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ORIGINAL ARTICLE

Rapid formation of antheraxanthin and zeaxanthin in seconds in microalgae and its relation to non-photochemical quenching

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Abstract

The violaxanthin (V)–antheraxanthin (A)–zeaxanthin (Z) (VAZ) cycle was deemed a non-second-scale process of photoprotection in higher plants and microalgae, but the validity of this view has not been confirmed. To test this view, we explored responses of the VAZ cycle and the relationship between the VAZ cycle and non-photochemical quenching (NPQ) under highlight at second and minute scales in *Heterosigma akashiwo* and *Platymonas* sp. Both A and Z were generated in *H. akashiwo* during 15 s of light exposure, whereas only A rapidly accumulated within 15 s of exposure in *Platymonas* sp. The above results, together with a time-dependent sigmoidal relationship between the VAZ cycle (de-epoxidation state, A/Chl *a*, and Z/Chl *a*) and NPQ, proved that the VAZ cycle was a second-scale process related to NPQ. In addition, we found that not all NPQ was dependent on the VAZ cycle and suggested that NPQ model should be carefully modified due to the species-specific proportions of de-epoxidation-dependent NPQ.

Keywords Xanthophyll · Second-scale · Raphidophyte · Green algae · Photoprotection · Model

Introduction

The violaxanthin (V)–antheraxanthin (A)–zeaxanthin (Z) (VAZ) cycle is a ubiquitous xanthophyll-dependent process for energy dissipation in higher plants, green algae, *Chromera*, eustigmatophytes, chrysophytes, Phaeophyceae, and raphidophytes (Demmig-Adams et al. 2012; Goss and Lepetit 2015). By the catalysis via V de-epoxidase (VDE), V was de-epoxidized to A (one epoxy group) and finally formed Z (epoxy-free) under high light intensity in the presence of a proton gradient across thylakoid membranes (Goss and Jakob 2010; Goss and Lepetit 2015). Both A and Z can serve as energy quenchers or aggregation enhancers of the peripheral light-harvesting complex II (LHCII) for photoprotection (Goss and Lepetit 2015).

Variation in light can occur on the time scale of seconds in the upper layers of the water column (Ferris and Christian 1991; Schubert et al. 2001). Microalgae must respond to this fast light variation to protect themselves against potential photoinhibition under high light (Goss and Jakob 2010). However, the full formation of Z from V required 5–15 min (Yamamoto 1979; Hager 1980). Erickson et al. (2015) also reviewed that the green alga *Chlamydomonas reinhardtii* accumulated Z in a manner corresponding to the slower phase of the fast component of non-photochemical quenching (NPQ) qE, i.e., over a few minutes, whereas a rapid increase in qE was attributed to a proton gradient and the presence of lutein. These results suggested that the xanthophyll cycle in VAZ-containing plants did not participate in the photoprotective response to sudden exposure to high light. In contrast to the above results, an NPQ model based on *Arabidopsis* indicated that the rapid generation of qE depended on the proton gradient but was modulated by Z in 10–200 s (Jahns and Holzwarth 2012). Considerable

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studies on the time course of Z accumulation and NPQ formation have been done since 1979 or 1980 (e.g., Demmig-Adams et al. 1989; Adams et al. 1999; Dimier et al. 2009; Blommaert et al. 2017). It has been found the retention of Z even in leaves that showed no sustained depressions in F_v/F_m allowed the ultra-fast engagement of NPQ upon illumination in leaves (Demmig-Adams et al. 1989; Adams et al. 1999). However, no direct evidence for the second-scale responses of the VAZ cycle was provided in previous studies. Therefore, it is hard to confirm whether the xanthophyll cycle in VAZ-containing microalgae contributed to their fast responses to high light.

In addition, the concentrations of de-epoxidated pigments were generally correlated with NPQ to explain the mechanism of photoprotection, and the relationship was most likely to be linear (e.g., Goss et al. 2006; Dimier et al. 2009; Blommaert et al. 2017). Hennige et al. (2013), however, found a time lag between NPQ and the de-epoxidation of pigments. These studies were conducted on the time scales of minutes or hours; thus, we did not know exactly whether the levels of these de-epoxidated pigments were correlated to NPQ in seconds, especially for the VAZ-containing algae, due to the lack of second-scale experiments.

In the present study, we hypothesized that VAZ cycle-containing microalgae can synthesize A and Z in seconds after exposure to high light and that the contents of A and Z are correlated with NPQ. To test this hypothesis, *Heterosigma akashiwo* (a raphidophyte) and *Platymonas* sp. (green algae) were selected as testing algae. Both raphidophytes and green algae (except prasinophyceae and zygnematophyceae) have been proven to have a complete VAZ cycle that can be triggered under high light (Goss and Lepetit 2015). The contents of four pigments (V, A, Z, and Chl *a*) were determined after 0 s, 15 s, 120 s, and 900 s of exposure to high light for the comparison of de-epoxidated responses at second and minute scales in these algae. NPQ and the rate constants for first-order kinetics of de-epoxidation were calculated accordingly. Two inhibitors, dithiothreitol (DTT) and NH_4Cl, were added to investigate the role of VDE and the proton gradient in the de-epoxidation of pigment and the induction of NPQ (Yamamoto and Kamite 1972; Goss et al. 1998). Our study will provide a fundamental and physiological understanding of the rapid responses of the xanthophyll cycle to high light in VAZ-containing microalgae and may help modify the NPQ model.

