (J.F. Gmel) Kuntze, Menyanthaceae) are ubiquitous aquatic plants, originally from SE North America but its use as an ornamental has now distributed it worldwide throughout temperate and tropical habitats. The Menyanthaceae are members of the Asterales and so the family is not part of the archaic basal group of angiosperms that includes the...
Nymphaeaceae [1-3]. The Blue Water Lily (Nymphaea caerulescens) and the Dwarf Water Lily are not closely related and might not be expected to have similar physiological characteristics due to a shared habitat and superficially similar appearance of the surface leaves. Unlike Nymphaea caerulescens, N. aquatica has morphologically different floating leaves as well as mature functional submerged leaves. The leaves of some aquatic plants with distinctly different emergent or floating leaves compared to their fully submerged leaves are known to have different photosynthetic physiologies [4,5].

Some aquatic vascular plants such as Isoetes species and Littorella uniflora have a form of CAM (Crassulacean Acid Metabolism) known as Submerged Aquatic Macrophyte (SAM) metabolism [4,6-14]. Very little information is available on photosynthesis of water-lilies or plants with a nymphoid (water lily-like) morphology (true water lilies: Nymphaeaceae; dwarf water lilies: Menyanthaceae; Lotus lily (Nelumbo nucifera); Nelumboaceae) [4,14-18]. Keeley and Morton [4] found no significant nocturnal carbon fixation in Nuphar polysepalum (Nymphaeaceae) in their survey of SAM-physiology in aquatic plants. The blue water lily, Nymphaea caerulea, is also not a SAM plant [19] despite circumstantial evidence from its 13C/12C ratio [19]. Longstreth [20] reported that no example of SAM physiology had yet been found in floating or emergent leaves of aquatic plants and it does not appear that any such plants have since been identified.

Known SAM metabolism plants have a physiology closer to facultative CAM plants than obligate CAM species because they only exhibit SAM metabolism under certain conditions [21-26]. The semi-aquatic fern ally Stylites sp. (now part of Isoetes) has no functional stomata and relies on CO₂ from its roots and a SAM metabolism [8]. The fern ally, Isoetes species have functional stomata when growing aerially (where it behaves as a typical C3 plant) but not under water. Submerged leaves of Isoetes species exhibit SAM metabolism with nocturnal C4 carbon fixation and using CO₂ from its roots and not the water column [6,7,10,13]. Another fern ally, Littorella uniflora growing on moist mud in the air does not exhibit the SAM metabolism characteristics of submerged plants [9,13]. Thus some SAM metabolism plants obtain much of their CO₂ supply from their roots rather than from the water column or the atmosphere but there might not necessarily be a definite connection between the presence of SAM metabolism and use of CO₂ obtained by their roots. Lobelia dortmanni lacks stomates but nevertheless is an example of a submerged aquatic vascular plant that does not have SAM/CAM metabolism [27].

In the case of aquatic plants such as N. aquatica which has both floating and emergent leaves it would be reasonable to expect considerable physiological differences between floating and emergent leaves [28-30]. If an aquatic plant with surface leaves and submerged leaves connected by aerenchyma had SAM metabolism it would have four potential sources of inorganic carbon: (1) atmospheric CO₂ during the day, (2) atmospheric CO₂ fixed as C4 acids at night, (3) CO₂/HCO₃⁻ from the water column and/or (4) CO₂ arising from the roots buried in sediment and delivered to the leaves through the aerenchyma [15]. In the case of N. aquatica the submerged leaves could be getting their CO₂ supply from mechanisms (3) and (4) above, the later by thermosmotic air flow from the surface leaves to the roots and back to the submerged leaves [15,16,20,31-34].

Gas flow through the petioles, stems and leaves of aquatic plants, particularly species of Nymphoides, Nymphaeaceae, Nelumbo and mangroves has long been a source of fascination but is not well understood [30]. Pressure changes (as much as 1 kPa ~ 3 kPa) and mass flow are basically caused by thermal and humidity gradients (thermosmosis) but there are conflicting findings concerning their effects on the physiology of aquatic and amphibious plants. In N. aquatica and Nymphaea caerulea the direction of flow is from young leaves, through their petioles to the rhizome and then up the petioles of the older leaves to the mature leaves carrying some CO₂ from the mud to the mature leaves: this would account for the anomalous 13C/12C ratio found by Troxler and Richards [19].

Measurements of photosynthesis based on fluorescence methods (Pulse Amplitude Modulation-PAM fluorometry) measure the actual light reactions of photosynthesis and do not involve gas exchange measurements. PAM fluorometers can be used to monitor photosynthesis in terrestrial plants and in most photo-oxygenic algae: the key advantage of the technique is that PAM fluorometry directly measures the light reactions of PSII. PAM fluorometry is also non-destructive and large amounts of data can be collected very quickly [18,35-42]. PAM fluorometers measure the photons of light that are emitted as far-red fluorescence (~690 nm) from a flash of LED diode blue or red light or quartz halogen light and so actually measure the light reactions by subtraction (absorbed minus fluorescent photons).

Two other very important parameters calculated by PAM methods are the Electron Transport Rate (ETR) and non-photochemical quenching (NPQ). The ETR is an estimate of the number of electrons passing through Photosystem II (4 electrons pass through PSII per O₂ produced in photosynthesis from 2H₂O) and so can be used as an estimate of the Photosynthetic Oxygen Evolution Rate (POER). This is a high estimate of gross photosynthesis...
(Pg) because it does not take into account oxygen consumption by photorespiration or possible Mehler reactions \[^{[43]}\]. PAM measurements cannot measure \(O_2\) consumption by photorespiration, mitochondrial respiration or by Mehler reactions. Non-Photochemical Quenching is related to the magnitude of the Proton Motive Force (PMF) that exists across the thylakoids in chloroplasts and the loss of absorbed energy as waste heat \[^{[37,44-47]}\]. The high variability of NPQ and poorly fitting kinetics makes it less useful than is sometimes supposed as a measure of photosynthetic stress \[^{[18,40,41,43,44,49]}\].

PAM fluorometers can perform measurements of the light reactions of photosynthesis very quickly \[^{[18,35-39,42,48,50,51]}\] and measure the light reactions directly and are not limited by \(O_2\) and \(CO_2\) diffusion problems \[^{[18,40]}\]. The air ventilation system makes attempts to estimate photosynthesis and respiration rates in Nymphoid plants based on gas exchange using an oxygen electrode or an IRGA problematic because of difficulties in identifying the pool of gas being used as a source of \(CO_2\). Whole-plant measurements need to be made which makes them difficult to do experimentally on anything but the smallest vascular plants. Consider the case of an IRGA probe attached to a water lily or lotus lily leaf in situ: the observed gas exchange would reflect \(CO_2\) fluxes in the interconnected aerenchyma of the whole plant, not respiration and net photosynthesis of that particular leaf \[^{[15,16,20,27,28,30-34,52]}\].

Snir et al. \[^{[14]}\] used PAM fluorometers to measure photosynthesis of emergent leaves of \(Nuphar lutea\) but did not focus on the SAM metabolism issue. Ritchie \[^{[18]}\] measured photosynthesis of the floating leaves of \(Nymphaea caerulea\). It was shown that the diurnal pattern of titratable acid in the leaves of \(Nymphaea caerulea\) was not consistent with SAM/CAM physiology and accumulation of C4 acids was not great enough to support substantial CAM physiology \[^{[18]}\].

The aim of the present study was to use PAM techniques to investigate photosynthesis in floating leaves and submerged leaves of \(N. aquatica\) and systematically determine whether either leaf type expressed significant SAM/CAM physiology. It will be shown that there is a strong diurnal effect on photosynthesis but no SAM physiology despite having fully functional surface and submerged leaves. The diurnal light curve kinetics data will be used to estimate the photosynthetic oxygen evolution rate (POER) over the course of daylight and hence model photosynthesis of a water lily bed – an important habitat for primary production in lakes, ponds and wetlands, particularly in monsoonal climates such as SE-Asia, and Northern Australia.

