Determination of Photosynthesis-Irradiance-Temperature Relationship Using the Phenoplate

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Introduction

Photosynthesis, the core physiological process in most aquatic and terrestrial ecosystems, is sensitive to both light intensity and temperature. Chlorophyll *a* fluorescence, which is widely used to probe the photochemistry of PSII, can provide critical information about the energy conversion processes in photosynthesis. This energy conversion, as well as the energy dissipation mechanisms are highly dependent on the light quanta (intensity), light quality (spectrum) but also importantly on the temperature of the system. *E.g.* it is reasonable to assume that few plants, algae or corals will have the same Y(II) and NPQ at 30 °C and 10 °C. Conversion of energy is a function of temperature, among other factors, therefore determination of photosynthetic parameters needs to be done at precisely controlled temperatures and light intensities. Furthermore, it can be argued that it is insufficient to know the relationship of photosynthesis and irradiance (PI) at a single temperature since most photosynthetic organisms experience significant temperature changes during the day, hence altering the PI relationship. The system presented in this Application Note allows for simultaneous determination of the PI relationship at multiple temperatures.

The Phenoplate approach, integrates the Maxi-Imaging-PAM and a thermocycler, to allow fast and dynamic control of temperature prior, during and post chlorophyll *a* fluorescence measurement. It allows for assessments of Photosystem II efficiency (Y(II)) and non-photochemical quenching (NPQ) of up to 384 simultaneous samples in a dynamically controlled thermal environment. In this paper we demonstrate how this simple system can be used to describe in detail the photosynthesis-irradiance-temperature relationship for electron transfer rate (ETR) and NPQ.

The thermocycler used in these experiments is a standard piece of equipment found in most laboratories and is used to bring samples to target temperatures at very quick rates (common temperature change rates are of 3°C s^{-1}), maintain the samples to these new temperatures for 10 minutes and during a subsequent PAM measurement. This procedure has been shown to produce results that corelate well with extended temperature treatments of 24h or longer (England et al., 2024). As demonstrated in previous studies, this system can assess complex interactions between light and temperature on NPQ (Herdean et al., 2023; Herdean et al., 2022) in microalgae. Furthermore, the impact of microalgae and bacteria, as well as fitness of corals, seaweed and higher plants can similarly be assessed (England et al., 2024; Matthews et al., 2023).

A recent innovation of the system was to integrate oxygen sensors that are monitored by the PAM, this has been detailed in a new publication (England et al., 2024) and it allows for real-time measurements of both photophysiology and metabolic parameters, broadening the scope of the method.

A key enabling feature of the Phenoplate is high throughput. We have successfully measured 384-well and 96-well plates, which enabled to collect robust data with high number of replicates measured simultaneously, while being exposed to temperature gradients, and rapid light curve measurements. Furthermore, in addition to temperature gradients we have demonstrated that chemical gradients can be added to the plate to test the impact of the chemicals on microalgae at different temperatures (Herdean et al., 2022). Lastly, the system offers a portable alternative for field studies where the impact of temperature needs to be assessed. This offers an alternative to the coral bleaching automated stress system (Evensen et al., 2023) commonly used to measure response kinetics of corals to increased temperatures. It also offers an alternative to the PhenoChip which draws on similar principles but is aimed at single cell analysis (Behrendt et al., 2020).

Material and Methods

Biological material. Corals (*Acropora kenti*) were maintained in aquariums under blue light and shortly before the Phenoplate measurement they were fragmented in to smaller samples of approximately 4X8 mm. Seaweeds (*Ulva lactuca*), were collected from various beaches in Sydney (Australia) and maintained for

multiple days in cylindrical photobioreactors. Leaves from adult small-leaved lime tree (*Tilia cordata*) were collected from the university campus before performing the experiment. Microalgae (*Chlorella vulgaris*) was grown in continuous light at 23 ºC in cell culture flasks in standard laboratory incubators.