Materials and methods

Algal culture

Heterosigma akashiwo (strain number CCMA-369) was purchased from the Center for Collections of Marine Algae (CCMA) at Xiamen University. *Platymonas* sp. was isolated from the prawn-culturing pond in Qingdao. Both species were stored in the First Institute of Oceanography, Ministry of Natural Resources, China. They were cultured at a constant temperature of 21 ± 1 °C under a 12 h light:12 h dark cycle. Culture media were enriched with f/2 medium without the addition of silicate (Guillard 1975) and had an initial pH and salinity of 7.8 and 30.5. The light intensity was 79–90 µmol photons m^{-2} s^{-1}, which was provided by five 25 W energy-saving white light tubes (MXF1-Y25W, Zhejiang Yankon Group Co., Ltd., China). The light density was measured by a Phyto-PAM instrument (Heinz Walz GmbH, Germany) equipped with a spherical micro-quantum sensor (US-SQS/B-D).

Experimental design and the measurement of pigments and NPQ

Both algae were cultured in the conditions listed in the section on algal culture with an initial density of 131 ± 22 cells mL^{-1} for *H. akashiwo* and 112 ± 15 cells mL^{-1} for *Platymonas* sp. They were collected after 10 days of culture. The final cell density was 527497 ± 61384 cells L^{-1} for *Platymonas* sp. and 286677 ± 72919 cells L^{-1} for *H. akashiwo*. To test the responses of xanthophyll-related pigments and NPQ to high light exposure, the two algae were acclimated in darkness for 20 min to determine the content of the xanthophyll pool and to open reaction centres to obtain F_m. To clarify the role of VDE and the proton gradient in the de-epoxidation of pigment and the induction of NPQ (Yamamoto and Kamite 1972; Goss et al. 1998), DTT (final concentration, 2 mM), and NH_4Cl (final concentration 10 mM) were added at the start of dark exposure. There were nine biological replicates for each alga: three for a control (without the addition of DTT and NH_4Cl), three for DTT treatments (only DTT was added), and three for NH_4Cl treatments (only NH_4Cl was added).

For pigment analysis, after dark acclimation, 10 mL of algae culture was immediately filtered onto a 25 mm GF/F membrane (Whatman, GE Healthcare UK Ltd., UK) under dim light (< 5 µmol photons m^{-2} s^{-1}). The case of 0 s of exposure to high light was considered. For 15 s and 120 s of exposure to high light, 10 mL of algae culture was immediately transferred to a filter bowl after dark acclimation. Before algal transfer, the filter bowl was equipped on a filter head with a 25 mm GF/F membrane on the head. Light (537 µmol photons m^{-2} s^{-1}) was provided by a 10 W accent light (C8-T6, Supfire Co., Ltd., China) that was hung up on the filter bowl in advance. The algae cultures were filtered under the high light intensity, and the exposure time included the filtering time. The filtering time was 6 s for *H. akashiwo* and 10 s for *Platymonas* sp. For the experiments of 15 s, the high light exposure and algal filtering started at the same time. When filtering finished, the high

light exposure continued until the end of 15 s. For 900 s of high light exposure, after dark acclimation, 10 mL of algae culture was immediately transferred into a 9 cm Petri dish under high light (537 µmol photons m^{-2} s^{-1}). Light was provided by white-light belts (5630, Zhongshan Wancai Co., Ltd., China). After 900 s of illumination with high light, algae were filtered onto a 25 mm GF/F membrane under a light intensity of 537 µmol photons m^{-2} s^{-1}, which was provided by the 10 W accent light used in 15 s and 120 s light exposures. The exposure time of 900 s also included the filtering time. Light intensities were measured by a spherical micro-quantum sensor (US-SQS/B-D) equipped in a Phyto-PAM instrument (Heinz Walz GmbH, Germany). The light spectra of white-light belts and accent lights were similar, with a maximum peak of relative intensity (0.20–0.25) at approximately 450 nm, and a weaker peak of relative intensity (0.10–0.15) at approximately 550 nm.

After filtering, membranes were immediately frozen in liquid nitrogen and stored at − 80 °C. The extraction and detection of pigment and the calculation of pigment content were performed according to Gao et al. (2018). The de-epoxidation state of pigments was calculated as in Dimier et al. (2009):

$$\text{De-epoxidation state} = \frac{A + Z}{V + A + Z} \quad (1)$$

where V, A, and Z are the mol ratios of violaxanthin, antheraxanthin, and zeaxanthin to Chl *a* (mol: 100 mol Chl *a*).

To compare the varying rates at which de-epoxidation state and pigment contents change with exposure time, an equation for first-order kinetics was used to compare different varying rates at different time intervals. The equation was revised based on Falkowski (1983):

$$k1 = -\frac{\ln \frac{R_{t-1}}{R_t}}{t_i} \quad (2)$$

where $k1$ was a first-order rate constant (min^{-1}), R_{t-1} was the shorter exposure time, and R_t was the longer exposure time. That is, if R_{t-1} was 0 s, R_t was 15 s; if R_{t-1} was 15 s, R_t was 120 s; and if R_{t-1} was 120 s, R_t was 900 s. t_i was the time interval between R_{t-1} and R_t. Because we calculated the actual $k1$ between two adjacent exposure times, the R value at equilibrium was not extracted from R_{t-1} and R_t as described in Falkowski (1983).

