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Delayed fluorescence as an indicator of the influence of the herbicides Irgarol 1051 and Diuron on hard coral *Acropora digitifera*

Masakazu Katsumata[a], Ichiro Takeuchi[b,c,*]

[a] Central Research Laboratory, Hamamatsu Photonics K.K., 5000 Hirakuchi, Hamakita, Hamamatsu, Shizuoka 434-8601, Japan
[b] Graduate School of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan
[c] Center of Advanced Technology for the Environment, Graduate School of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan

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ABSTRACT

We examined the effect of two herbicides (Irgarol 1051 and Diuron) on symbiotic dinoflagellates in the hard coral *Acropora digitifera* using delayed fluorescence (DF), specifically assessing changes in molecular membrane transport, *i.e.* inflow and outflow rates, and the binding of the herbicides to target proteins in photosystem II. The DF approach is rapid (*e.g.* measurement time, 60 s) and non-invasive, and can provide data on the extent of a photosynthetic system and the activity of its electron carriers. The DF of *A. digitifera* is inhibited 2 h after exposure to 1 μg/L of either Irgarol or Diuron. Analysis of DF inhibition over time by a compartment model suggests that Irgarol exposure results in a relatively higher inflow rate and lower outflow rate than does Diuron exposure. This suggests that Irgarol exposure more strongly inhibits photosynthesis and that the coral symbiotic dinoflagellates recover less from inhibition.

1. Introduction

The global ban on tributyltin on 1 January 2008 prompted research on and the development of alternative antifouling paints. While copper is highly toxic to many marine organisms, several algae are tolerant to it, so most copper-based antifouling paints are fortified with additional 'booster' biocides (Dafforn et al., 2011). Approximately 10 chemicals, including Irgarol 1051 (hereafter Irgarol) and Diuron, are currently used worldwide as 'boosters' (Dafforn et al., 2011). These two chemicals were originally used as agricultural herbicides (Jones and Kerswell, 2003), and they have been detected in seawater samples from port areas worldwide (see Basheer et al., 2002; Okamura et al., 2003; Harino et al., 2005; Lam et al., 2005; Balakrishnan et al., 2012; Ali et al., 2013). Understanding the effects of such herbicides and other inhibitors on corals and their symbiotic algae is crucial. One promising technique is determining the effective quantum yield of chlorophyll fluorescence, which is an estimate of photosynthesis efficiency, using ratios of chlorophyll fluorescence (*e.g.* $\Delta F/F_m'$) (Jones et al., 2003; Jones and Kerswell, 2003).

Delayed fluorescence (DF) is a special type of chlorophyll fluorescence; it occurs only in photosynthetic systems (cells, chloroplasts or photosystems) under dark conditions after light irradiation (Strehler and Arnold, 1951). DF can be described as a back reaction of photosynthesis (Lavorel, 1975; Gerhardt and Krause, 1984; Jursinic, 1986; Schmidt and Senger, 1987; Goltsev et al., 2009). It is based on a charge recombination in the photosynthetic electron transport reactions, specifically *via* the quinone B (Q_B) site in photosystem II, which is a target of triazine- or phenylurea- type herbicides (*i.e.* Irgarol or Diuron). Time decays of the DF signal indicate notable fast decay component and a slower decay component (or second peak). A hypothesis for the origin of the slow component is the slower recombination of photosynthetic charge on some electron carriers that are distant from P680 in the photosystem II reaction centre (Lavorel, 1975; Gerhardt and Krause, 1984; Jursinic, 1986; Schmidt and Senger, 1987; Bürger and Schmidt, 1988; Katsumata et al., 2008; Goltsev et al., 2009).

A schematic view of fast and slow components of DF in photosystems is outlined based on the above literature and presented in Fig. 1. Early electron acceptors, quinone Q_A and Q_B, are close to P680 in photosystem II and so the charge recombination between P680 and Q_A or Q_B would be observed as the fast component. Subsequent carriers, *i.e.* plastoquinone in the thylakoid membrane (plastoquinone pool), plastocyanin, and ferredoxin in photosystem I, are positioned relatively far from photosystem II and therefore would be observed as the slow component. In fact, research on the slow component suggested that it may be related to the plastoquinone pool (Lavorel, 1975; Gerhardt and Krause, 1984; Nakamoto et al., 1988; Goltsev et al., 2009), and our previous research on the induction of the slow component by strong illumination supports the link between the slow component, the cyclic

* Corresponding author at: Graduate School of Agriculture, Ehime University, 3-5-7 Tarumi, Matsuyama, Ehime 790-8566, Japan.
E-mail addresses: katumata@crl.hpk.co.jp (M. Katsumata), takeuchi@agr.ehime-u.ac.jp (I. Takeuchi).