Instruments. Measurements were done using a Maxi Imaging-PAM (Heinz Walz GmbH) and a thermocycler (ABI Veriti, Applied Biosystems, Waltham, MA, USA). Validation of the temperature was done using a thermal camera (model FLIR C2 (Teledyne FLIR, LLC, Wilsonville, OR, USA). For each experiment the Imaging PAM and the thermocycler were started at the same time and the instruments remained synchronised throughout the entire experiment.

Heat treatment. The temperature profile of the heat treatment was done by programming the thermocycler as follows:

1. Hold the entire 96-well plate at 18 ºC (or growth temperature of the biological sample),
2. Initiate temperature gradient: 15, 17, 19, 21, 23, 25 ºC and hold for 30 minutes. For covering the expanded range of 6 to 54 ºC we performed 3 separate measurements using 3 different gradients.

PAM protocol. A standard Rapid Light Curve was programmed in the instrument, with the only distinction that the first 2 steps were programmed to last for 10 minutes and to be at 0 PAR, followed by subsequent steps of 60 seconds, each at an increasing light intensity. This protocol provides Fv/Fm values at growth temperature, Fv/Fm values after 10 minutes of temperature treatment, and complete RLC dataset at multiple temperatures.

Important considerations for optimum results:

1. *Validation of the temperatures*. Thermocyclers are designed to work with sealed plates and with a heated cover, this is not possible in this experimental design. Therefore, the temperature shown in the thermocycler software may not always reflect the real temperature of the samples. To assure the results are interpreted correctly the user should measure the temperatures generated in the wells using thermometers or a thermal camera.
2. *Validation of light intensity*. While the Imaging PAM provides very uniform illumination, it is advised to measure the actinic light intensity that reaches each well using a quantum sensor. If illumination is not equal in all 96 wells, ETR requires recalculation for each well.
3. *Sample health*. For microalgae samples the best results are obtained if the samples are in exponential growth and have been resuspended in fresh media 6-24h prior to the measurements.
4. *Multiple sample measurements*. Additional care needs to be taken if diverse samples are to be measured simultaneously to avoid overexposure or underexposure of part of the samples. In the case of liquid samples, it is advised to adjust the samples concentrations before the experiment in such a way that there will be similar number of cells, or similar quantity of chlorophyll *a* in all wells measured at the same time.
5. *PAM settings optimization*. For every species it is advised to optimize the PAM settings by determining the optimum intensity of the saturation pulse (SP) by testing various settings until any additional increase in SP intensity does not yield an additional increase in Fm or Fv/Fm. Choosing the correct light intensity range for a rapid light curve can be done by prior testing of similar samples before running a comprehensive experiment.

Variations of experimental design:

1. Rapid light curves can easily be performed a multitude of temperatures in a time effectively manner, as shown in this paper.
2. NPQ induction curves during temperature changes can reveal how different NPQ components become active or inactive at precise temperatures, see (Herdean et al., 2023).
3. Critical temperature of the photosynthetic machinery can be quickly determined by gradually increasing the temperature and measuring F_0. The method has been previously been developed using and Imaging PAM and thermocouples (Harris et al., 2023).

Example Measurements

A wide variety of sample types can be used in the Phenoplate, from microalgae to plants to corals. Sample preparation varies depending on the sample type (Figure 1) but can involve cutting or just simply dispensing a liquid sample in the well plate.

There are two “standard” choices of measurements in most PAM fluorometers: rapid light curves (RLC) or induction + recovery curves, commonly used for NPQ measurements. In the following examples we will only show rapid light curves. In Figure 2, RLC measurements

were carried out on coral samples and the results can be visualised as individual curves (Fig. 2A) or elegantly assembled into a heat map (Fig. 2B) which better shows the relationship between photosynthesis, light and temperature. Such an analysis provides a temperature coordinate for maximum photosynthesis, in the case of the corals measured in Figure 2 we can now establish that maximum photosynthesis (maximum ETR) occurs at 225 µmol photons m^{-2} s^{-1} at 26.5 ºC. Similarly, we can see how NPQ increases with light intensity in a temperature dependent manner (Figure 2C and D), and we can establish that maximum NPQ was observed at 1000 µmol photons m^{-2} s^{-1} at 28 ºC.

