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Efficacy of botanical pesticide for rotifer extermination during the cultivation of *Nannochloropsis oculata* probed by chlorophyll *a* fluorescence transient

L.T. ZHANG[*,**], R. XU[*,***], and J.G. LIU[*,**,+]

Key Laboratory of Experimental Marine Biology, Center for Ocean Mega-Science, Institute of Oceanology,
Chinese Academy of Sciences, 266071 Qingdao, China[*]
Laboratory for Marine Biology and Biotechnology, Qingdao National Laboratory for Marine Science
and Technology, 266071 Qingdao, China[**]
University of Chinese Academy of Sciences, 100049 Beijing, China[***]

Abstract

Nannochloropsis is widely used in aquaculture as a feed source. However, large-scale cultivation of *Nannochloropsis* usually fails due to rotifer contamination. In order to identify an effective technique for reducing rotifer contamination, the effect of *Brachionus plicatilis* contamination on photosynthetic characteristics in *Nannochloropsis oculata* and the efficacy of the celangulin (CA):toosendanin (TSN) (1:9) combination for rotifer extermination were investigated using chlorophyll *a* fluorescence transient. *B. plicatilis* could directly devour microalgal cells and sharply reduced *N. oculata* density to very low levels. *B. plicatilis* also inhibited activities of PSII reaction centers, both acceptor and donor side, thereby damaging the photosynthetic performance of surviving *N. oculata* cells. However, the CA:TSN (1:9) combination could completely eliminate *B. plicatilis*, thereby preventing rotifers from devouring microalgae cells and protecting the photosynthetic performance of the surviving algal cells against rotifers damage. Therefore, the binary combination of CA:TSN (1:9), is considered to be a good candidate of botanical pesticide for controlling rotifer contamination.

Additional key words: biological contamination; JIP-test; light utilization; microalgal cultivation.

Introduction

Microalgae are potentially suitable biomass feedstock for human health food (*e.g.*, proteins, pharmaceuticals), animal feed, and the production of bioenergy, as their efficiency of light utilization is high, and they have fast growth rates and the ability to accumulate a high quantity of lipids (Day *et al.* 2012, Rawat *et al.* 2013, Huang *et al.* 2014a,b). Moreover, the cultivation of microalgae can remove nitrogen and phosphorous compounds from wastewater, and can be used for CO_2 capture from industrial flue gases (Li *et al.* 2008). Sustained, large-scale biomass production, is a prerequisite for making these potential applications of microalgae possible.

Biological contamination, as a major hindrance to mass cultivation, impedes industrial-scale cultivation of microalgae (Xu *et al.* 2019). During large-scale cultivation, microalgae are susceptible to zooplankton contaminations, such as rotifers, ciliates, and cladocerans (Huang *et al.* 2014a, Acosta *et al.* 2015). Rotifer contamination is especially serious threat to the large-scale microalgal biomass production. Rotifers are able to survive in extreme environments and reproduce rapidly. Rotifers can devour microalgal cells and sharply reduce algal biomass to very low levels in just a few days (Huang *et al.* 2014a,b). Consequently, large-scale cultivation of microalgae usually fails due to rotifer contamination.

Several methods have been investigated, with limited success, for rotifer contamination control during microalgal cultivation. Chemical control is considered to be a feasible method for rotifer extermination (Huang *et al.* 2014a). It seems that ammonium bicarbonate is the most effective agent for exterminating rotifers. The ammonium toxicity is related to nonionized ammonia, which can exterminate

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[+]Corresponding author; phone/fax: +86-532-82898709, e-mail: jgliu@qdio.ac.cn

Abbreviations: ABS/RC – the specific energy fluxes for absorption; CA – celangulin; ET_0/RC – the specific energy fluxes for electron transport; MA_{IP} – maximal amplitude of fluorescence in the I–P phase; OJIP transient – chlorophyll *a* fluorescence transient; RC – reaction center; RC/CS_0 – the amount of active PSII RCs per excited cross section; TR_0/RC – the specific energy fluxes for trapping; TSN – toosendanin; V_J – relative variable fluorescence at J-step; W_K – relative variable fluorescence at K-step; δ_{R0} – the efficiency of the reduction of end electron acceptors at the PSI acceptor side; φ_{E0} – the quantum yield of electron transport.