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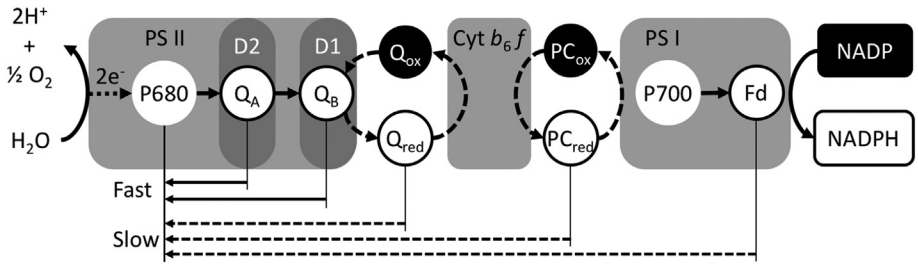

Fig. 1. Schematic view of fast and slow components of DF in photosystems. **PS II**, photosystem II complex. **Cyt b_6 f**, cytochrome b_6 f complex. **PS I**, photosystem I complex. **P680**, photosystem II primary donor chlorophyll. **P700**, photosystem I primary donor chlorophyll. **Q_A**, quinone Q_A. **Q_B**, quinone Q_B. **$Q_{red/ox}$**, reduced/oxidized plastoquinone in thylakoid membrane. **$PC_{red/ox}$**, reduced/oxidized plastocyanin. **Fd**, ferredoxin in photosystem I. **NADPH/NADP**, reduced/oxidized nicotinamide adenine dinucleotide phosphate.

electron transfer pathway of photosystem I and the plastoquinone pool (Katsumata et al., 2009). Irgarol and Diuron are competitive inhibitors at Q_B on the D1 protein, and concentrations of the herbicides, Q_B and the D1 protein itself will affect inhibition of slow component of DF Integrated. Note that it is electron transport by the latter carriers that is closely related to NADPH production and is necessary to produce biomass and growth (*i.e.* carbon dioxide fixation). Therefore, the slow component of DF integrated can be an indicator of growth inhibition of typical oxygenic photosynthetic organisms.

On a larger scale, since the amount of DF directly corresponds to the number of active (growing) algal cells, rapid, non-invasive and simple methods based on DF have been developed to detect changes in algal growth brought about by exposure to inhibitors, including those affecting photosystem II (Katsumata et al., 2006, 2008, 2009, 2010; Yamagishi et al., 2016). The reports suggest that DF can be used to evaluate both the extent of a photosynthetic system and the activity of the electron carriers in it. They also present relatively later ranges for the onset of DF (*e.g.* later than 0.6 s after excitation) that can be used as quick indicators of algal growth inhibition. Finally, DF is reported to be useful in evaluating other effects of toxic chemicals on algae, *i.e.* herbicides or heavy metals on green algae (*Desmodesmus subspicatus* and *Acetabularia acetabulum*), and cyanobacteria (*Microcystis aeruginosa*) (Bürger and Schmidt, 1988; Scordino et al., 1996; Berden-Zrimec et al., 2007; Leunert et al., 2013).

Most hermatypic corals, including *Acropora*, possess symbiotic dinoflagellates, *Symbiodinium*, commonly known as zooxanthellae. While dinoflagellates exhibit a wide variety of feeding modes from photoautography to heterotrophy, *Symbiodinium* associated with corals are photosynthetic (Muller-Parker et al., 2015). *Symbiodinium* are divided into several distinct phylogenetic groups (= clades; designated A to I) based on genetic analysis, and each clade is further divided into numerous subclades and types (Muller-Parker et al., 2015; Yamashita and Koike, 2015). In the Indo-Pacific, the vast majority of corals possess clades C and D of *Symbiodinium* (Muller-Parker et al., 2015; Keshavmurthy et al., 2017). Flexibility in symbiotic associations has also been observed in the early growth stage of *Acropora* infected by *Symbiodinium* from the surrounding environment, known as horizontal transmission (Yuyama and Higuchi, 2014; Yamashita and Koike, 2015). Coral bleaching is induced by a breakdown of the symbiotic relationships between corals and *Symbiodinium* and/or loss of photosynthetic pigments (see Yamashita and Koike, 2015).