To better understand how temperature influences maximum photosynthesis and NPQ, we can extract these values from every light curve and plot them against the temperature that they were determined at (Figure 3A and C). In the presented dataset we can see how diverse the response of plants, seaweed and green algae are. The ETR and NPQ heat maps also show that *T. cordata* has precise temperature and light boundaries for these two mechanisms (Figure 3B and D)

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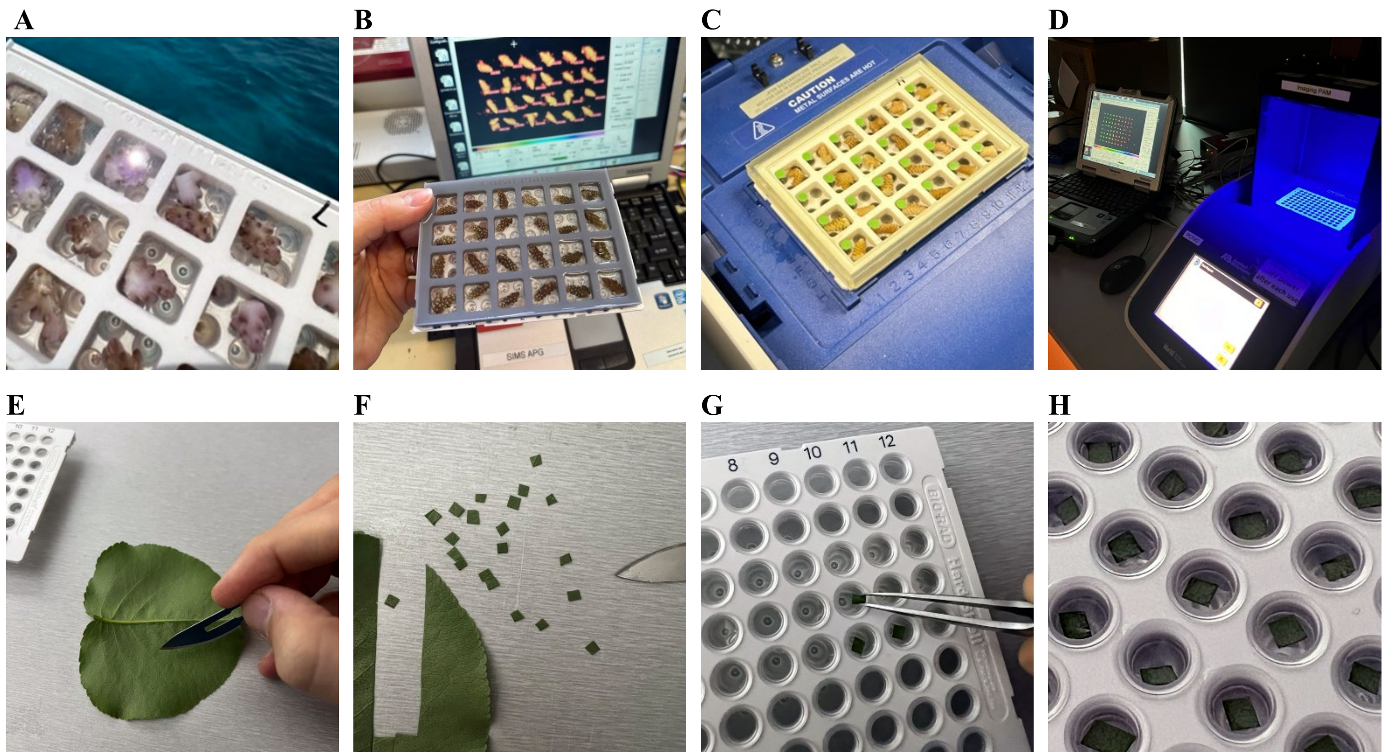