2. Materials and Methods

2.1 Experimental Materials

Dwarf Water Lily (\(Nymphoides aquatica\) (J.F. Gmel) Kuntze, Menyanthaceae) and the Blue Egyptian Water Lily (\(Nymphaea caerulea\), Savigny) which was the subject of a previous photosynthesis study \[^{[18]}\] are grown as decorative plants in circular earthenware lily pond bowls (≈30 L) on the Phuket campus of Prince Songkla University Phuket campus, Phuket Province, Thailand (Lat. 7°53’N, Long. 98°24’E) and collected for experiments in June to September 2016 within 30 m of the laboratory. The lily ponds were not fertilised, rely on rainwater most of the year and are topped up with tap water in the dry season. The water depth was only 100 mm ~ 150 mm. Phuket has a monsoon tropical climate and the experimental period was during the wet season (average precipitation: June 286 mm/month). Daylight lengths were about 12 h 15 min per day. Solar time for Phuket was -28 min 6s from Thailand Standard Time (GMT+7h) on 22 June 2016 \[^{[53]}\]. Emergent and submergent leaves of adult plants were used. Small submergent seedlings (leaf size ≈ 5 mm ~ 20 mm) were also available but in limited amounts not large enough for assays of acid accumulation. Leaves were removed using scissors and kept floating on water in Petri dishes then blotted dry on moist filter paper immediately before PAM measurements. Leaves had to be used soon after cutting otherwise they rapidly lost turgor and photosynthesis dropped dramatically. For photosynthesis measurements during daylight plants were used within 15 to 30 min after collection and were kept in the light before measuring photosynthesis (see Appendix Table) in contrast to the protocol as used previously for Blue Water Lily, \(Nymphaea caerulea\) \[^{[18]}\]. The protocol for night-time measurements followed previous practice of keeping them in the dark for about 10 min before measurement but care was taken to minimise the time between collection and measurement in the light of experience with leaves collected during daylight (see Appendix Table). The leaves were collected in a black bucket with a lid to minimise exposure to light.

2.2 PPFD Irradiance in Phuket

Information on the daily 400 nm ~ 700 nm PPFD irradiance experienced in Phuket has been published elsewhere \[^{[18,40,41]}\]. The method for calculating irradiances is described in Ritchie \[^{[18,54]}\] using the SMARTS software \[^{[55,56]}\]. The calculated midday irradiance at the summer solstice (22 June) was 2115 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) \[^{[18,40,41]}\]. The present study was made during the wet season and so days were typical-
ly overcast with midday irradiances about 2/3 of full sunlight (≈ 1450 μmol photons m⁻² s⁻¹ PPFD) but irradiance on cloudy days was as low as 500 μmol photons m⁻² s⁻¹ PPFD.

2.3 Chlorophyll Determinations

A small hole-punch (9.7 mm diameter) was used to collect 73.9 × 10⁶ m² buttons of N. aquatica leaf tissue as described for Nymphaea caerulea leaves [18]. Chlorophyll was extracted in Mg carbonate-neutralized ethanol and assayed using a Shimadzu UV-1601 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and assayed using the equations of Ritchie [57]. Chl a was calculated as μg Chl a m⁻² of projected leaf surface area and μg Chl a g⁻¹ FW (Table 1A) and the Chl b/a was also calculated. Chl b/a ratio is more logical than the more commonly quoted Chl ratios because Chl a is the primary pigment.

2.4 Pulse Amplitude Modulation Fluorometry

Light saturation curve measurements were made on the adaxial surfaces of floating N. aquatica leaves using a Junior PAM portable chlorophyll fluorometer (Gademann Instruments GmbH, Würzburg, Germany) fitted with a 1.5 mm diameter optic fibre and a blue diode (465±40 nm) light source. PAM parameters (Y, rETR & NPQ) were calculated using the standard settings for rapid light curves (default absorptance factor, Abₜ = 0.84, PSI/PSII allocation factor = 0.5) (Heinz Walz GmbH, Effeltrich, Germany) to calculate the relative Electron Transport Rate (rETR) [35,37,51]. Sets of PAM light curve measurements each took about 88 s to complete with 10 s between saturating flashes of light (0.8 s duration). The actinic light values were in order of increasing intensity and the standard Walz rapid light curve protocol was used (9 levels of light). Only one light saturation experiment was run on each leaf to avoid confounding effects of multiple experimental treatments and invalid estimates of Ei. The non-linear least squares fit routines (Microsoft-EXCEL) used in the present paper are available on Research Gate [58].

2.5 Absorptance Measurements Using the Reflectance-Absorptance-Transmittance (RAT) Monitor

Absorptances of vascular plants are often considerably different to the standard value of Abₜ = 0.84 [59,60] and so it is better to measure them experimentally. Our absorptance values for N. aquatica are shown in Table 1B. As found here, experimentally measured absorptance values for blue light (Abₜ465nm) are typically found to be substantially different to the default value (in various aquatic plants [56,59], Nymphaea caerulea [62] and Wolffia arrhiza [48]).

2.6 Experimental Protocol

The routine protocol used for rapid light curves in our laboratory was to measure light curves in situ on the intact plant (Oil Palm [42]) or to cut leaves and place them in moistened filter paper in Petri dishes in a black bag for no more than about 10 min before performing a rapid light curve (Orchids [40,63], Pineapple [41], Nymphaea caerulea [18] and Davallia angustata [49]). Longer dark preincubation protocols on cut leaves were found to be unsuitable for N. aquatica.

A single rapid light curve takes about 2 ½ to 3 minutes to perform and so eight replicate leaves collected at one time take about 20 minutes to process. It was noticed that Eₜ and ETRₜ decreased with each successive leaf in a batch if the leaves were kept in the dark. A series of experiments shown in the Appendix Table showed that cut leaves decreased rapidly in photosynthesis if kept in the dark. Both the Eₜ and POERₜ decreased in the leaves kept in the dark and so not only did photosynthesis decrease but the shape of the P vs. E curves also changed. Collecting the leaves and measuring them as soon as possible after collection and keeping them in the light was found to be the most satisfactory protocol for measuring rapid light curves on N. aquatica in the light. Leaves collected in the night-time were cut and placed in the dark following previous standard protocols such as for Nymphaea caerulea [18]. Effective Yield and ETR decreased rapidly in dehydrated leaves and so flaccid leaves were rejected.

2.7 Calculation of Photosynthetic Electron Transport Rates and Other Parameters

It is found experimentally that if fluorescence yield (Y) is plotted against irradiance (E) it follows a simple exponential decay function of the form y = e⁻sx [18,39-42,48,49,63,64]. The WinControl software calculates relative ETR (rETR) based on a default leaf absorptance factor (Abₜ = 0.84). Absorptance measurements determined experimentally can then be used to recalculate actual ETR (ETR = rETR × Abₜ465nm/Abₜ).

Since effective Y vs. Irradiance (E) obeys a simple exponential decay function [18,39-42,48,49,63,64], plots of ETR vs. E obey an exponential function of the form y = x e⁻sx. This equation is known as the Waiting-in-Line model [39,65]. Equation (1) below is a form of the Waiting-in-Line equation that is easier to fit using iterative least squares methods:
ETR = \frac{ETR_{max}E}{E_{opt}} e^{-1E/E_{opt}}

(1)

where, following previous conventions (refs above), ETR is the Electron Transport Rate as mol e⁻ m⁻² s⁻¹, \( E_{opt} \) is a scaling constant for the maximum height of the curve, E is the Irradiance (µmol photons m⁻² s⁻¹ 400 nm ~ 700 nm PPFD) and \( E_{opt} \) is the optimum irradiance that gives maximum ETR. The maximum photosynthetic efficiency (Alpha, \( \alpha_0 \)) is the initial slope of the curve at \( E = 0 \) (\( \alpha_0 = e ^{\times E_{max}/E_{opt}} \)). Perhaps a more realistic expression is the photosynthetic efficiency at optimum irradiance (\( \alpha_{E_{opt}} = ETR_{max}/E_{opt} \)). The half-maximum photosynthesis (ETR_{half-max}) is reached at 0.231961 times \( E_{opt} \) and photosynthesis is also inhibited by 50% at 2.67341 times \( E_{opt} \). Four electrons are moved through PSII for each O₂ produced in photosynthesis and so an ETR of 4 µmol e⁻ m⁻² s⁻¹ is roughly equivalent to a Photosynthetic Oxygen Evolution Rate (POER) of 1 µmol O₂ m⁻² s⁻¹.