In this study we report on our first application of the DF technique to evaluate the influence of two herbicides (Irgarol and Diuron) on the symbiotic dinoflagellates in the hard coral *Acropora digitifera* (Dana, 1846), in terms of molecular membrane transport (*i.e.* inflow and outflow rates), and their binding to the target protein in photosystem II.

2. Material and methods

2.1. Sample preparation

Colonies of *Acropora digitifera* previously collected from the reef flat of Sesoko Island were cut into small branches (approximately 1 cm long) at the Sesoko Station, Tropical Biosphere Research Center, the University of the Ryukyus. The branches were maintained in an aerated aquarium supplied with flowing seawater for about 4 weeks. Then they were transported to Ehime University at Matsuyama, Ehime from Okinawa within 24 h, where they were acclimated for about 1–2 weeks in a *ca.* 36-L aquarium (26 °C) that had been filled with artificial seawater, LIVEsea salt (Delphis Inc., Itami, Hyogo, Japan), and kept under an LED light (PowerShot; Kotobuki Co. Ltd., Matsubara, Osaka, Japan).

One day before the exposure test, the branches were transferred individually into glass petri dishes that had been washed and cleaned using acetone, hexane, hydrochloric acid (HCl), and distilled water according to the protocol of Aono and Takeuchi (2008). Each dish was filled with *ca.* 80 mL of artificial seawater and maintained at 27.5 °C with a 12 h dark: 12 h light (LED bulb) photoperiod. PPFD (photosynthetic photon flux density) at the center of the petri dish's cover (outer surface) was set at 50 μmol m^{-2} s^{-1}.

2.2. Measurement of DF signal

The DF signals from each branch before (as 0 h) and 1–24 h after exposure were measured by a high sensitivity luminometer (type-6110, Hamamatsu Photonics K.K., Hamamatsu, Shizuoka, Japan). A branch, in measurement dish, was placed in the luminometer dark box under the excitation light source, as shown in Fig. 2. The branch was left undisturbed for 60 s and then the excitation light (680 nm, 20 μmol m^{-2} s^{-1}, 5 s) irradiated the branch. The excitation light was turned off and the detector above the branch sent the DF signal to a personal computer, where the signal was recorded every 100 ms for 0.1–60 s.

The DF signals from uninhibited *A. digitifera* show typical decay kinetics, with an exponential-like fast decay component for approximately 0.1–10 s after excitation, and an afterglow-like (or bi-exponential-like) slow decay component beyond 10 s after excitation. The fast decay component is related to charge recombination in photosystem II, and the slow decay component is related to various other types of charge recombination, including those of cyclic electron transport pathways through photosystem I (Lavorel, 1975; Nakamoto et al., 1988; Goltsev et al., 2009). To evaluate the effect of herbicides on the fast and slow components, the DF signals for 0.1–10.0 s were integrated as F-DFI (DF Integrated of fast component), and those for 10.1–60.0 s were integrated as S-DFI (DF Integrated of slow component). The F-DFI and the S-DFI of each exposure time (*i.e.* 0, 1, 2, 4, 6, 8, and 24 h) are referred to as F- or S-DFI_t (where t is the exposure time).

2.3. Exposure test conditions

The two herbicides Irgarol (CAS 28159–98-0; triazine type) and Diuron (CAS 330-54-1; phenylurea type) are well known inhibitors of photosynthesis that block electron transport at photosystem II. The effect of each herbicide was evaluated by comparing DF behaviour over time after exposure. The test procedure is depicted in Fig. 3. Before the exposure test, the initial DF of each branch in seawater was measured. Then the branch was transferred into a prepared petri dish that had been filled with approximately 80 mL of one of the following: the control solution (*i.e.* seawater), control solution with solvent (acetone),