Figure 1. Sample preparation for Phenoplate analysis. The Multi Taxa Phenoplate (MTP) adapter previously described (England et al., 2024) is a 3D printed 24-well adapter which transforms the 96-well plate in to a 24-well plate to allow measurements of coral and other larger samples (Panel A-B). The corals are placed on to the MTP which is sealed with a rectangular glass that helps to separate the temperatures generated by the thermocycler in the wells and the environmental temperature. Furthermore, the glass lid can be fitter with optodes (oxygen sensor dots, OXSP5, Pyroscience, Aachen, Germany) that enables simultaneous measurements of chlorophyll *a* fluorescence and oxygen evolution (Panel C). Once the samples are prepared and the temperature gradient is programmed into the thermocycler, the Imaging PAM can be placed above the plate and the measurement can be started (Panel D). Microalgae are the easiest samples to measure using this system and the approach has been thoroughly demonstrated to yield robust results (Herdean et al., 2023; Herdean et al., 2022; Matthews et al., 2023). Measurements of plants can be done using the MTP or directly using a 96-well plate. Leaves can be cut in to ~2X2 mm small pieces using a surgical scalpel or a sharp blade (Panels E and F), and the small leaf samples can be place in the 96 wells which have been filled with water for improved temperature transfer (Panels G and H); seaweed samples were processes similarly.