Non-photochemical quenching (NPQ) is reputed to be a measure of the quenching of the photochemistry of photosynthesis or the energy absorbed by the photosynthetic apparatus that is not lost as fluorescence nor is it used in photosynthetic electron transport \([44,45,47]\). Only NPQ and not variable fluorescence NPQ (qN) is quoted in the present study. NPQ is calculated by the WinControl software using the equations described by Genty et al. \([44]\), van Kooten and Snel \([45]\) and Brestic and Zivcak \([47]\). NPQ can be described by simple exponential saturation curves (NPQ = \( NPQ_{max} \times (1-e ^{-kE}) \)) where, \( NPQ_{max} \) is the maximum NPQ at maximum irradiance and \( k \) is an exponential constant and E is the irradiance: the \( ½ \) irradiance point giving \( ½ \) of \( NPQ_{max} \). \( NPQ_{1/2} \) can be used to describe the shape of the curve. \( NPQ_{1/2} \) saturation values rather than \( k_{NPQ} \) are quoted in Table 2 following previous conventions \([42,48,49,63]\) Microsoft Excel® Software Files to fit yield, ETR, qP and NPQ vs. Irradiance and calculate the asymptotic errors of the fitted parameters are publically available on the internet \([49]\).

### 2.8 Titratable Acid

Titratable acid was measured in a freshweight basis (FW) based on methods described previously for Nymphaea caerulea leaves \([18]\) for Davallia angustata \([49]\) and for the orchid Vanda sp. \([63]\). Surface and submerged leaves of adult plants were sampled on a 24 h cycle, routinely boiled and then the extracts stored frozen (−20 °C) before extraction. Sufficient supplies of the very small seedling leaves were not available for titration studies. Acid was extracted in 30 mL of distilled water by heating the leaves in a hot-water bath for 30 min. After cooling, the free acid was titrated using 5 mol m⁻³ NaOH using a standard pH meter (PH-230SD, Lutron Electronic Enterprise Co. Ltd, Taipei, Taiwan) because \( N. \) aquatica produced a compound which interfered with the phenolphthalein indicator used in previous studies (≈ 30 mg L⁻¹) \([18,40,41,49,66]\). For comparative purposes, Table 1A provides the projected leaf surface area/g FW, leaf water content (g g⁻¹FW) and the freshweight/dry weight ratio of the leaves used in the present study. Dry weights of leaves were determined on standard cut leaf disks of known surface area and fresh-weight dried to constant weight at 80 °C as for \( N. \) caerulea and \( D. \) angustata \([18,49]\). Titratable acid was calculated as mol H⁺ g⁻¹ FW: this could be converted into mol H⁺ m⁻³ per unit tissue water using the data in Table 1A. Buttons of \( N. \) aquatica leaves cut with the cork borer were also used to calculate the relationship of fresh weight per unit projected surface area and water content per unit projected leaf surface area. These could be used to calculate mol H⁺ m⁻².

### 2.9 Statistics

Significant differences were found using one-way ANOVA and the Tukey Test criterion using Microsoft Excel® for the ANOVA and to write the Tukey Test routines. Zar \([67]\) was used as the standard statistical reference book.

### 3. Results

#### 3.1 Basic Information on Leaf Material

Table 1A shows the Chlorophyll a (Chl a) and water content of \( N. \) aquatica leaves used in the present study. The Chl a content of seedling leaves and submerged leaves of adult plants were not significantly different on a projected surface area basis (p > 0.05) but were only about ½ that found in surface leaves. There was a small but statistically significant differences in the Chl b/a ratio between all the leaves (surface leaves: 0.2487±0.0029 (n=48); submerged leaves: 0.2727±0.0044 (n=10); seedling leaves: 0.2860±0.0074 (n=12)). Despite the adult plant submerged leaves being noticeably thinner than floating leaves, their water contents were not significantly different on a freshweight basis.

#### 3.2 Titratable Acid

Round disks of leaves obtained with the cork borer had a surface area/FW ratio of 5.17±0.28×10⁻³ (n = 22) m² g⁻¹ FW for surface leaves and 7.73±0.49×10⁻³ m² g⁻¹ FW (n = 22) for submerged leaves. FW/DW ratio and water content of the material used for the acid content measurements was added to the other measurements of FW/DW ratios
and leaf water contents obtained in the course of other parts of the study to give overall values (Table 1A). These data were used to convert titratable acid on a freshweight basis to titratable acid per unit projected surface area of the leaves [19].

### 3.3 Light Absorption Properties of Leaves

Table 1B shows the experimentally measured Reflectance, Transmission and Absorptance properties of *N. aquatica* leaves under blue light (465 nm) using the RAT monitor. Adult surface leaves of *N. aquatica* are effectively optically black in blue light: they absorb nearly all incident blue light (Abt%465 nm = 97.0±0.8) and reflectance and transmission total only about 2%. Reflectance of seedling and submerged leaves is also very low (2% or less) but due to the noticeable transparency of the leaves to light, transmission is higher than in the surface leaves. Abt%465nm was not significantly different in seedling leaves vs. submerged leaves (90.7±1.9% vs. 91.9±2.42% respectively). Absorptances of adult leaves are ≈15% higher and submerged leaves are ≈9% higher than the default Absorptance used by the WinControl software (AbtF = 0.84) and so the actual ETR is considerably higher than relative ETR (rETR).

### 3.4 Rapid Light Curves

Figure 1 shows plots of Photosynthesis (as POER) vs. Irradiance of submerged seedling, adult submerged and surface leaves of *Nymphaea caerulea* leaves collected at local midday (11:32 solar time). The Waiting-in-Line equation was fitted using non-linear least-squares fitting of Equation (1). PAM light curve data is based on 12 leaves for the seedling and 8 leaves for the submerged leaves and 8 for surface leaves and 9 different irradiance levels. Means ±95% confidence limits of Eopt and ETRmax and POERmax of the fits are included in Table 2 along with the other statistics calculated from the rapid light curves. Relative ETR (rETR) was corrected to actual ETR using the RAT data in Table 1B and recalculated on a chlorophyll a (Chl a) basis using the Chl a m−2 data in Table 1A. ETR is expressed on a leaf surface area basis as mol e−1 m−2 s−1 whereas photosynthesis on a Chl a basis is expressed as mol O2 g−1 Chl a s−1. The surface leaves saturated at high irradiances: the maximum photosynthesis on a surface area basis was high (Eopt = 923±68 µmol photon m−2 s−1; ETRmax = 58.5±2.5 µmol e−1 m−2 s−1; POERmax = 76.1±3.3 µmol O2 g−1 Chl a s−1) based on 9 different light intensities (thus 9 × 8 = 72 data points for surface and submerged leaves). Correlation coefficients were all r > 0.7467 giving p << 0.001 for all the P vs. E curve fits. The asymptotic photosynthetic efficiency (αo) for surface leaves was 0.172±0.015 e−/photon (Table 2). Submerged adult leaves are morphologically different to surface leaves and they have much lower chlorophyll a content on a surface area basis (Table 1B). Table 2 and Figure 2 show that they have substantially lower photosynthesis (ETRmax = 58.5±2.5 µmol e−1 m−2 s−1; POERmax = 76.1±3.3 µmol O2 g−1 Chl a s−1) but the optimum irradiance is also much lower (Eopt = 419±64 µmol photon m−2 s−1) resulting in a crucial change in shape of the POER vs. E curve. Photosynthetic efficiency was very low on a surface area basis (αo = 0.0978±0.0178 e− photon−1) compared to surface leaves

### Table 1A Chlorophyll a and Water Content of *Nymphoides aquatica* leaves

|                     | Fresh weight / Dry weight Ratio | H2O mL/g Fresh weight |
|---------------------|--------------------------------|------------------------|
| Seedling leaves     | 12.50±0.78 (n=10)              | 0.920±0.005 (n=10)     |
| Submerged leaves    | 9.82±0.45 (n=39)               | 0.884±0.005 (n=39)     |
| Surface leaves      | 8.64±0.82 (n=63)               | 0.888±0.005 (n=63)     |