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most of the rotifers (Méndez and Uribe 2012). However, growth inhibition of microalgae caused by ammonia also occurs (Yuan *et al.* 2011). Other reagents, such as hexane and toluene, were also shown to be toxic to rotifers (Ferrando and Andreu-Moliner 1992, Gama Flores *et al.* 1999, Sarma *et al.* 2001, Marcial and Hagiwara 2007). However, they have a broad-spectrum impact that can injure microalgae and other aquatic organisms and potentially threaten human health (Jeyaratnam 1990, Hanazato 2001, Huang *et al.* 2014a). Botanical pesticides are good choice for controlling rotifer contamination because they are effective, safe, and environment-friendly (Champagne *et al.* 1992, Huang *et al.* 2014a). Our previous study showed that celangulin (CA) and toosendanin (TSN) derived from *Celastrus angulatus* and *Melia azedarach*, respectively, decreased rotifer density and fecundity, although had no toxic effects on microalgal cell growth of *Chlorella* culture (Huang *et al.* 2014a). Furthermore, the botanical pesticide of CA:TSN combination, at a 1:9 ratio, showed an excellent potential and was economically feasible for rotifer extermination in microalgal cultures (Huang *et al.* 2014b). However, the efficacy of the CA:TSN (1:9) combination needs further investigation.

Nannochloropsis is widely used in aquaculture as a feed source and has been proposed as an alternative source of fish oil due to its high content of polyunsaturated fatty acids, especially eicosapentaenoic acid (Shi *et al.* 2008). However, large-scale cultivation of *Nannochloropsis* usually fails due to *Brachionus plicatilis* contamination. In the present study, the effect of rotifer (*B. plicatilis*) contamination on photosynthetic characteristics in *Nannochloropsis oculata* and the efficacy of the CA:TSN (1:9) combination for rotifer extermination were probed using chlorophyll *a* fluorescence transient.

Materials and methods

***Nannochloropsis oculata* and rotifers**: The alga *Nannochloropsis oculata* and rotifer *Brachionus plicatilis* were obtained from the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China. The rotifers were continuously cultured in the laboratory at 26 ± 1°C in filtered seawater (salinity of 32‰, pH 8.0). Rotifers were fed daily on *N. oculata*, which were cultured in L1 medium (Guillard and Hargraves 1993) at an average light intensity of 80 μmol(photon) m^{-2} s^{-1} under a 14/10-h light/dark photoperiod using fluorescent lamps.

The botanical pesticides celangulin (CA, *Xi'an Fengzu Biotechnology Co. Ltd.*, China) and toosendanin (TSN, *Xi'an Fengzu Biotechnology Co. Ltd.*, China) were prepared as stock solutions in distilled water at concentrations of 10 mg L^{-1} and 0.5 mg L^{-1}, respectively. The synergistic combination of CA/TSN at the 1:9 ratio (1 mg L^{-1}) was obtained by mixing the stock solutions of CA and TSN and distilled water at a volume ratio of 45:98:357 (Huang *et al.* 2014b).

Test protocol: *N. oculata* at the exponential growth stage was cultured in L1 medium at 26 ± 1°C. Light intensity of 80 μmol(photon) m^{-2} s^{-1} was provided by fluorescent lights with a 14/10-h light/dark photoperiod. Rotifers were added to the algal culture to reach an initial density of five individuals per mL. After 5 h, the botanical pesticide, CA:TSN (1:9) combination (0.006 mg L^{-1}), was added to series of flasks containing the algae or algae plus rotifer cultures. A rotifer-free algal culture was used as the control. Each test group included eight replicates. All the parameters were determined in the laboratory daily at 10:00 h.

Measurement of densities of microalgae and rotifers: Microalgal density was measured by cell counting using a hemocytometer and inverted optical microscope (*37XB*, *Shanghai Optical Instrument*, China). The rotifer density was estimated using a zooplankton counter under a dissection microscope (*JSZ5B*, *Yongxin*, China). The morphology of the rotifer was observed and photographed using a light microscope (*Ci-POL*, *Nikon*, Japan).