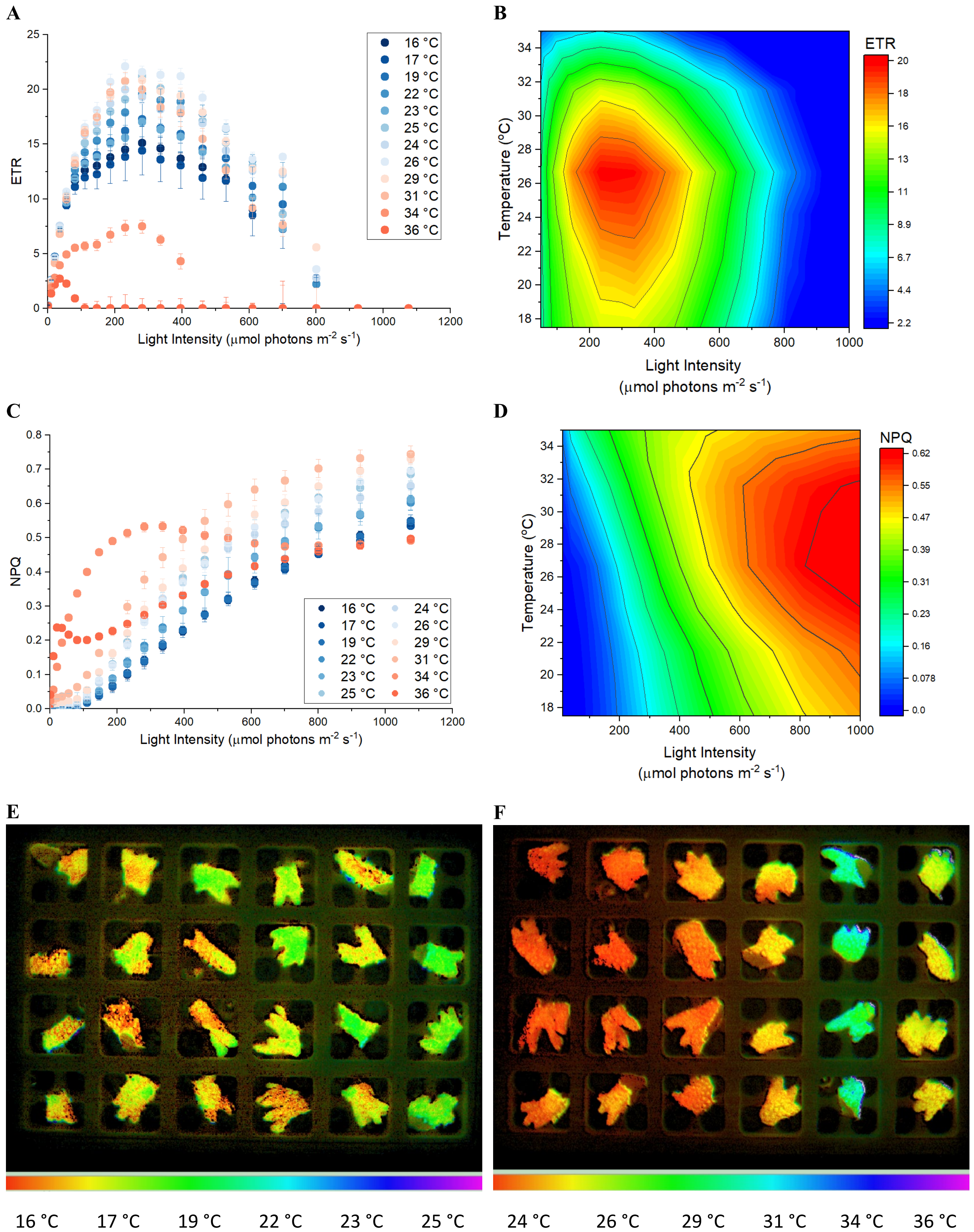

Figure 2. Phenoplate Rapid Light Curve of corals using the MTP adapter. Panel A shows averages of 4 biological replicates at each temperature. Panel B shows assembled ETR data in to a heat-map made using OriginPro. Panel B shows NPQ derived from the RLC and Panel D shows its corresponding heat map. Panels E and F show example NPQ/4 images from the RLC measurement of the coral fragments measured at various temperatures (shown below). Every column consisting of 4 coral fragments was maintained for 10 minutes at growth temperature in darkness, followed by 10 minutes at the shown temperatures at the bottom of the figure, and followed by the rapid light curve during which the samples were maintained at the indicated temperatures.