### Table 1B Reflectance – Absorptance–Transmittance (RAT) of *Nymphoides aquatica* leaves

|                     | %Reflection (465 nm) | %Transmission (465 nm) | %Absorptance (465 nm) |
|---------------------|---------------------|------------------------|------------------------|
| Seedling leaves     | 0.49±0.52 (n=12)    | 8.84±1.69 (n=12)       | 90.7±1.9 (n=12)        |
| Submerged leaves    | 2.18±1.44 (n=12)    | 5.88±1.77 (n=12)       | 91.9±2.4 (n=12)        |
| Surface leaves      | 2.47±0.59 (n=20)    | 1.64±0.40 (n=20)       | 97.0±0.8 (n=20)        |
but on a Chl a basis was significantly higher than for surface leaves \( (\alpha_0 = 0.261 \pm 0.046 \text{ O}_2 \text{ photon}^{-1} \text{ g Chl a m}^{-2}) \). Seedling leaves are very small and thin even compared to subsurface leaves of adult plants (Table 1A). Table 2 and Figure 2 show that they have the lowest photosynthetic rate \( (\text{ETR}_{\text{max}} = 1.765 \pm 0.162 \mu\text{mol e}^{-}\text{m}^{-2}\text{s}^{-1}; \text{POER}_{\text{max}} = 4.946 \pm 0.455 \mu\text{mol O}_2 \text{ g}^{-1} \text{ Chl a s}^{-1}) \) and also a very low optimum irradiance \( (E_{\text{opt}} = 104 \pm 14 \mu\text{mol photon m}^{-2}\text{s}^{-1}) \) resulting in no substantial photosynthesis above about \( \frac{1}{4} \) of sunlight. \( \alpha_0 \) was much lower than mature submerged leaves growing adjacently \( (\alpha_0 = 0.0460 \pm 0.0076 \text{ e}^{-} \text{ photon}^{-1}; 0.129 \pm 0.021 \text{ O}_2 \text{ photon}^{-1} \text{ g Chl a m}^{-2}) \) and extremely low compared to surface leaves. The \( \alpha_0 \) of floating leaves is about 80% higher than submerged leaves on a surface area basis. On a Chl a basis, however, \( \alpha_0 \) is highest in submerged leaves compared to adult surface leaves (Table 2). This is because surface leaves have a much higher Chl a content (Table 1A).

NPQ was calculated by the WinControl software based on the equations of Genty et al. \(^{[44]}\), van Kooten and Snel \(^{[45]}\) and Brestic and Zivcak \(^{[47]}\) (Table 2). \( \text{NPQ}_{\text{max}} \) was calculated using non-linear least squares methods fitting to a simple exponential saturation model \(^{[53]}\). The number of valid data points may be substantially fewer than the measurements made in routine rapid light curves, for example in submerged leaves 72 yield determinations were made but only 64 produced estimates of NPQ (Table 2) (the Walz software gives an ERROR value if NPQ cannot be evaluated because of a division-by-zero error). NPQ results were highly variable and so it was difficult to determine \( \text{NPQ}_{\text{max}} \) or its exponential kinetic parameter (\( k_{\text{NPQ}} \) and hence \( \frac{1}{2} \) saturation point) by curve fitting. \( \text{NPQ}_{\text{max}} \) values were not very accurately measurable (seedling leaves \( \approx 1.415 \), submerged leaves \( \approx 1.2 \), surface leaves \( \approx 1.81 \)) with \( \frac{1}{2} \) saturation irradiances of \( \approx 41, 69 \) and \( 214 \mu\text{mol photons m}^{-2}\text{s}^{-1} \) respectively. The kinetics NPQ could not be determined very accurately because of the limitations of the fits to a simple exponential saturation model: the maxima could be determined with reasonable accuracy (Table 2) but most NPQ values were measured at irradiances well below the \( \frac{1}{2} \) saturation point.

![Figure 1](image-url). Plot of Photosynthetic Oxygen Evolution Rate (POER) vs. Irradiance of submerged seedling, adult submerged and surface leaves of *Nymphoides aquatica* leaves collected at local solar midday corrected from Thailand Standard Time. PAM light curve data are based on 12 surface, 8 submerged and 12 seedling leaves and 9 different irradiance levels. Means ±95% confidence limits of \( E_{\text{opt}} \) and \( \text{POER}_{\text{max}} \) of the fits are included in Table 2 along with the other statistics calculated from the rapid light curves.
above the half-saturation point and so the curvature to the exponential saturation curves could not be determined very accurately.

3.5 Temporal Changes in Photosynthesis

PAM measurements of photosynthetic parameters in surface and submerged *N. aquatica* show pronounced diel behaviour (Figures 2, 3 and 4) with generally higher $E_{\text{opt}}$, $\text{ETR}_{\text{max}}$, $\text{POER}_{\text{max}}$ and $\alpha_0$ during the day and low values in the night. Diel differences in $E_{\text{opt}}$ and $\text{POER}_{\text{max}}$ are much more pronounced in surface leaves compared to submerged leaves (Figure 2).

Figure 2 shows the $\text{POER}_{\text{max}}$ and $E_{\text{opt}}$ irradiance of surface and submerged *N. aquatica* leaves collected over the course of a day at 3 h intervals. The same 24 h measurements are presented on the left and right on the graph in order to show a complete diurnal cycle. Estimates of $\text{POER}_{\text{max}}$ (primary Y-axis) and $E_{\text{opt}}$ (secondary Y-axis) are based on rapid light curves conducted at 3 h intervals over the course of 24 h. Both types of leaves have a $\text{POER}_{\text{max}}$ and $E_{\text{opt}}$ at about midday. There is a strong diurnal effect on $\text{POER}_{\text{max}}$ and $E_{\text{opt}}$ on surface leaves with maxima at about midday and minimal values at night. The diurnal effect is less apparent in submerged leaves. There is a strong correlation between $\text{POER}_{\text{max}}$ and $E_{\text{opt}}$ in both submerged and floating leaves. Means ±95% confidence limits ($n = 8,72$: eight leaves, 9 light levels in the rapid light curves).

Figure 2. Photosynthetic Oxygen Evolution Rate ($\text{POER}_{\text{max}}$) and Optimum irradiance ($E_{\text{opt}}$) of surface and submerged *Nymphoides aquatica* leaves collected over the course of a day. Light period 6:00 to 18:15 solar time. The 24 h measurements are repeated twice on the graph (left and right) in order to show a complete diurnal cycle. Estimates of $\text{POER}_{\text{max}}$ (primary Y-axis) and $E_{\text{opt}}$ (secondary Y-axis) are based on rapid light curves conducted at 3h intervals over the course of 24 h. Both types of leaves have a $\text{POER}_{\text{max}}$ and $E_{\text{opt}}$ at about midday. There is a strong diurnal effect on $\text{POER}_{\text{max}}$ and $E_{\text{opt}}$ on surface leaves with maxima at about midday and minimal values at night. The diurnal effect is less apparent in submerged leaves. There is a strong correlation between $\text{POER}_{\text{max}}$ and $E_{\text{opt}}$ in both submerged and floating leaves. Means ±95% confidence limits ($n = 8,72$: eight leaves, 9 light levels in the rapid light curves).
are strongly correlated. A plot of all POER\text{max} values vs. $E_{\text{opt}}$ had a correlation of 0.9240 and POER\text{max} appears to be directly proportional to $E_{\text{opt}}$ giving an overall $\alpha_{E_{\text{opt}}}$ value of $0.07120\pm0.0021$ O$_2$ photon$^{-1}$ m$^{-2}$ g$^{-1}$ Chl a or an $\alpha_0$ value of $0.1935\pm0.0056$ O$_2$ photon$^{-1}$ m$^{-2}$ g$^{-1}$ Chl a.

Figure 4 shows that the maximum of the parameter used to express Non-Photochemical Quenching (NPQ\text{max}) varies over the diurnal cycle for both surface and submerged leaves. Daily maximum NPQ\text{max} values were $\approx1.42$ for submerged seedling leaves, $\approx1.20$ for submerged leaves and $\approx1.81$ for surface leaves. Minimum NPQ\text{max} values at night were $\approx0.8$. The diel patterns of NPQ\text{max} over the course of 24 h of submerged leaves were different to those of surface leaves. There are limitations to the usefulness of NPQ measurements because of their limited reproducibility in $N$. aquatica and so should not be over-interpreted. There was a methodological difficulty with measurements in the present study for leaves collected during daylight. Photosynthetic electron transport of cut $N$. aquatica leaves was severely reduced in leaves given a routine dark preincubation treatment (see above and Appendix Table).