Chl *a* fluorescence (OJIP) transients of *N. oculata* cells during the incubation were measured with a *Handy PEA* fluorometer combined with a liquid-phase adapter (*Hansatech*, UK). All measurements were performed with cells dark-adapted for 10 min at room temperature.

JIP-test: OJIP transients were analyzed with the JIP-test (Strasser *et al.* 2000, Appenroth *et al.* 2001, Oukarroum *et al.* 2009, Yusuf *et al.* 2010, Zhang *et al.* 2011). The amount of active PSII reaction centers (RCs) per excited cross section (RC/CS_0), maximal amplitude of fluorescence in the I–P phase (MA_{IP}), relative variable fluorescence at J-step (V_J), and at K-step (W_K) were calculated to reflect the state of photosynthetic electron transport chains. The specific energy fluxes (per RC) for absorption (ABS/RC), trapping (TR_0/RC), and electron transport (ET_0/RC) were calculated to reflect the efficiency of active PSII RCs. The quantum yield of electron transport (φ_{E0}) and the efficiency of the reduction of end electron acceptors at the PSI acceptor side (δ_{R0}) were calculated to reflect the efficiency of photosynthetic electron transport.

Statistical analysis: *SPSS 17.0* was used for statistical analyses. The variation of each parameter was tested by the one-way analysis of variance (*ANOVA*) procedure. Least significant difference (LSD) was used to analyze differences between treatments and control. Differences were considered significant at a probability level of $p<0.05$.

Results

Algal cell density and rotifer density: The cell density of *N. oculata* increased from the initial 24.5 × 10^6 cells mL^{-1} to 61.0 × 10^6 cells mL^{-1} under normal conditions (control group) during a 5-d incubation period (Fig. 1*A*). When exposed to rotifers (*B. plicatilis*), the cell density of *N. oculata* declined sharply after 2 d of incubation (Fig. 1*A*). The algal cell density decreased to 4.8 × 10^6 cells L^{-1} after exposure to rotifers for 5 d. However, the cell density of the *N. oculata* cultures containing both rotifers and the botanical pesticide binary combinations of CA:TSN (1:9) increased to 58.1 × 10^6 cells mL^{-1} within 5 d (Fig. 1*A*), and no significant differences were found compared to *N. oculata* cultures without rotifers (control group, *p*>0.05).

Population density of *B. plicatilis* in *N. oculata* cultures increased sharply after 2 d of incubation (Fig. 1*B*). During a 5-d incubation period, the density of rotifers increased from the initial five individuals per mL to 92 individuals per mL in *N. oculata* cultures. The growth of *B. plicatilis* could be completely inhibited by the CA:TSN (1:9) combination. All the rotifers in the *N. oculata* culture died after exposure to the CA:TSN (1:9) combination for 3 d (Fig. 1*B*). Morphological results (Fig. 1*C–E*) showed that the *B. plicatilis* shrank considerably when exposed to the CA:TSN (1:9) combination for 1 d (Fig. 1*D*), and then were fractured after 2 d (Fig. 1*E*). These results showed that *B. plicatilis* was quite susceptible to the CA:TSN (1:9) combination.