For NPQ measurement, algal cultures were transferred to a 24-well plate (Corning Incorporated, USA) after dark acclimation of 20 min. The bottom of the plate was blacked in advance using permanent marker pens. We filled plate wells with fresh culture medium, and no NPQ signal was detected in the well using Image-PAM with a MAXI measuring head equipped with an IMAG-MAX/L illumination unit (Heinz Walz GmbH, Germany). The bottom-blacked plate can thus be used in measurements of NPQ. Each well indicates a biological replicate. A saturating light was applied for 200 ms to acquire F_m. After that an actinic light (537 µmol photons m^{-2} s^{-1}) was applied for 15 s to obtain F'_m. NPQ was calculated following the following equation in Maxwell and Johnson (2000):

$$\text{NPQ} = \frac{F_m - F'_m}{F'_m} \quad (3)$$

The same measuring protocols were used to measure NPQ after 120 s and 900 s of exposure to actinic light. If NPQ values were lower than zero, they were recorded as zero. The NPQ values at 0 s of light exposure were also considered zero.

Proportions of A- and/or Z-dependent NPQ at one exposure time were calculated using the following equation:

$$\text{Proportions of de-epoxidation-dependent NPQ}(\%) = \frac{\text{NPQ}_{\text{control}} - \text{NPQ}_{\text{inhibition}}}{\text{NPQ}_{\text{control}}} \quad (4)$$

where $NPQ_{control}$ was the NPQ value in the control group and $NPQ_{inhibition}$ was the NPQ value in DTT or NH_4Cl treatments. DTT and NH_4Cl treatments were considered one treatment in the calculation because changes in both A/Chl *a* and Z/Chl *a* were inhibited so that these quantities remained at their levels before light exposure in the two inhibitor treatments (Figs. 1 and 2). This resulted in six replicates for calculating the proportions at each exposure time.

Statistical analysis

Graphics were generated by SigmaPlot 12.5 (Systat Software Inc., San Jose, USA). An one-way ANOVA (homogeneity of variances was satisfied) or a Kruskal–Wallis *H* test (homogeneity of variances was not satisfied) was used using

Table 1 Initial xanthophyll pool (mol 100 mol Chl a^{-1}) at 0 s of light exposure ($n = 3$, mean ± SD, V: violaxanthin; A: antheraxanthin; Z: zeaxanthin)

Alga	V/Chl *a*	A/Chl *a*	Z/Chl *a*	(V + A + Z)/Chl *a*
H. akashiwo	14.506 ± 0.523	0.164 ± 0.039	0.177 ± 0.040	14.846 ± 0.656
Platymonas sp.	9.413 ± 0.174	0.155 ± 0.018	0.362 ± 0.018	9.929 ± 0.162

SPSS 16.0 (SPSS Inc., Chicago, USA) to show whether exposure time was a significant factor that affected the de-epoxidation state, pigment contents, NPQ, and proportion of de-epoxidation-dependent NPQ. An independent samples *t* test was conducted in SPSS 16.0 to compare changes in the above variables between different exposure times once the exposure time was considered a significant factor. An independent samples *t* test was also used to compare changes in rate constants of first-order kinetics between different time intervals. To clarify the relationships between NPQ and de-epoxidation state, A/Chl *a* and Z/Chl *a*, the data were fitted by a sigmoidal equation using SigmaPlot 12.5. For all statistical analysis, $p < 0.05$ was considered statistically significant.

Results

At 0 s of light exposure (V + A + Z)/Chl *a* (14.846 ± 0.656 mol 100 mol Chl a^{-1}) in *H. akashiwo* was higher than that of *Platymonas* sp. (9.929 ± 0.162 mol 100 mol Chl a^{-1}) (Table 1). In only the control did *H. akashiwo* and *Platymonas* sp. show significant increases in the de-epoxidation state of xanthophyll pigments [(A + Z)/(V + A + Z)] and pigment contents (A/Chl *a* and Z/Chl *a*) with the extension of light exposure. These parameters were significantly restricted to the initial level in the DTT and NH_4Cl treatments (Figs. 1 and 2).

Within the initial 15 s exposure to high light, the de-epoxidation state in the control increased about three times from 0.023 ± 0.004 to 0.074 ± 0.011 in *H. akashiwo* (Fig. 1a) and about two times from 0.052 ± 0.003 to 0.092 ± 0.019 in *Platymonas* sp.(Fig. 2a). The first-order rate constant for formation of the de-epoxidation state [$k1_{(A+Z)/(V+A+Z)}$]