### 3.6 Titratable Acid and SAM/CAM Properties

Figure 5 shows the titratable acid of $N$. aquatica surface and submerged leaves collected over the course of a day. In surface leaves, titratable acid does not accumulate at night and decrease during the day as would be expected in a SAM/CAM plant. The lowest titratable acid was found at 08:33 (24.0$\pm$4.4 mol H$^+$ m$^{-3}$, n = 8) and the maximum value at 02:33 (31.0$\pm$9.1 mol H$^+$ m$^{-3}$, n = 8). These are not significantly different ($p>0.05$). This result is similar to that found previously in $Nymphaea caerulea$ [18] and contrary to what would be found if CAM physiology operated in the surface leaves of $N$. aquatica. In the case of submerged leaves, there is a more apparent diurnal

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### Table 2. Comparison of Photosynthesis of $Nymphoides$ aquatica leaves using PAM for Plants at 11:33 solar time.

| Parameter Class       | Parameter            | Seedling Leaves (n=12) | Submerged Leaves (n=8) | Surface Leaves (n=8) |
|-----------------------|----------------------|------------------------|------------------------|----------------------|
| Quantum Yield         | Yield vs. Irradiance | 0.9797 (n=108)         | 0.9562 (n=72)          | 0.9708 (n=72)        |
| Photosystem II        | Pearson's r (n)      |                        |                        |                      |
|                       | Yield ($Y_{\text{max}}$) | 0.657 ±0.025            | 0.670 ±0.048           | 0.549 ±0.024         |
|                       | Yield (k)            | 0.0717 ±0.008           | 0.0166 ±0.0023         | 0.0021 ±0.0002       |
|                       | ($\mu$mol photon m$^{-2}$ s$^{-1}$)$^{-1}$ |                        |                        |                      |
| Photosynthesis        | ETR vs. Irradiance   | 0.7792                  | 0.7467                 | 0.9305               |
|                       | Pearson's r (r)      |                        |                        |                      |
|                       | $E_{\text{opt}}$     | 104 ±14                 | 419 ±64                | 923 ±68              |
|                       | ($\mu$mol photon m$^{-2}$ s$^{-1}$) |                        |                        |                      |
|                       | ETR\text{max} Surface Area Basis | 1.76 ±0.16             | 14.0 ±1.5              | 58.5 ±2.5            |
|                       | ($\mu$mol e$^{-}$ m$^{-2}$ s$^{-1}$) |                        |                        |                      |
|                       | POER\text{max} Chl a Basis | 4.95 ±0.46             | 38.1 ±4.0              | 76.1 ±3.3            |
|                       | ($\mu$mol O$_2$ g$^{-1}$ Chl a s$^{-1}$) |                        |                        |                      |
|                       | PS Efficiency ($\alpha_0$) Surface Area Basis | 0.0460 ±0.0076 | 0.0958 ±0.0178 | 0.172 ±0.015 |
|                       | ($e$ photon$^{-1}$) |                        |                        |                      |
|                       | PS Efficiency ($\alpha_0$) Chl a Basis | 0.129 ±0.021 | 0.261 ±0.046 | 0.226 ±0.019 |
|                       | ($O_2$ photon$^{-1}$ m$^{-2}$ g$^{-1}$ Chl a) |                        |                        |                      |
| Non-Photochemical Quenching | NPQ | 0.7979 (n=82) | 0.5332 (n=64) | 0.7446 (n=45) |
|                       | Pearson's r (n)      |                        |                        |                      |
|                       | qNPQ 1/2 saturation | 41.0 ±9.2               | 68.8 ±24.3             | 214 ±60              |
|                       | ($\mu$mol photon m$^{-2}$ s$^{-1}$) |                        |                        |                      |
|                       | NPQ\text{max}       | 1.42 ±0.11              | 1.20 ±0.12             | 1.81 ±0.29           |
Figure 3. Photosynthetic Efficiency ($\alpha_0$) of *Nymphoides aquatica* submerged and surface leaves collected over the course of a day based on the POER and $E_{opt}$ data shown in Figure 2 and Table 2. Light period 6:00 to 18:15 solar time. For surface leaves, the photosynthetic efficiency is at a maximum in the middle of the day and is lower at night. Photosynthetic efficiencies of submerged plants are higher than the surface leaves during the night and morning but significantly decreases in the afternoon. Means ±95% confidence limits.

Figure 4. Non-Photochemical Quenching (NPQ) calculations on *Nymphoides aquatica* submerged and surface leaves collected over the course of a day, light period 6:00 to 18:15 solar time using the same data set as used for Figures 2 and 3. The asymptotic maximum (NPQ$_{max}$) were estimated using non-linear least-squares fitting of simple exponential saturation curves to NPQ vs. Irradiance. The largest differences between NPQ$_{max}$ values were found during the morning in the surface and submerged leaves. Means ±95% confidence limits.
cycle of leaf acidity that it could be argued might be consistent with SAM/CAM physiology. The lowest titratable acid was found at 17:33 (19.0±4.9 μmol g\(^{-1}\) FW: 16.9±3.7 mol H\(^+\) m\(^{-3}\), n = 8) and the maximum value was at 11:33 (38.2±6.8 μmol g\(^{-1}\) FW: 34.0±5.1 mol H\(^+\) m\(^{-3}\), n = 8). These acidity values are significantly different (p< 0.05) but imply a maximum diurnal change on leaf acidity of only 19.0±7.6 μmol g\(^{-1}\) FW or 17.0±6.3 mol H\(^+\) m\(^{-3}\) based on the water content of the leaves.

3.7 Estimating Primary Productivity

Primary productivity (P\(_g\)) is normally expressed as gC per m\(^2\) per hour or per day and so the ETR data on a leaf surface area was converted into gC m\(^{-2}\) h\(^{-1}\) based on 4e\(^-\) ≡ 1O\(_2\) ≡ 1C. P\(_g\) of *N. aquatica* leaves (gC m\(^2\) h\(^{-1}\)) over the course of a day was estimated using Equation (1) by first taking the calculated irradiance (E) for the summer solstice (22 June) at Phuket and then using the estimates of E\(_{\text{max}}\) and E\(_{\text{opt}}\) made during the course of the day (Figure 2). For example, taking the midday POER\(_{\text{max}}\) of 14.63 μmol O\(_2\) m\(^{-2}\) s\(^{-1}\) (76.1 μmol O\(_2\) g\(^{-1}\) Chl a s\(^{-1}\)) for floating leaves (Table 2) this is equivalent to a maximum carbon fixation rate of 0.631 gC m\(^{-2}\) h\(^{-1}\) on a leaf surface area basis. As noted in the introduction POER gives a high estimate of gross photosynthesis (P\(_g\)) because it cannot take into account photorespiration. The results, calculated as described by Ritchie \(^{[18]}\), shown in Figures 6a and b, were integrated using the trapezium rule to estimate cumulative and total daily P\(_g\). Total daily irradiance on a clear day would be ≈57.4 mol photon m\(^{-2}\) d\(^{-1}\) (daily maximum PPFD 2100 μmol photon m\(^{-2}\) s\(^{-1}\)): total P\(_g\) increased rapidly during the morning but levelled off and strongly declined during the middle of the day because of photoinhibition during the middle of the day, followed by resumption of high photosynthesis in the afternoon. In the case of the submerged leaves, because of their much lower ETR\(_{\text{max}}\) than surface leaves and their low E\(_{\text{opt}}\) values, photosynthesis over the course of a day was much lower than the surface leaves and was very low in the middle of the day from 9:00 to 15:00 solar time (Figure 6a). The PAM data gives an estimation of the maximum rate of photosynthesis of ≈0.53 g C m\(^{-2}\) h\(^{-1}\) at about 08:00 solar time in the morning, a significant decrease to only ≈0.14 g C m\(^{-2}\) h\(^{-1}\) during the middle of the day, followed by a rise to ≈0.51 g C m\(^{-2}\) h\(^{-1}\) in the late afternoon (16:30). The PAM data gives an estimation of total daily photosynthesis of ≈3.7±0.6 g C m\(^{-2}\) d\(^{-1}\) or 19.5±2.9 g C g\(^{-1}\) Chl a d\(^{-1}\) for surface leaves but only ≈0.338±0.051 g C m\(^{-2}\) d\(^{-1}\) (≈3.68±0.61 g C g\(^{-1}\) Chl a d\(^{-1}\)) for submerged leaves because of their much lower E\(_{\text{opt}}\) and POER\(_{\text{max}}\) (Figure 2).