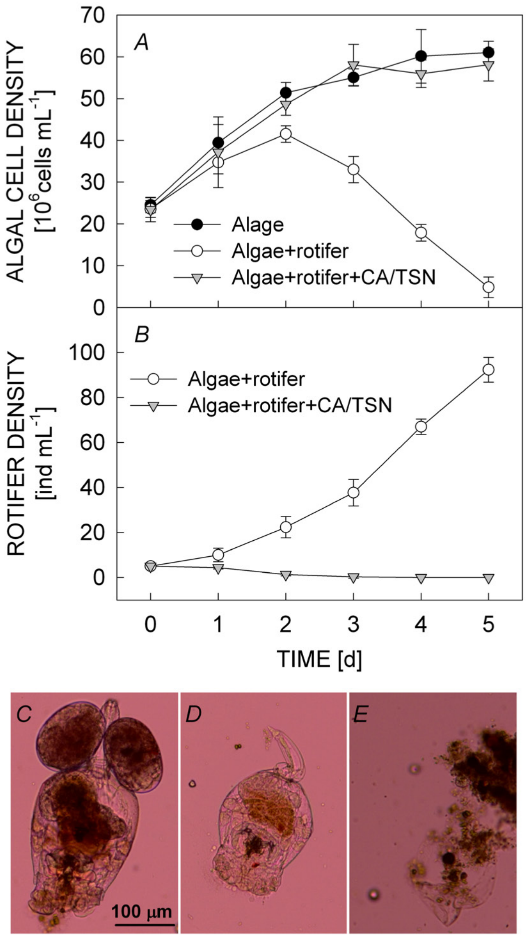

Fig. 1. (*A*) The effect of *Brachionus plicatilis* on the cell density of *Nannochloropsis oculata* in the absence or presence of the binary combinations of CA:TSN (1:9). (*B*) Changes in population density of the *B. plicatilis* in *N. oculata* cultures in the absence or presence of the CA:TSN (1:9) combination. (*C–E*) Morphological appearance of *B. plicatilis*: (*C*) the rotifers in the absence of CA:TSN (1:9) combination; the rotifers after exposure to CA:TSN (1:9) combination for 24 h (*D*) or 48 h (*E*). Means ± SD of five replicates are presented.

Chl *a* fluorescence transient combined with the JIP-test: The *N. oculata* cells showed a typical OJIP Chl fluorescence transient (the O-, J-, I- and P-steps are marked in the plot) in the control group (Fig. 2*A*). The fluorescence intensity increased significantly after 1 d of incubation, and then stayed at a relatively stable level. The fluorescence intensity of *N. oculata* cultures began decreasing after exposure to *B. plicatilis* for 2 d (Fig. 2*B*). However, during the incubation, the variation of Chl *a* fluorescence transient (OJIP transient) in *N. oculata* cultures containing both rotifers and the CA:TSN (1:9) combination did not exhibit any significant differences compared to the control group (Fig. 2*C*).

Photosynthetic parameters derived from the JIP-test analysis of Chl *a* fluorescence transients were analysed in order to clarify the detailed changes of photosynthetic behaviors in *N. oculata* cultures with or without rotifers, and in the absence or presence of botanical pesticides (Figs. 3–5). According to the JIP-test, the amount of active PSII RC per excited cross section (RC/CS_0) in the *N. oculata* control group increased slightly during the

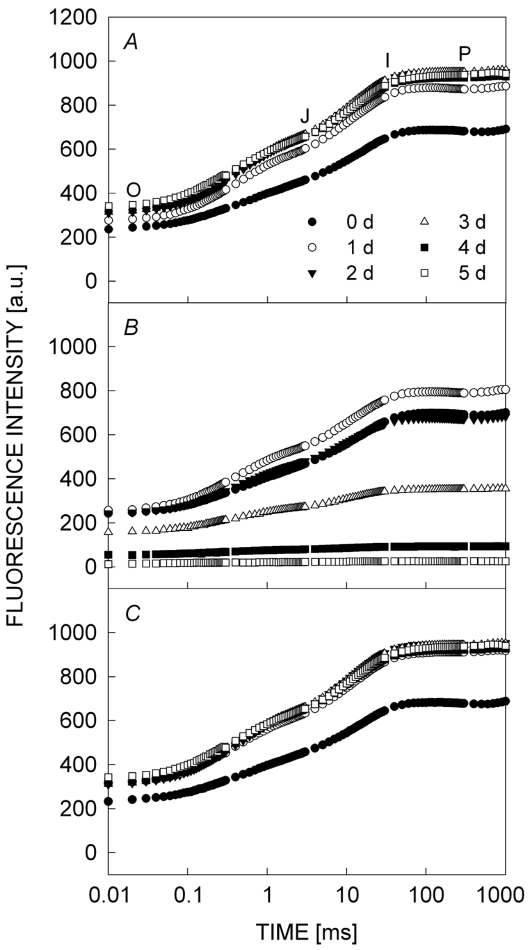

Fig. 2. The effect of *Brachionus plicatilis* on the chlorophyll *a* fluorescence transient (OJIP transient) of *Nannochloropsis oculata* in the absence or presence of the binary combinations of CA:TSN (1:9). (*A*) algae (control); (*B*) algae + rotifer; (*C*) algae + rotifer + CA/TSN. Each transient represents the average of eight replicates.