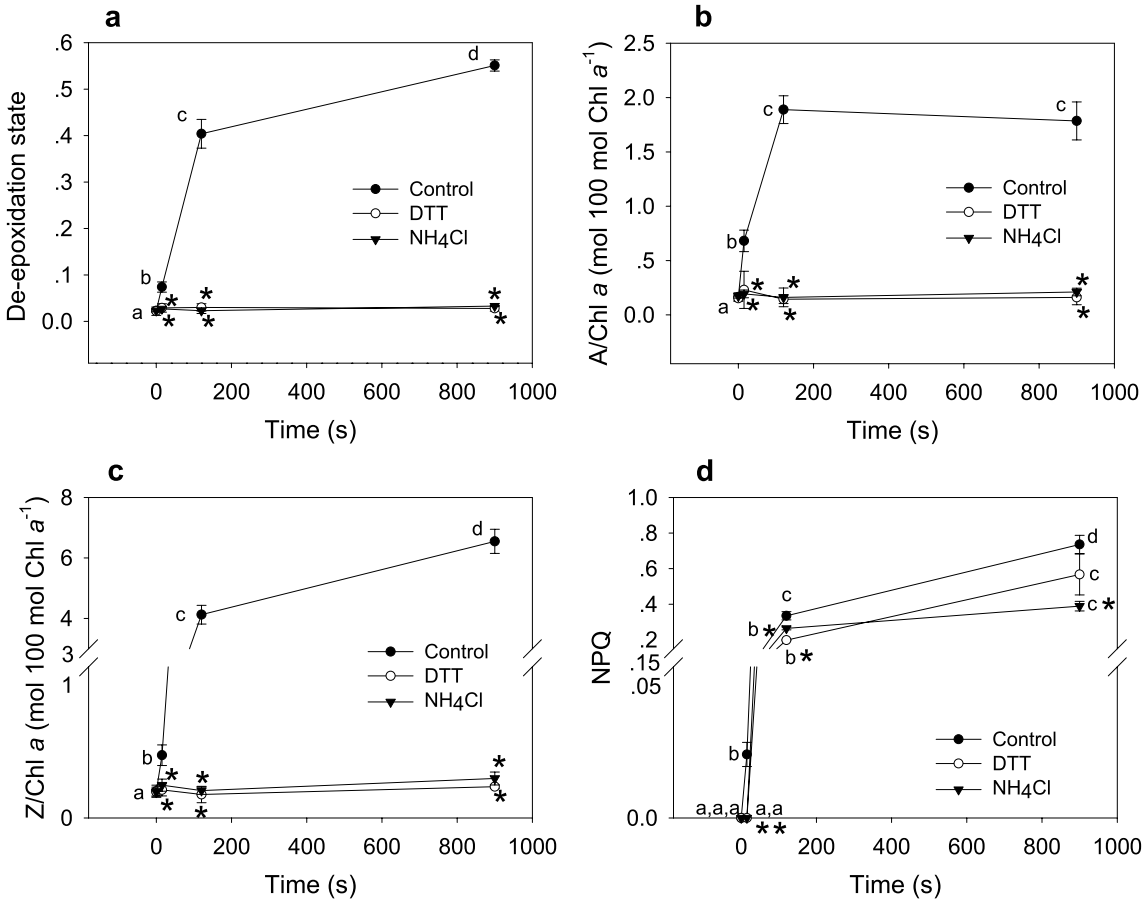

Fig. 1 Changes in de-epoxidation state (**a**), pigment contents of A/Chl *a* (**b**) and Z/Chl *a* (**c**), and NPQ (d) in the control (filled circle), DTT (unfilled circle), and NH_4Cl (filled triangle) treatments at different high light exposure times in *H. akashiwo*. ($n = 3$, mean ± SD, *V* violaxanthin; *A* antheraxanthin; *Z* zeaxanthin; *NPQ* non-photochemical quenching; *DTT* dithiothreitol). Different lowercase letters indicate significant differences with $p < 0.05$ between values for different exposure times in one treatment, *indicates significant changes with $p < 0.05$ between the inhibitor-added treatments and control