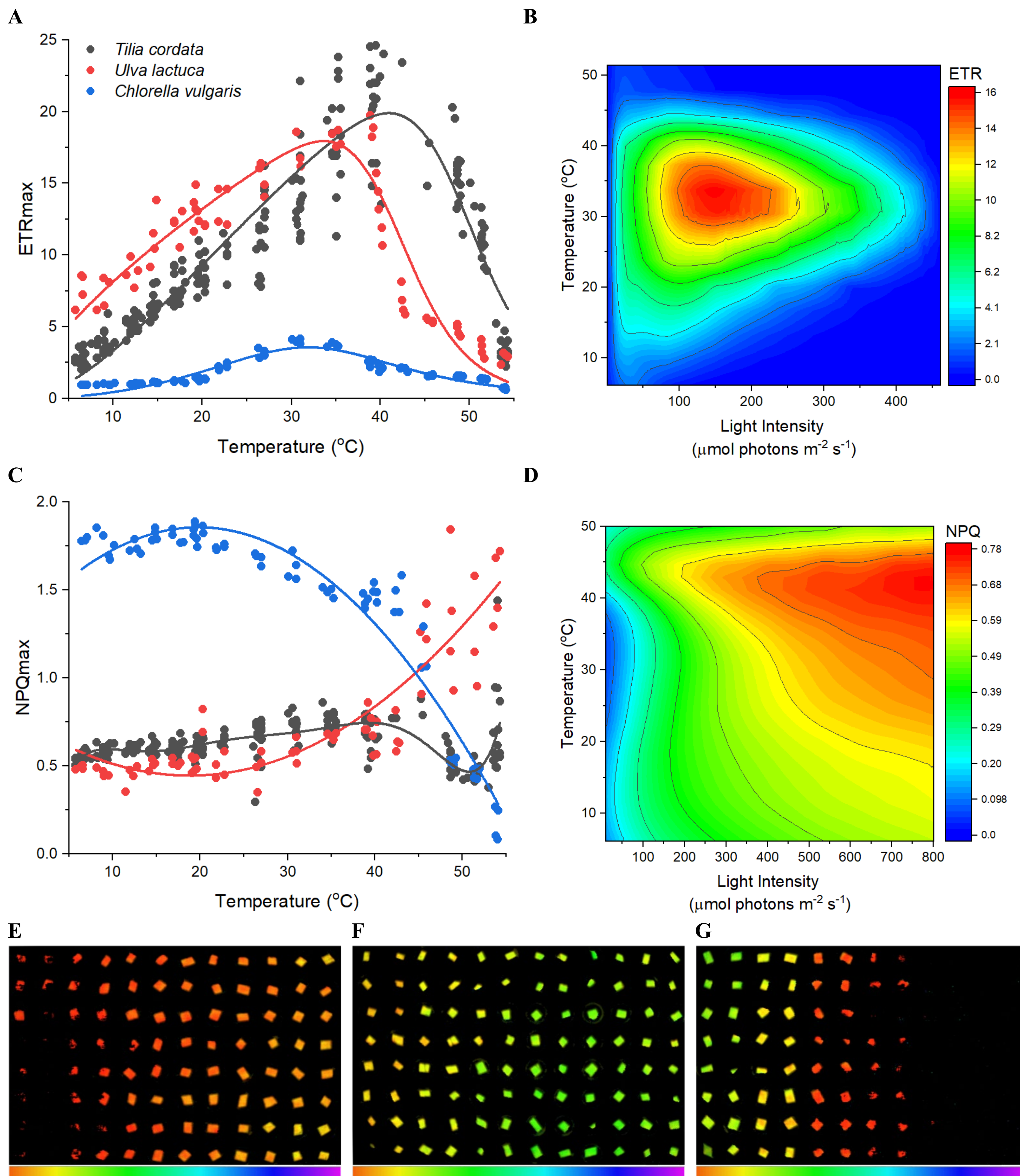

Figure 3. Phenoplate Rapid Light Curve of algae, seaweed and plants. Panel A, temperature resolved maximum electron transfer rated (ETRmax) of a green alga (*Chlorella vulgaris*), seaweed (*Ulva lactuca*) and a higher plant (*Tilia cordata*) shows how different taxa perform photosynthesis in a temperature dependent manner. Panel B shows a Photosynthesis-Irradiance-Temperature (PIT) heat map of *T. cordata*, which displays the temperature integrated photosynthesis irradiance relationship. The conditions for obtaining maximum electron transfer rate can be identified using this type of measurements; in the case of *T. cordata* shown in Panel B maximum photosynthetic energy conversion occurs at 33.5 °C at 140 μmol photons m^{-2} s^{-1}. Panel C shows the diverse response of NPQ to temperature, with *C. vulgaris* and *U. lactuca* showing opposite responses to elevated temperature; NPQ in *C. vulgaris* decreases with temperature, likely a sign of irreversible photo-thermal damage, whereas in *U. lactuca* NPQ increases with temperature indicating that photoprotective processes are active. *T. cordata* shows a gradual increase in NPQ, followed by a decrease at elevated temperatures, and continued with an increase after 50 °C. This final increase of NPQ at high temperatures is a likely sign of irreversible photo-thermal damage, which is the case in some microalgae (data not shown). Panel D shows the complete NPQ irradiance temperature (NPQ-I-T) heatmap for *T. cordata*. Panels E, F and G are representative images of Y(II) from the 6 to 20 °C gradient (E), 19 to 40 °C gradient (F), and 39 to 54 °C gradient (G).

Conclusion and outlook

The Phenoplate is a simple, yet powerful coupling of two widely available instruments that is gradually opening up high-throughput PAM measurements at controlled temperatures. Recent applications using the Imaging PAM (Behrendt et al., 2020; Harris et al., 2023; Herdean et al., 2022) have clearly demonstrated that measurement temperature is extremely important can if not accounted for interpretation of the fluorometry results can be incomplete. The Phenoplate approach has also revealed the diverse and surprising response of ETR and NPQ at low and high temperatures relative to growth temperature.

These diverse response of photosynthesis at various temperatures could be at least partly explained by:

- Altered (thylakoid) membrane fluidity. Processes that rely on some degree of ultrastructural changes of the thylakoid membrane will be favoured at higher temperatures rather than low temperatures.
- Optimal temperature is likely different for various enzymes involved in photosynthesis. The choice of measurement temperature may hide certain processes by maintaining them inactive due to non-optimal temperature of key enzymes involved in that process (e.g. state transition).

Lastly, combining the capability of an imaging fluorometer, the precise temperature control of a thermocycler, and oxygen measurements into one system creates a very powerful research tool. Furthermore, the Phenoplate and MPT were designed in such a way that takes advantage of readily available equipment in most labs, significantly reducing the costs of and accessibility of this research tool

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