**Figure 5.** Titratable acid of *Nymphoides aquatica* surface and submerged leaves collected over the course of a day, light period 6:00 to 18:15 solar time. Data are means based on 8 replicates. Error bars are ±95% confidence limits. In surface leaves titratable acid does not accumulate at night and decrease during the day as would be expected in a SAM/CAM plant. In the case of submerged leaves there is a more apparent diurnal cycle of leaf acidity.
Figure 6a. Estimated total Gross Photosynthesis ($P_g$; gC m$^{-2}$ day$^{-1}$) of *Nymphoides aquatica* leaves based upon $ETR_{\text{max}}$, POER$\text{max}$ and $E_{\text{opt}}$ data (Figure 2) inserted into Equation 1 and using the daily irradiance curve for a sunny day. Integration of the PAM data over time gives a total daily photosynthesis of about $3.71 \pm 0.56$ gC m$^{-2}$ day$^{-1}$ from a total daily irradiance of 57.4 mol photon m$^{-2}$ day$^{-1}$ (PPFD) for a clear day. Low POER$\text{max}$ and low optimum irradiance (Figure 2) results in high photoinhibition at high irradiances Gross photosynthesis by the submerged leaves and would be very low particularly during the middle of the day (9:00 to 15:00).

Figure 6b. Estimated total Gross Photosynthesis ($P_g$) of *Nymphoides aquatica* leaves calculated as for Figure 6a. $P_g$ of submerged leaves greatly increased under cloudy conditions or under a canopy of floating leaves. Total daily irradiance would be about 14.3 mol photon m$^{-2}$ for a cloudy day typical of the wet season. Reduced daily irradiance actually increased daily photosynthesis of surface leaves ($\approx 4.1 \pm 0.6$ gC m$^{-2}$ d$^{-1}$) because midday photoinhibition was much reduced compared to Figure 6a and greatly increased the photosynthesis of submerged leaves.
On a cloudy day measurements of PPDF irradiance at midday was only about 500 μmol photon m⁻² s⁻¹ PPDF (Apogee Instruments Quantum Meter MQ-220, Apogee Instruments, Lothan, UTAH 84321, USA). Figure 6b shows that if daily irradiance was reduced by 75%, rather than decreasing photosynthesis the total daily photosynthesis would be greatly increased because midday photoinhibition is decreased. For a daily irradiance of 14.3 mol photon m⁻² the total daily photosynthesis by the surface leaves was increased to 4.10±0.62 gC m⁻² d⁻¹ but photosynthesis of the submerged leaves increased to 1.16±0.17 gC m⁻² d⁻¹ or a quadrupling of their photosynthesis compared to a sunny day.

4. Discussion

Rapid light curves on N. aquatica ideally should be done on leaves in situ (as done for Oil Palm [42]). The 10 min dark pre-treatment routinely used in previous rapid light curve studies on pineapples, orchids, blue water lilies and Davallia angustata in previous studies in our laboratory was found to be unsuitable for N. aquatica see Appendix Table). The relationship of estimates of gross photosynthesis and net photosynthesis of anything but very small vascular plants such as Lemna and Wolffia is always problematic [48] but is especially the case for plants with aerenchyma. The respiration (and hence net photosynthesis) of the whole dwarf water lily plant would be very difficult to measure experimentally. In the present study leaves were cut from the plants and brought into the laboratory from outside the building and kept in the light in the laboratory for as short a time as practicable before being used for rapid light curves.

In chlorophyll content per unit surface area and absorptance properties the floating leaves of N. aquatica are comparable to that found for the floating leaf morphotype of Potamogeton sp. plants [5] and the floating leaves of the Blue Water Lily Nymphaea caerulea [18]. In their photosynthetic properties (Tables 1 and 2; Figures. 1, 2, 3, 4, 6a and 6b), the surface leaves of N. aquatica resemble those of Nymphaea caerulea [18,62] despite N. aquatica leaves having a marked wounding response encountered in the present study. The asymptotic photosynthetic efficiency (α₀) for surface leaves, on a surface area basis, was 0.172±0.015 e/photon (Table 2) is somewhat lower than a typical value found for C3 vascular plants [39,49,63] or CAM plants [40,41] but closely similar to that found previously in the Blue Water Lily [18]. Figure 2 (and analysis of POERₘₐₓ and Eₜₜₜₜ data, see above) shows that there is a strong overall correlation between POERₘₐₓ and Eₜₜₜₜ. A similar phenomenon was noted for the resurrection plant Davallia angustata [49]: POERₘₐₓ was appeared to be directly proportional to Eₜₜₜₜ resulting in an essentially constant α₀ on a Chl a basis over a daily cycle. Such an observation should not be taken as a universal: in another study it has been found that the POERₘₐₓ and Eₜₜₜₜ in the litoral weed species, Launaea sarmentosa exhibits no such close correlation [64].

Experimentally determined absorptances of Nymphaea caerulea leaves were not available when the study was published by Ritchie [18]. The Abt₄₅⁵ₐₗₜₜ values (Abt₄₅⁵ₐₗₜₜ = 98.2±0.2) found by Ritchie and Runcie [62] show that ETR and Pg of Nymphaea caerulea was underestimated by a factor of 98.2/84 or about 17% in the original study. Making allowances for this, the ETRₘₐₓ, POERₘₐₓ and α₀ of surface leaves of N. aquatica have considerably lower ETRₘₐₓ, POERₘₐₓ and photosynthetic efficiencies than Nymphaea caerulea [18] which would have a considerably higher photosynthetic rate than originally reported because its absorbance is above the default value of 0.84 [62].

The NPQₘₐₓ of mature submerged and surface leaves (Figure 4) are comparable to those found in other vascular plants (orichis [40,63], pineappples [41], blue water lily [18], Wolffia arrhiza [48], Oil Palm [42] and Davallia angustata [49]) but the high variability of NPQ means that NPQ data needs to be interpreted cautiously [47]. In the case of surface leaves NPQₘₐₓ tends to be maximum during the morning contrary to previous findings for Dendrobium orchids and pineapples [40,41] but in agreement with findings on Nymphaea caerulea [18]. Diel changes in Non-Photochemical Quenching (NPQ) have been noted in non-CAM plants [44,45,51] and in facultative CAM plants (Clusia hilariana [68], Clusia minor [24,25,26,69], Mesembryanthemum crystallinum [70] and obligate CAM species such as Kalanchoé daigremontiana and K. pinnata [66], Dendrobium orchids [40] and the Phuket pineapple [41]. In this study, lower NPQₘₐₓ values were found at dawn than in the rest of the day and during the night (Figure 4) (for Nymphaea caerulea see Ritchie [18]. High NPQₘₐₓ values were found in surface leaves in the morning until about midday then decreased (Figure 4); submerged leaves had a less conspicuous daylight/dark effect with minimal values at night and higher values during the day.

Diurnal cycling is a diagnostic feature of CAM physiology and is essential for CAM to function [21-26,40,41,66,69,71] but only certain diurnal cycling patterns are consistent with SAM/CAM physiology. The magnitude and diurnal patterns of leaf acidity found in the blue water lily [18] and the fern Davallia angustata [49] are not consistent with any significant CAM or SAM physiology. Obligate and facultative CAM plants and especially CAM-cycling type plants, use both the reservoir of CO₂ fixed as C4 acids during the previous night plus some atmospheric CO₂ taken
up while the stomates are still open in daylight as sources of CO₂ for the Calvin cycle. There is a wide spectrum of the expression of CAM physiology: some orchids express little or no significant CAM physiology (e.g. *Vanda sp.* [63]).