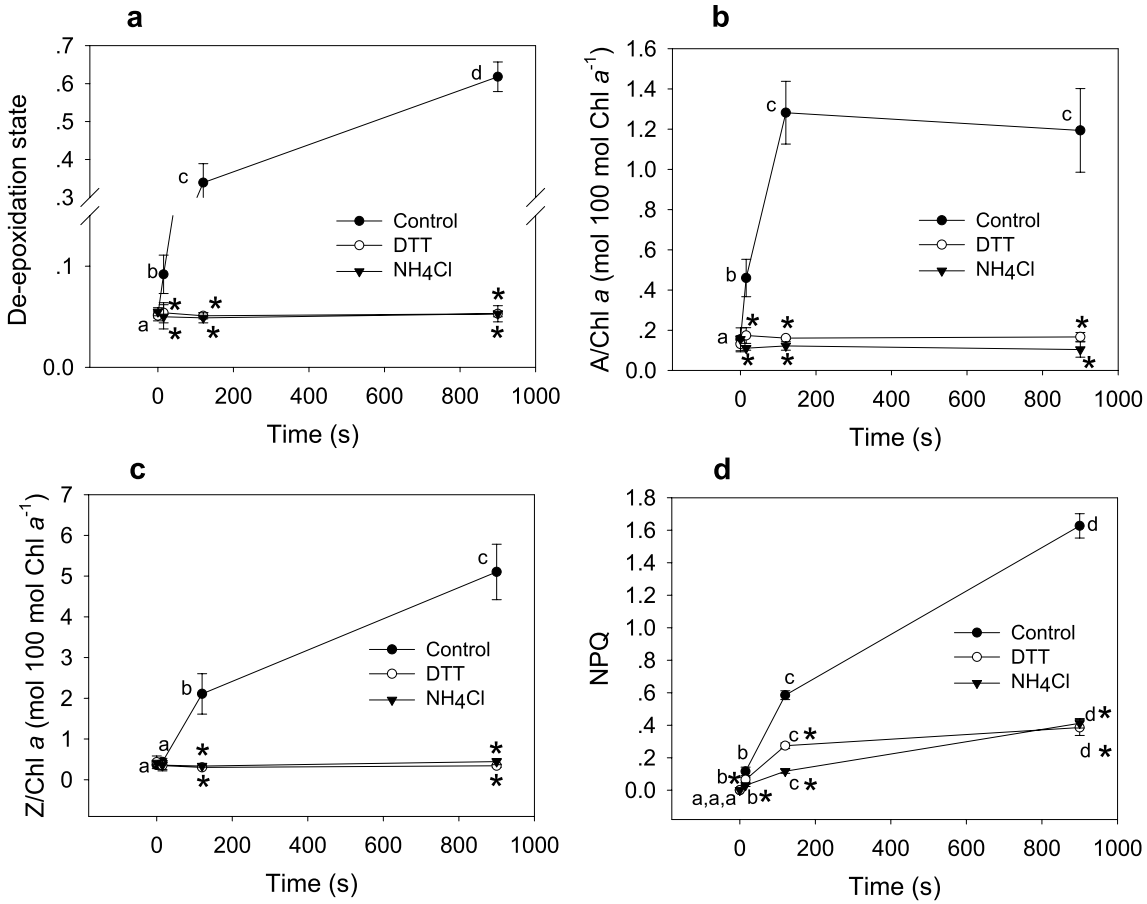

Fig. 2 Changes in de-epoxidation state (**a**), pigment contents of A/Chl *a* (**b**) and Z/Chl *a* (**c**), and NPQ (**d**) in the control (filled circle), DTT (unfilled circle), and NH_4Cl (filled triangle) treatments at different high-light exposure times in *Platymonas* sp. ($n = 3$, mean ± SD, *V* violaxanthin; *A* antheraxanthin; *Z* zeaxanthin; *NPQ* non-photochemical quenching; *DTT* dithiothreitol). Different lowercase letters indicate significant differences with $p < 0.05$ between values for different exposure times in one treatment, *indicates significant changes with $p < 0.05$ between the inhibitor-added treatments and control

within this period was higher than that of other time intervals, reaching up to 4.714 ± 1.218 min^{-1} in *H. akashiwo* and 2.238 ± 0.873 min^{-1} in *Platymonas* sp. (Table 2). At 120 s of light exposure, the de-epoxidation state of *H. akashiwo* and *Platymonas* sp. increased to 0.404 ± 0.031 and 0.339 ± 0.050, respectively, which were about five times and four times higher than the values at 15 s of light exposure. The first-order rate constants [$k1_{(A+Z)/(V+A+Z)}$] significantly decreased to 0.972 ± 0.052 min^{-1} in *H. akashiwo* and 0.748 ± 0.121 min^{-1} in *Platymonas* sp. in the time interval of 15–120 s. At 900 s of light exposure, the de-epoxidation state of *H. akashiwo* and *Platymonas* sp. increased to 0.551 ± 0.012 and 0.618 ± 0.039, respectively. The increments were less than two times higher than that at 120 s

Table 2 First-order rate constant (min^{-1}) over different intervals of exposure time ($n = 3$, mean ± SD, V: violaxanthin; A: antheraxanthin; Z: zeaxanthin)

Alga	Time interval	$k1_{(A+Z)/(V+A+Z)}$	$k1_{A/Chl\ a}$	$k1_{Z/Chl\ a}$
H. akashiwo	0–15 s	4.714 ± 1.218^{a}	5.743 ± 1.355^{a}	3.442 ± 1.576^{ab}
	10–120 s	0.972 ± 0.052^{b}	0.586 ± 0.063^{b}	1.316 ± 0.058^{a}
	120–900 s	0.024 ± 0.005^{c}	-0.005 ± 0.007^{c}	0.036 ± 0.002^{b}
Platymonas sp.	0–15 s	2.238 ± 0.873^{a}	4.320 ± 0.601^{a}	0.698 ± 1.038^{ab}
	10–120 s	0.748 ± 0.121^{b}	0.590 ± 0.086^{b}	0.872 ± 0.096^{a}
	120–900 s	0.047 ± 0.008^{c}	-0.006 ± 0.009^{c}	0.071 ± 0.015^{b}

[a,b,c]Different lowercase letters after values indicate significant differences with $p < 0.05$ between values for different time intervals

in both algae. Correspondingly, the lowest first-order rate constants [$k1_{(A+Z)/(V+A+Z)}$] were found in the time interval of 120–900 s and were 0.024 ± 0.005 min^{-1} in *H. akashiwo* and 0.047 ± 0.008 min^{-1} in *Platymonas* sp.

The A/Chl *a* ratios in the control increased about fourfold, from 0.164 ± 0.039 to 0.681 ± 0.099 mol 100 mol Chl a^{-1} in *H. akashiwo* (Fig. 1b), and about threefold, from 0.155 ± 0.018 to 0.460 ± 0.093 mol 100 mol Chl a^{-1} in *Platymonas* sp. (Fig. 2b), within the initial light exposure of 15 s. The highest first-order rate constants ($k1_{A/Chl\ a}$) occurred in the time interval of 0–15 s, with 5.743 ± 1.355 min^{-1} in *H. akashiwo* and 4.320 ± 0.601 min^{-1} in *Platymonas* sp. being found (Table 2). The A/Chl *a* ratios continued to increase to 1.889 ± 0.128 mol 100 mol Chl a^{-1} in *H. akashiwo* and 1.282 ± 0.156 mol 100 mol Chl a^{-1} in *Platymonas* sp. at 120 s of light exposure, both of which were about three times higher than those at 15 s. After that, the A/Chl *a* ratios did not further significantly increase at 900 s of light exposure. Correspondingly, the first-order rate constant ($k1_{A/Chl\ a}$) decreased from 0.586 ± 0.063 min^{-1} in the time interval of 15–120 s to − 0.005 ± 0.007 min^{-1} in the time interval of 120–900 s in *H. akashiwo* and decreased from 0.590 ± 0.086 to − 0.006 ± 0.009 min^{-1} in *Platymonas* sp.

For *H. akashiwo*, the Z/Chl *a* ratios in the control significantly increased threefold, from 0.177 ± 0.040 to 0.415 ± 0.068 mol 100 mol Chl a^{-1}, within the initial 15 s exposure to high light (Fig. 1c). It further increased to 4.125 ± 0.310 mol 100 mol Chl a^{-1} at 120 s of light exposure, which was about ten times higher than that at 15 s. At 900 s of light exposure, the Z/Chl *a* ratio was 6.549 ± 0.399 mol 100 mol Chl a^{-1}, which was less than two times higher than that at 120 s. For *Platymonas* sp., 15 s of light exposure did not significantly enhance the Z/Chl *a* ratios (Fig. 2c). After that, the Z/Chl *a* ratios increased nearly fivefold, from 0.436 ± 0.089 to 2.018 ± 0.497 mol 100 mol Chl a^{-1}, when the light exposure was extended from 15 to 120 s and further increased to 5.013 ± 0.681 mol 100 mol Chl a^{-1} at 900 s of light exposure. The first-order rate constants ($k1_{Z/Chl\ a}$) significantly decreased from 1.316 ± 0.058 min^{-1} in the 15–120 s interval to 0.036 ± 0.002 min^{-1} in the 120–900 s interval in *H. akashiwo* and decreased from 0.872 ± 0.096 to 0.071 ± 0.015 min^{-1} in *Platymonas* sp.(Table 2).