initial 2 d of incubation, and then did not change (Fig. 3*A*). When exposed to *B. plicatilis*, the RC/CS$_0$ of *N. oculata* cultures decreased sharply after 2 d of incubation. Compared to the control group, the RC/CS$_0$ in the rotifer group decreased by 97.8% after 5 d of incubation. The relative variable fluorescence at J-step (V$_J$) in the *N. oculata* control group increased slightly during the first day of incubation, and then did not change over time (Fig. 3*B*). When exposed to *B. plicatilis*, V$_J$ in *N. oculata* cultures increased after 4 d of incubation. Compared with the control group, V$_J$ in the rotifer group increased by 43.1% after 5 d of incubation. The relative variable fluorescence at the K-step (W$_K$) in the *N. oculata* control group did not change during the incubation (Fig. 3*C*). When exposed to *B. plicatilis*, W$_K$ in *N. oculata* cultures increased after 3 d of incubation. During the incubation, the maximal amplitude of fluorescence in the I–P phase (MA$_{IP}$) first decreased, and then increased in the *N. oculata* control group (Fig. 3*D*). When exposed to *B. plicatilis*, the MA$_{IP}$ in *N. oculata* cultures was not affected during the whole experimental period compared with the control group. Meanwhile, during the incubation, the RC/CS$_0$, V$_J$, W$_K$, and MA$_{IP}$ in *N. oculata* cultures containing both rotifers and the CA:TSN (1:9) combination were not significantly different compared to the control group (Fig. 3).

The light absorption flux per active reaction center (ABS/RC) and the trapping flux of excitation energy per active reaction center (TR$_0$/RC) in the *N. oculata* control group did not change during the entire experimental period (Fig. 4*A*,*B*). When exposed to *B. plicatilis*, ABS/RC and TR$_0$/RC in *N. oculata* cultures increased after 3 d of incubation. Compared with the control group, ABS/RC and TR$_0$/RC in the rotifer group increased by 117.2 and 44.0%, respectively, after 5 d of incubation. The electron transport flux per active reaction center (ET$_0$/RC) in the *N. oculata* control group decreased slightly during the first day of incubation, and then did not change over time (Fig. 4*C*). Regarding exposure to *B. plicatilis*, the ET$_0$/RC in *N. oculata* cultures decreased after 4 d of incubation. Compared with the control group, the ET$_0$/RC in the rotifer group decreased by 19.4% after 5 d of incubation. During the incubation, the ABS/RC, TR$_0$/RC, and ET$_0$/RC in *N. oculata* cultures containing both rotifers and the CA:TSN (1:9) combination were not significantly different compared to the control group (Fig. 4).

The quantum yield for electron transport (φ_{E0}) in the *N. oculata* control group decreased slightly during the first day of incubation, and then did not change over time (Fig. 5*A*). When exposed to *B. plicatilis*, the φ_{E0} in *N. oculata* cultures decreased after 3 d of incubation. The efficiency of the reduction of end electron acceptors at the PSI acceptor side (δ_{R0}) decreased first, and then increased in the *N. oculata* control group (Fig. 5*B*). When exposed to *B. plicatilis*, the δ_{R0} in *N. oculata* cultures was not affected during the entire experimental period compared to control group. During the incubation, the φ_{E0} and δ_{R0} in *N. oculata* cultures containing both rotifers and the CA:TSN (1:9) combination were not significantly different compared to the control group (Fig. 5).

Discussion

Large-scale cultivation of microalgae is susceptible to rotifer consumption (Huang *et al.* 2014a, Acosta *et al.* 2015). The cell density of *N. oculata* decreased sharply in the rotifer group (Fig. 1*A*) when the population density