The diurnal pattern of titratable acid in the leaves of *N. aquatica* surface leaves is not consistent with any significant CAM physiology (Figure 5) consistent with previous findings on *Nymphaea caerulea* [19]. Keeley and Morton [89] also found no significant CAM physiology in the Yellow American Water Lily *Nuphar polysepulum*. The magnitude of changes in titratable acid is also too low for any significant CAM/SAM physiology. The amount of titratable acid in *N. aquatica* leaves (≈20 to 50 μmol H⁺ g⁻¹ FW, Figure 5) is lower than previously found in *Nymphaea caerulea* plants which were growing in the same lily pond bowls on the Phuket campus (≈ 60 to 82 μmol H⁺ g⁻¹ FW [18]).

The acid content in the submerged *N. aquatica* leaves is also lower than found in *Nymphaea caerulea*. Classical CAM plants like *Dendrobiyum* orchids [60], pineapples [41] and *Clusia* sp. [24-26] and aquatic plants known to have SAM/CAM physiology (*Isoetes howellii* [13] and *Littorella uniflora* [9]) store ≈300 to over 1000 μmol H⁺ g⁻¹ FW on a diurnal cycle.

The surface leaves of *N. aquatica* do not show the diagnostic diurnal rhythm of nocturnal accumulation of acid in the leaves and metabolism of the stored C₄ acids during the day (Figure 5) that is found in classic CAM plants [46,41]. As is the case in the leaves of *Nymphaea caerulea* [19], the surface leaves of *N. aquatica* do not perform CAM but the possibility remained that perhaps the submerged leaves performed some SAM/CAM physiology. Figure 5 does show that in submerged leaves there is some evidence for accumulation of acid at night and in the morning and a decrease in the afternoon but the labile organic acid pool of submerged leaves *N. aquatica* is too small to support significant CAM/SAM physiology. At midday the accumulated acid in submerged leaves was found to be 38.23±6.76 μmol H⁺ g⁻¹ FW (n = 8) or using the conversion factors in Table 1A, 34.0±5.1 mol m⁻³ or 4.945±(0.802) × 10⁻⁵ mol H⁻¹ m⁻². At 17:33 at the end of daylight the titratable acid had fallen to 19.03±4.92 μmol H⁺ g⁻¹ FW or 16.90±3.69 mol H⁻¹ m⁻³ on a leaf water basis or 2.462±(0.560) × 10⁻³ mol H⁻¹ m⁻². The difference in acid content in the leaves over this 6h period is only 19.2±7.6 μmol H⁺ g⁻¹ FW; 17.0±6.3 mol H⁻¹ m⁻³ or 2.48±0.93 μmol H⁺ m⁻². Assuming the accumulated acid is malate (a diprotic C₄ acid) then at midday the leaves had =17 mol malate m⁻² which fell to ≈8.5 mol malate m⁻³ at sunset, which would be able to supply 1.24±(0.47) × 10⁻⁵ mol CO₂ m⁻² for photosynthesis. From Figures 6a and b it can be estimated that the total carbon fixation by underwater leaves of *N. aquatica* from midday to sunset in full daylight can be no more than about 13.7(±2.1) × 10⁻³ mol C m⁻² based on a C:O₂ ratio of 1 and integrating over time by the trapezium rule. This is much higher than the amount of CO₂ potentially stored in the vacuoles of the leaves and would supply only enough CO₂ for about 33±13 min of photosynthesis in full daylight. Photosynthesis on a cloudy afternoon by submerged leaves would total about 57.8(±8.7) × 10⁻³ mol C m⁻² and so the leaves would exhaust the vascular CO₂ pool in only 7.7±3.1 min. Ritchie [18] calculated that vacuolar acid in *Nymphaea caerulea* could only supply enough CO₂ to account for only about 17 minutes of photosynthesis of the plant in full daylight.

Another approach to the problem shows that the amount of organic acid inside the submerged leaves of *N. aquatica* is not sufficient to support any significant CAM-like physiology. If we assume that floating leaves cover 90% of a pond surface, then the average light received by an understory of submerged leaves would be an irradiance of only about 10% of full sunlight [72] totalling about 5.74 mol photons m⁻² d⁻¹. Daily carbon fixation would be about 1.4 gC m⁻² d⁻¹ or a total of 0.7±0.1 gC m⁻² (59±8 mmol C m⁻²) on a sunny afternoon. The leaves would exhaust the organic acids stored in the submerged leaves in only 8±3 minutes.

SAM/CAM physiology only occurs in submerged *Isoetes* and *Littorella* species [8,7,9,10,13]. Smits et al. [73] found that in terms of HCO⁻₃ usage the underwater leaves of Nymphaeid seedlings are quite different to adult floating leaves but we have shown that submerged *N. aquatica* leaves do not store enough C₄ acids in their vacuoles to support SAM/CAM physiology. Photosynthesis of both submerged and surface leaves of *N. aquatica* shows definite diel cycles in most PAM parameters but the cycling pattern is not what would be expected in a CAM plant (Figures 2 to 4). Table 2 and Figures 2 and 3 show that surface leaves of *N. aquatica* are “sun leaves” with high rates of photosynthesis in full sunlight [43] but Nymphoid plants are not C₄. Smits et al. [73] determined that the CO₂ compensation point of *Nymphaea alba*, *Nuphar lutea* and *Nymphoides peltata* leaf disks varied from 6.6 to 13.5 mmol CO₂ m⁻³. These are typical C₃ values, well above the essentially zero values found in C₄ plants.

PAM fluorometers measure the light reactions of photosynthesis and provide only indirect information about the Calvin Cycle, ATP and NADPH+H⁺ status or the source of the CO₂ used for carbon fixation. Slesak et al. [74] showed that in *Mesembryanthemum crystallinum* under conditions of high light, but little or no available CO₂, there is a build-up of NADPH+H⁺ and H₂O₂ resulting in significant photosystem damage. The lack of suppression
of NPQ found in the present study of N. aquatica during the middle of the day agrees with previous observations on Nymphaea caerulea \[^{18}\] and contrasts results on true CAM plants \[^{40,41}\].

\(E_{\text{opt}}\) of N. aquatica of \(\approx 850-1000 \mu \text{mol photon m}^{-2} \text{s}^{-1}\) PPFD (Table 2 and Figure 2, Appendix Table) classifies N. aquatica as a sun plant: Nymphaea caerulea saturates at similar irradiances \(\approx 1000 \mu \text{mol photon m}^{-2} \text{s}^{-1}\) \[^{18}\]. Figures 6a and b are plots of \(P_{d} \) \(N. \) aquatica using our ETR\(_{\text{max}}\) and \(E_{\text{opt}}\) data over the course of daylight (Figure 3). Irradiance reached over 2100 \(\mu \text{mol photon m}^{-2} \text{s}^{-1}\) at midday in Phuket resulting in substantial photoinhibition during the middle of the day. Compared to Nymphaea caerulea, midday inhibition is more severe in N. aquatica resulting in an estimated daily total gross photosynthesis of \(\approx 3.71\pm 0.51 \text{ gC m}^{-2} \text{ d}^{-1}\) on a full sun day but \(4.1\pm 0.6 \text{ gC m}^{-2} \text{ d}^{-1}\) on a cloudy day compared to more than \(6 \text{ gC m}^{-2} \text{ d}^{-1}\) in Nymphaea caerulea \[^{18}\] when the original estimates are corrected to actual ETR rather than relative ETR (rETR) \[^{62}\].

PAM fluorometers give no information on respiration: oxygen electrode, \(^{14}\)C or IRGA methods are necessary to make estimates of respiration and hence net photosynthesis from PAM data \[^{98,75,79}\]. Measurement of respiration of excised leaf disks and pieces of petiole of a water lily is \textit{in principle} straightforward but misleading because the respiration of the whole plant, including the petioles, roots and rhizome is required including anaerobic respiration \textit{in situ} \[^{15,16,33,52}\]. Solid, liquid and gas phases, the internal ventilation system and aerobic and anaerobic compartments within the mud substrate all combine to make it very difficult to make estimates of respiration (and hence net photosynthesis) in a Nymphoid aquatic plant \textit{in situ}. Inferences can be made about the magnitude of respiration of the whole plant if growth (Net Photosynthesis) is measured and good estimates are made of gross photosynthesis by a range of different methods.