The NPQ in *H. akashiwo* and *Platymonas* sp. significantly increased with the duration of light exposure in control, DTT, and NH_4Cl treatments (Figs. 1d and 2d). For *H. akashiwo* (Fig. 1d), the NPQ in the control increased from 0 to 0.021 ± 0.004 with the initial 15 s of light exposure. It further increased to 0.335 ± 0.023 at 120 s of light exposure and 0.736 ± 0.051 at 900 s of light exposure. Compared with the control treatment, the addition of inhibitors significantly suppressed the NPQ to 0 at 15 s of light exposure and to 0.198 ± 0.003 in the DTT treatment and 0.264 ± 0.008 in the NH_4Cl treatment at 120 s of light exposure. At 900 s of light exposure, the average NPQ values in the NH_4Cl treatment were also lower than those in the control. For *Platymonas* sp. (Fig. 2d), the NPQ in the control significantly increased from 0 to 0.116 ± 0.026 in the initial 15 s of light exposure, further increased to 0.585 ± 0.027 at 120 s of light exposure, and finally reached 1.627 ± 0.075 at 900 s of light exposure. Compared with the control, the addition of DTT significantly suppressed the NPQ to 0.065 ± 0.015 and 0.274 ± 0.017 at 15 s and 120 s of light exposure, respectively, and the addition of NH_4Cl significantly suppressed the NPQ to 0.030 ± 0.007 and 0.117 ± 0.013 at 15 s and 120 s of light exposure. At 900 s of light exposure, approximately 76% of NPQ was inhibited in two inhibitor-added treatments relative to the control.

When the relationships between pigment-related parameters (de-epoxidation state, A/Chl *a*, and Z/Chl *a*) and NPQ were fit, a significant sigmoidal relationship was found in the controls of *H. akashiwo* (Fig. 3a) and *Platymonas* sp. (Fig. 3b), but not in the two inhibitor-added treatments (Fig. 3c–e). Both the de-epoxidation state and Z/Chl *a* increased with increases in NPQ except those of the 900 s of light exposure, but the A/Chl *a* ratios increased with the increases in NPQ only when the light exposure was less than 120 s. In *H. akashiwo*, the average proportion of de-epoxidation-dependent NPQ was 1 at 15 s of light exposure and decreased to ca. 0.35 with increasing time (Fig. 4a). In *Platymonas* sp., the proportion showed time-independent changes, but a high proportion of ca. 0.6 to 0.8 was found at all time points (Fig. 4b).

Discussion

In our study, two distinguishing results were found: (1) the VAZ cycle can be triggered rapidly in microalgae within 15 s of exposure to high light but showed species-specific features and (2) the relationship between the VAZ cycle and NPQ can be fitted by a sigmoidal curve, but the relationship was only valid when VDE was not inhibited and a proton gradient was present.

Activation of the VAZ cycle on the time scale of seconds

Previous studies demonstrated that the full formation of Z from V (Yamamoto 1979; Hager 1980) and the accumulation of Z (Erickson et al. 2015) take minutes to occur. Similarly, the formation of Z has been reported in *H. akashiwo* when the alga was exposed to high light for several generations (Rodríguez et al. 2006; Butrón et al. 2012) and for several hours (Hennige et al. 2013). In contrast to previous studies, our study demonstrated that Z can be rapidly synthesized on the second time scale in *H.*