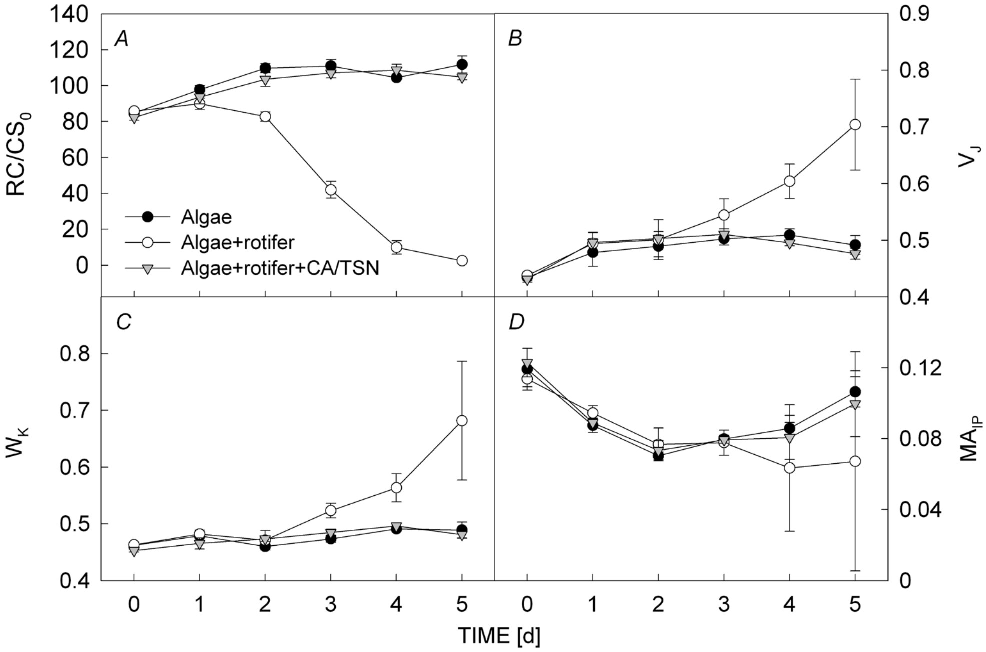

Fig. 3. The effect of *Brachionus plicatilis* on the amount of active PSII reaction centers per excited cross section, RC/CS$_0$ (*A*), relative variable fluorescence at J-step, V$_J$ (*B*) and at K-step, W$_K$ (*C*), maximal amplitude of fluorescence in the I–P phase, MA$_{IP}$ (*D*) in *Nannochloropsis oculata*, in the absence or presence of the binary combinations of CA:TSN (1:9). Means ± SD of eight replicates are presented.

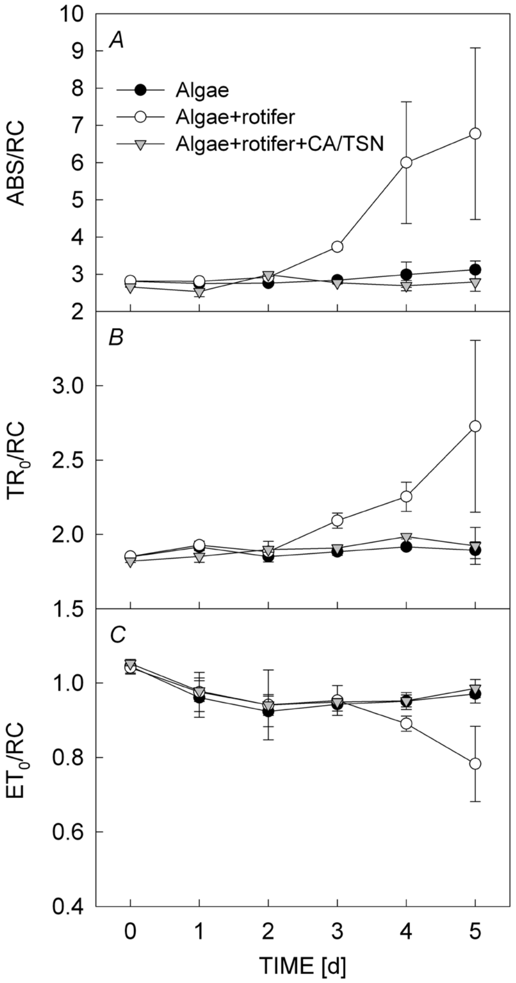

Fig. 4. The effect of *Brachionus plicatilis* on the specific energy fluxes for absorption, trapping, and electron transport per active reaction center (RC) in *Nannochloropsis oculata* in the absence or presence of the binary combinations of CA:TSN (1:9). The light absorption flux per active reaction center, ABS/RC (*A*), the trapping flux of excitation energy per active reaction center, TR$_0$/RC (*B*), and the electron transport flux per active reaction center, ET$_0$/RC (*C*). Means ± SD of eight replicates are presented.

of *B. plicatilis* increased sharply after 2 d of incubation (Fig. 1*B*). The finding suggests that *B. plicatilis* can devour and damage *N. oculata* cells, and sharply reduce a microalgal biomass to very low levels in just a few days.