The POER achievable by N. aquatica on a sunny day (\(\approx 3.71\pm 0.56 \text{ gC m}^{-2} \text{ d}^{-1}\); Figure 6a) is comparable to Nymphaea caerulea \[^{18}\], wetland communities \[^{77}\] and well-kept field C3 crops or pastures \[^{93}\]. This is despite the relatively low photosynthetic efficiency of N. aquatica (Table 2; Figure 3) (compare to Nymphaea caerulea water lily \[^{18}\], Oil Palm \[^{42}\] and Davallia angustata \[^{49}\]). POER for a N. aquatica pond in Phuket at the summer solstice in the wet season with overcast days with \(\approx 500 \mu \text{mol photon m}^{-2} \text{s}^{-1}\) PPFD (or 1/4 full sunlight) would be about 10% higher (\(4.1\pm 0.6 \text{ gC m}^{-2} \text{ d}^{-1}\)) due to less photoinhibition in the middle of the day \[^{54}\]. Shading (75%) due to cloud cover which would reduce daily irradiance to \(\approx 143 \mu \text{mol photon m}^{-2} \text{ d}^{-1}\) but the reduced irradiance would quadruple POER of submerged N. aquatica leaves to \(1.16\pm 0.17 \text{ gC m}^{-2} \text{ d}^{-1}\).

Shading of 90% actually increases the photosynthesis of submerged leaves even further (\(1.4\pm 0.2 \text{ gC m}^{-2} \text{ d}^{-1}\)). The photosynthesis of a N. aquatica water lily bed with essentially 100% floating leaf cover would be considerably above the production by the floating leaves alone (\(3.7 \text{ gC m}^{-2} \text{ d}^{-1}\)) because of an additional contribution by the submerged leaves to add up to as much as \(\approx 5 \text{ gC m}^{-2} \text{ d}^{-1}\).

A water lily pond covered by Nymphoid floating leaf species has important structural differences to most photosynthetic plant communities \[^{18,72}\] because it acts as a single flat leaf absorbing nearly all incident light (Table 1B) \[^{72,78-81}\] but photoinhibition is a limitation for productivity for both floating and especially for submerged leaves. Even in a plant that rates as a sun plant based on its photosynthesis vs. irradiance curves (Figures 1 and 2), N. aquatica photosynthesis is greatly reduced at high irradiances during the middle of a sunny day and daily photosynthesis on a cloudy day turns out to be about the same as a sunny day because of reduced midday photoinhibition (Figures 6a and b).

**Abbreviations**

\(\alpha_{\text{sp}}\), photosynthetic efficiency at asymptotically zero Irradiance; \(\alpha_{\text{optg}}\), photosynthetic efficiency at optimum Irradiance; E, Irradiance (\(\mu \text{mol photon m}^{-2} \text{s}^{-1}\)) 400-700 nm PPFD; ETR, electron transport rate; PAM, Pulse Amplitude Modulation fluorometry; PPFD Photosynthetic Photon Fluence Density (400 – 700 nm); POER, Photosynthetic Oxygen Evolution Rate; PSI, Photosystem I; PSII, Photosystem II; SAM, Submerged Aquatic Macrophyte.

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**Conflicts of Interest**

The authors declare no conflicts of interest.

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Appendix Table Legend:

Experiments on cut leaves incubated in the dark showed that \( E_{opt} \) and \( ETR_{max} \) decreased over time if the leaves were kept in the dark. All values are means ± 95% confidence limits. \( Fm' \) is the fluorescence in the light acclimated state after a flash of actinic light and is used to calculate the Yield \( (Y = 1 - Fo/Fm') \) where, \( Fo \) is the fluorescence in the modulated measuring light (Genty et al. [44]; Brestic and Zivcak [47]). \( Y_{max} \) is maximum Yield \( (Y) \) fitted from a \( Y \) vs. Irradiance rapid light curve 0 to 1300 \( \mu \text{mol photon m}^{-2} \text{s}^{-1} \). \( Y_k \) is the exponential constant fitted to the simple exponential decay curve fitted to the \( Y \) vs. Irradiance data; \( Y_{0.5} \) is the irradiance at which \( Y \) was ½ maximum. \( r \) is the correlation coefficient, all \( r \) values were significant at \( p << 0.001 \). \( E_{opt} \) is the optimum irradiance of photosynthetic electron transport in \( \mu \text{mol photon m}^{-2} \text{s}^{-1} \) of the fitted waiting-in-line relationship of POER vs. Irradiance. \( POER_{max} \) is the maximum photosynthetic electron transport rate (\( \mu \text{mol} \text{O}_2 \text{ g}^{-1} \text{ Chl a} \text{ s}^{-1} \)) at the \( E_{opt} \) irradiance value in Alpha \( (\alpha_0) \) is the photosynthetic efficiency at zero irradiance. The results show that photosynthesis in \( N. aquatica \) is very vulnerable to a wounding effect on excised leaves and so pre-incubation (light or dark) before rapid light curves is not appropriate in this species. Furthermore, since both \( POER_{max} \) and \( E_{opt} \) both change over time the shape of the \( P \) vs. \( E \) curves of cut leaves changed over time. Preincubation in the dark is worse than cutting the leaves and keeping them in the light in the laboratory. Measuring rapid light curves as soon as possible after collection and keeping in the light was the best option for this species.

| Experiment       | \( Fm' \) | \( Y_{max} \) | \( Y_k \) | \( Y_{0.5} \) | \( r & P \) | \( E_{opt} \) | \( POER_{max} \) | \( Alpha \ (\alpha_0) \) | \( r & P \) |
|------------------|----------|--------------|----------|--------------|----------|---------|------------|-------------------|----------|
| zero dark        | 1236     | 0.6544       | 0.001696 | 408.7        | 0.9683   | 714.5   | 96.3       | 1.465             | 0.8945   |
| \( n = 8 \)      | ± 185    | ± 0.0236     | ± 0.000169 | ± 40.8       | << 0.001 | ± 68.7  | ± 4.9      | ± 0.159           | << 0.001 |
| 15 min dark      | 1494     | 0.6098       | 0.005072 | 136.6        | 0.8931   | 619.3   | 46.0       | 0.808             | 0.8220   |
| \( n = 8 \)      | ± 198    | ± 0.0487     | ± 0.000858 | ± 23.1       | << 0.001 | ± 70.8  | ± 3.0      | ± 0.107           | << 0.001 |
| 30 min dark      | 1362     | 0.5950       | 0.005420 | 127.9        | 0.9609   | 554.2   | 39.8       | 0.780             | 0.9720   |
| \( n = 8 \)      | ± 107    | ± 0.0371     | ± 0.000702 | ± 16.6       | << 0.001 | ± 24.5  | ± 1.1      | ± 0.041           | << 0.001 |
| 1h dark          | 1701     | 0.6185       | 0.007189 | 96.4         | 0.9217   | 474.8   | 35.0       | 0.801             | 0.9002   |
| \( n = 8 \)      | ± 221    | ± 0.0450     | ± 0.00111 | ± 14.9       | << 0.001 | ± 44.8  | ± 1.7      | ± 0.085           | << 0.001 |
| 2h dark          | 867      | 0.6058       | 0.007938 | 87.3         | 0.9307   | 432.8   | 30.4       | 0.763             | 0.9486   |
| \( n = 16 \)     | ± 352    | ± 0.0226     | ± 0.000696 | ± 7.7        | << 0.001 | ± 21.2  | ± 0.92     | ± 0.044           | << 0.001 |
| Light Treatment  |          |              |          |              |          |         |            |                   |          |
| Control          |          |              |          |              |          |         |            |                   |          |
| 0 h Light        | 947      | 0.6295       | 0.001300 | 533.1        | 0.98554  | 855.8   | 119        | 1.505             | 0.9811   |
| \( n = 8 \)      | ± 108    | ± 0.0121     | ± 0.000075 | ± 30.8       | << 0.001 | ± 47.2  | ± 3.1      | ± 0.092           | << 0.001 |
| 1h Light         | 1229     | 0.6533       | 0.001829 | 379.1        | 0.9757   | 732.5   | 93.5       | 1.388             | 0.9754   |
| \( n = 8 \)      | ± 143    | ± 0.0200     | ± 0.000151 | ± 31.4       | << 0.001 | ± 38.3  | ± 2.6      | ± 0.082           | << 0.001 |