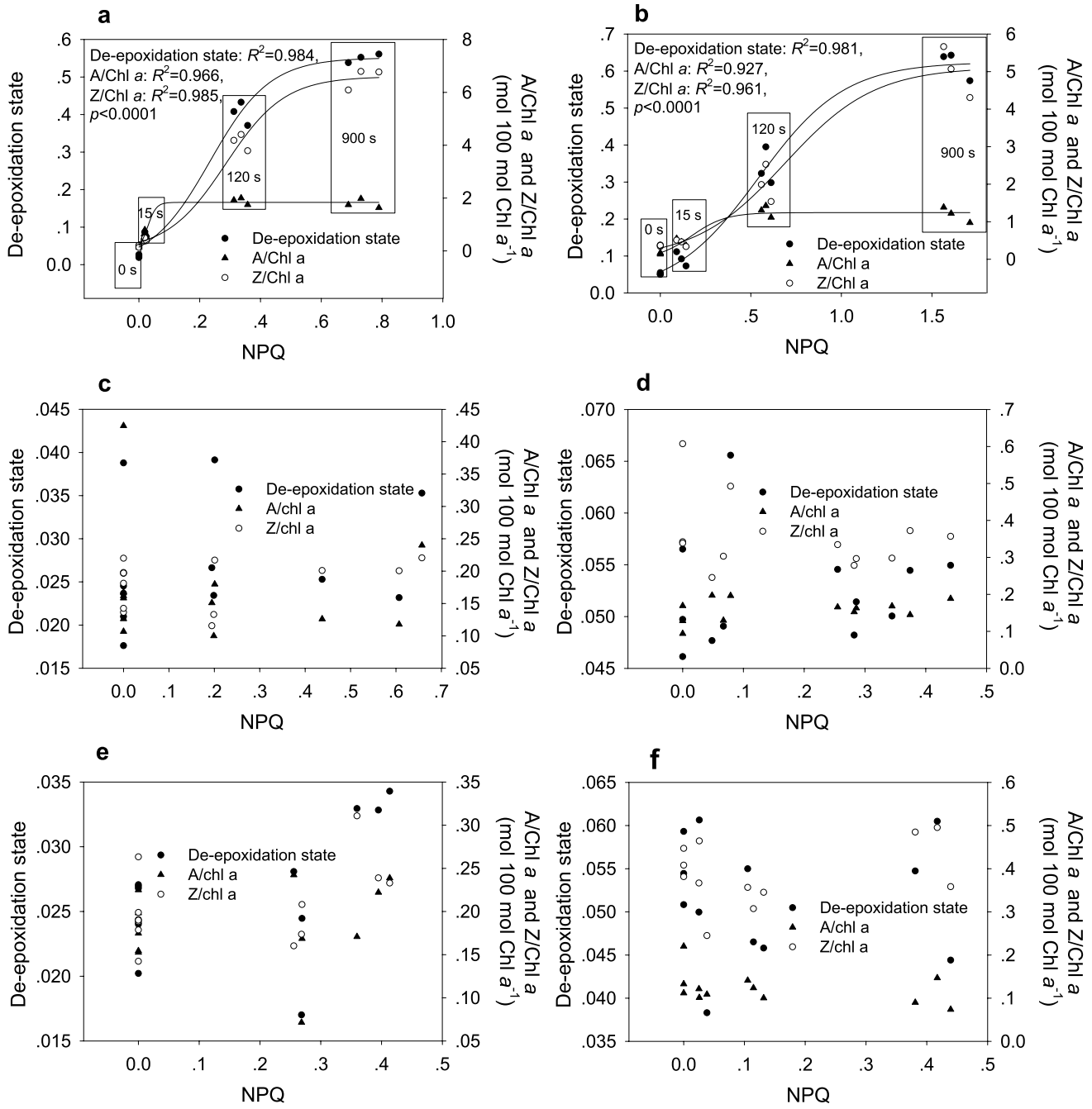

Fig. 3 Relationship between NPQ and the VAZ cycle (filled circle: de-epoxidation state, filled triangle: A/Chl *a*, unfilled circle: Z/Chl *a*) in the control (**a**, **b**), DTT (**c**, **d**) and NH_4Cl treatments (**e**, **f**) of *H. akashiwo* (**a**, **c**, **e**), and *Platymonas* sp. (**b**, **e**, **f**) ($n = 12$, *NPQ* non-photochemical quenching, *V* violaxanthin; A antheraxanthin; *Z* zeaxanthin, *DTT* dithiothreitol)

akashiwo; thereby, showing that the xanthophyll cycle participated in the response of *H. akashiwo* to sudden exposure to high light. However, the formation of Z was not detected until the exposure time of high light reached 120 s in *Platymonas* sp. Blommaert et al. (2017) suggested that algae with a high xanthophyll pool showed fast xanthophyll kinetics in high light. In our study, the xanthophyll pool [(V + A + Z)/Chl *a*] in *Platymonas* sp. was smaller than that in *H. akashiwo* (Table 1), which resulted in relatively slow xanthophyll kinetics in *Platymonas* sp. Therefore, light exposure for 15 s was likely to be not sufficient to induce the formation of Z. In addition, in our study, after filtering was finished, the real light intensity that the algae received was plausibly higher than

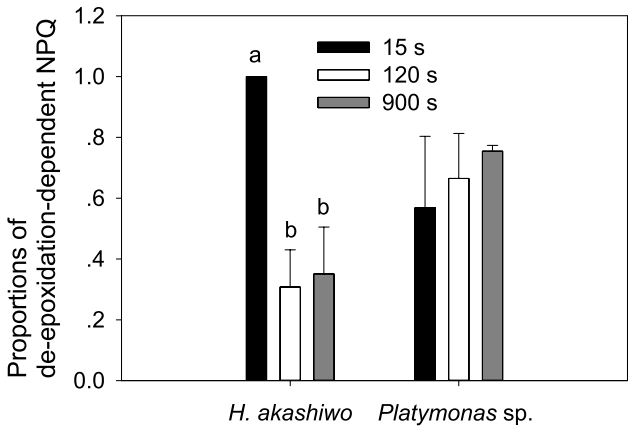

Fig. 4 Proportions of de-epoxidation-dependent NPQ at 15 s (dark rectangular), 120 s (white rectangular), and 900 s (gray rectangular) of exposure to high light ($n=6$, mean ± SD, *NPQ* non-photochemical quenching). Different lowercase letters indicate significant differences with $p<0.05$ between values among different time points in one algae

that during the filtering process. This is because there was no water column covering the filter membrane once filtering had finished. Light, therefore, would not be attenuated as it did in the water column. In the 15 s experiment, the time of *H. akashiwo* exposure to the un-attenuated high light was 9 s, and the time for *Platymonas* sp. was 5 s. The reduced exposure time of *Platymonas* sp. to un-attenuated high light might be an alternative explanation for the insignificant changes in Z/Chl *a* in the 15 s experiment.