The growth of microalgae mainly depends on the daytime photosynthetic production and biomass loss (Torzillo *et al.* 1991). Photosynthesis is one of the most stress-sensitive physiological processes in plants (Berry and Björkman 1980). In order to further analyze the effects of *B. plicatilis* contamination on the growth of *N. oculata*, *in vivo* OJIP transient was used as an indicator of photosynthetic performance (Strasser *et al.* 2000, 2004; Yusuf *et al.* 2010). Compared to the *N. oculata* control group (Fig. 2*A*), the OJIP fluorescence intensity in the rotifer group decreased gradually after 2 d of incubation (Fig. 2*B*), which suggests that *B. plicatilis* contamination could decrease the photosynthetic performance of the surviving algal cells.

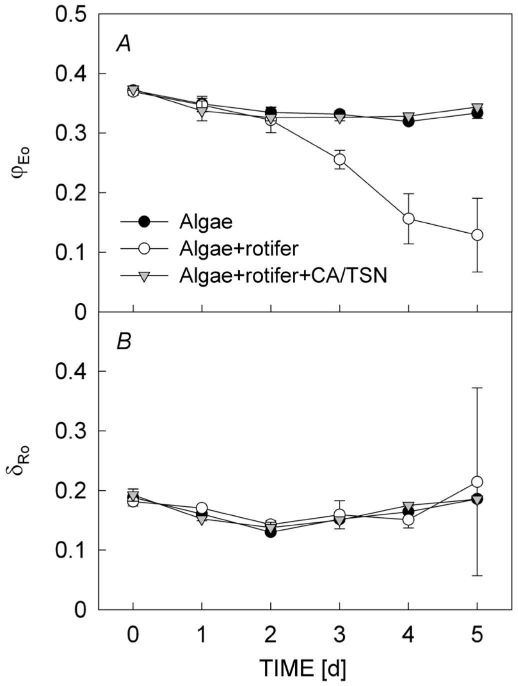

Fig. 5. The effect of *Brachionus plicatilis* on the quantum yield for electron transport, φ_{E0} (*A*) and the efficiency for reduction of end electron acceptors at the PSI acceptor side, δ_{R0} (*B*) in *Nannochloropsis oculata*, in the absence or presence of the binary combinations of CA:TSN (1:9). Means ± SD of eight replicates are presented.

Specifically, the RC/CS$_0$ of the *N. oculata* cultures decreased sharply after 2 d of incubation when exposed to *B. plicatilis* compared to the control group (Fig. 3*A*). The finding suggests that rotifer contamination led to the inactivation of PSII reaction centers, and decreased the number of active PSII reaction centers. The RC/CS$_0$ represents the quantity of active PSII reaction centers in the measured excited cross section (Strasser *et al.* 2004) of liquid-phase samples (microalgae). Thus, the RC/CS$_0$ values of *N. oculata* cultures (Fig. 3*A*) were strongly influenced by the change in the algal cell density (Fig. 1*A*). According to the JIP-test, the increase in V_J represents the momentary maximum accumulations of Q_A^-, resulting in the overreduction of PSII acceptor side (Zhang *et al.* 2011, Zhang and Liu 2016). The enhanced K-step indicates increased damage to the PSII donor side (Yusuf *et al.* 2010, Zhang *et al.* 2017). The MA_{IP} reflects the pool size of end electron acceptors (Yusuf *et al.* 2010, Zhang and Liu 2016, Zhang *et al.* 2017). In the present study, *B. plicatilis* contamination in *N. oculata* cultures led to overreduction of the PSII acceptor side after 4 d of incubation (Fig. 3*B*), damaged the donor side of PSII after 3 d of incubation (Fig. 3*C*), but did not affect the pool size of end electron acceptors during the entire experimental period (Fig. 3*D*). All the results suggest that *B. plicatilis* contamination first decreased the activity of PSII reaction centers, and then damaged the donor side of PSII, finally causing an overreduction of PSII acceptor side in *N. oculata* during the incubation.