The synthesis of A in *H. akashiwo* and *Platymonas* sp. can be triggered in seconds. Rapid transformation of diadinoxanthin (Dd) to diatoxanthin (Dt) was also found based on a first-order kinetic rate constant (Olaizola et al. 1994; Olaizola and Yamamoto 1994; Lohr and Wilhelm 1999; Macintyre et al. 2000; Bidigare et al. 2014). A similar phenomenon can be easily understood because both the transformation from V to A and that from Dd to Dt were one-step de-epoxidations from the initial molecule in their corresponding xanthophyll cycles. However, our results also showed that A can be rapidly accumulated only when the light exposure time was shorter than 120 s (Figs. 1b and 2b). Rapid accumulation of A was also found in *Arabidopsis thaliana* and the green alga *Mantoniella squamata* (Frommolt et al. 2001; Johnson et al. 2008). The accumulation of A in *M. squamata* was partly due to the slow transformation of A to Z (Frommolt et al. 2001). In our study, in the time interval of 120–900 s, the negative values of the first-order kinetic rate constant of A in the two algae suggested that the transformation from A and Z was faster than the transformation from V and A, which suppressed the accumulation of A.

Relationship between the VAZ cycle and NPQ induction

Goss and Lepetit (2015) reviewed that A can replace Z as a direct quencher in NPQ mechanisms, although there might be different roles of A at the stromal and the luminal sides of the thylakoid membrane. Our study also suggested that A can participate in the induction of NPQ in *H. akashiwo* and *Platymonas* sp. due to its increase with NPQ in less than 120 s of exposure. The sigmoidal fitting curve of the A, Z and de-epoxidation state in the NPQ induction in our study was similar to one found for *Arabidopsis thaliana* when NPQ formation rates were plotted against Z/Chl *a* (Johnson et al. 2008).

Previous studies showed that the fast NPQ component qE can be triggered in seconds (Müller et al. 2001) and that the initial rise in qE could be attributed to a proton gradient and lutein (Erickson et al. 2015). Our study showed that the initial rise in qE might also be related to the xanthophyll cycle because NPQ and the increases in de-epoxidized xanthophyll pigment were correlated. Before 900 s of light exposure, both the Z(A)-dependent part of qE and the Z-dependent, slow inducible component qZ likely contributed to the NPQ increases (Nilkens et al. 2010; Jahns and Holzwarth 2012; Erickson et al. 2015). A slow photoinhibition component of NPQ (qI) occurred during long-term exposure to high light (Müller et al. 2001; Nilkens et al. 2010), perhaps contributing to the NPQ changes when the light exposure reached 900 s. However, the role of Z in qI is presently unclear (Jahns and Holzwarth 2012).

We also found that NPQ kept increasing even if the VAZ cycle was inhibited by the addition of DTT or NH4Cl. The results showed that not all NPQ was dependent on the VAZ cycle in *H. akashiwo* and *Platymonas* sp. The low proportion of de-epoxidation-dependent NPQ found after 120 s of light exposure in our study confirmed the finding in Hennige et al. (2013) that increases in de-epoxidated xanthophyll pigments are not the primary pathway of photoprotection in *H. akashiwo*. For green algae, it has been reported that several macro- and micro-green algae do not exhibit A/Z-dependent NPQ (e.g., Quaas et al. 2015; Christa et al. 2017) due to the action of other quenching mechanisms, such as the direct energy quenching accomplished by harvesting of a complex stress-related protein (LHCSR3) in *Chlamydomonas* (Tian et al. 2019). Quaas et al. (2015) indicated that NPQ heterogeneity in their study was not related to phylogeny of algae. The genus *Platymonas* belongs to Chlamydomonadales, Chlorophyceae (Guiry 2020); for the algae, more than 50% of NPQ was de-epoxidation dependent in our study. In the study of Quaas et al. (2015), however, for the species in Chlamydomonadales, V de-epoxidation was absent or unrelated to the establishment of NPQ. Our results, therefore, agreed

with the conclusion of Quaas et al. (2015), at least at the level of Chlamydomonadales. In addition, we admit the likely role of state transitions (qT) in the de-epoxidation-independent NPQ induction in the green alga *Platymonas* sp. due to the importance of qT for photoprotection in green algae (Wobbe et al. 2016).

Christa et al. (2017) suggested that broad biodiversity studies on photoprotective mechanisms are needed. Our results showed that the A/Z-independent NPQ occurred not only in green algae but also in raphidophytes at varying proportions, thus confirming the suggestion by Christa et al. (2017). We suggested that other patterns regarding the de-epoxidation-dependent NPQ in these algae need to be further investigated.

Implications in NPQ model

Holzwarth et al. (2009) and Jahns and Holzwarth (2012) proposed an NPQ model that described a two-site quenching mechanism for NPQ based on *Arabidopsis*. NPQ, which strictly depends on PsbS, occurred in 1–5 min at the Q1 site; NPQ, which strongly depends on the formation of Z, occurred in 10–15 min at the Q2 site (Holzwarth et al. 2009). Their results also showed that Z modulated the generation of qE, and Jahns and Holzwarth (2012) redefined the time span to be 10–200 s. Our results partly agreed with the model and presented direct evidence of pigments to confirm the participation of the Z in the generation of fast NPQ. However, our results showed that A and Z are crucial for *H. akashiwo* within 15 s of light exposure, which differed from the above NPQ model and revealed the dependence of NPQ on de-epoxidated pigments on the time scale of seconds. In addition, according to the model, Z-dependent NPQ should be low in the short term but become high with prolonged light exposure. However, our study showed that this de-epoxidation-dependent NPQ was high in the short term but low in the long term in *H. akashiwo*, and for *Platymonas* sp., this kind of NPQ was uniformly high irrespective of exposure time. These differences proved that different species may have different patterns of NPQ induction in relation to de-epoxidated pigments and further suggested that the present NPQ model should be modified according to species once more evidence was available.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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