Compared with controls, the ABS/RC and TR$_0$/RC increased (Fig. 4*A*,*B*), but the ET_0/RC decreased (Fig. 4*C*) when cultures were exposed to *B. plicatilis*, which indicates that the balance between photosynthetic light absorption and energy utilization was disturbed (Zhang *et al.* 2011, 2017) by the inhibition of activities of reaction centers and PSII donor side after 3 d of incubation (Fig. 3*A*,*C*) due to rotifer contamination in *N. oculata* cultures. The fact that the quantum yield for electron transport (φ_{E0}) in *N. oculata* cultures decreased when exposed to *B. plicatilis* for 3 d (Fig. 5*A*) also supports the above suggestion. The imbalance between photosynthetic light absorption and energy utilization inevitably leads to the overexcitation of the PSII reaction centers (Vass 2011, Zhang *et al.* 2011). Under photoinhibitory conditions, overexcitation of PSII reaction centers due to rotifer contamination would inevitably enhance the generation of reactive oxygen species (Zhang *et al.* 2011) that further damaged the algal cells.

Rotifers have been shown to release undefined chemical secretions into water during their lifetime, which affects the growth of microalgae (Yang *et al.* 2005, Huang *et al.* 2014a). Therefore, regarding the algae + rotifer group in the present study, we concluded that *B. plicatilis* can inhibit the growth of *N. oculata* in the following ways: (*1*) by directly devouring algal cells; (*2*) by releasing undefined chemical secretions that damage the photosynthetic performance of surviving *N. oculata* cells. Once a culture was contaminated, *B. plicatilis* rapidly reduced the algal concentration and cleared the entire culture within just a few days.

The binary combinations of CA:TSN (1:9) have been considered to be a good potential botanical pesticide for controlling rotifers in microalgal cultures (Huang *et al.* 2014b). All the *B. plicatilis* in culture died when exposed to the CA:TSN (1:9) combination (Fig. 1*B*), suggesting that the CA:TSN (1:9) combination can eliminate *B. plicatilis* in *N. oculata* cultures. Meanwhile, the CA:TSN (1:9) combination did not affect the growth of *N. oculata* (Fig. 1*A*).

The RC/CS_0, V_J, and W_K in *N. oculata* cultures exposed to both rotifers and the CA:TSN (1:9) combination were not significantly different compared to the control group (Fig. 3), indicating that the CA:TSN (1:9) combination can protect the PSII reaction centers, acceptor side, and donor side of surviving algal cells against rotifer damage. Similarly, in *N. oculata* cultures, the CA:TSN (1:9) combination prevented the establishment of an imbalance between photosynthetic light absorption and energy utilization, thereby protecting PSII reaction centers of surviving algal cells from overexcitation (Figs. 4, 5) during the entire experimental period.

It has been suggested that toosendanin could directly inhibit the digestive enzymes of rotifer (Huang *et al.* 2017). We concluded that CA:TSN (1:9) combination also restricted digestive enzymes and thus decreased the population density of rotifers (Fig. 1). Therefore, the CA:TSN (1:9) combination can prevent *B. plicatilis* from devouring *N. oculata* cells and can protect the photosynthetic performance of the surviving *N. oculata* cells against *B. plicatilis* contamination.

Conclusion: Rotifer *B. plicatilis* could directly devour *N. oculata* cells and sharply reduce an algal density to very low levels. *B. plicatilis* also inhibited the activities of PSII reaction centers, acceptor side and donor side in surviving *N. oculata* cells, and led to the imbalance between photosynthetic light absorption and energy utilization. However, the CA:TSN (1:9) combination could eliminate *B. plicatilis*, thereby preventing *B. plicatilis* from devouring *N. oculata* cells and protecting photosynthetic performance of the surviving *N. oculata* cells against rotifers damage. Meanwhile, the CA:TSN (1:9) combination did not affect the growth of *N. oculata*. Therefore, the botanical pesticide, the binary combination of CA:TSN (1:9), is a good candidate of botanical pesticide for controlling rotifer contamination.

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