

# Responses of photosystems I and II of *Acutodesmus obliquus* to chemical stress caused by the use of recycled nutrients

Dominik J. Patzelt<sup>1,2</sup> · Stefan Hindersin<sup>2</sup> · Martin Kerner<sup>2</sup> · Dieter Hanelt<sup>1</sup>

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**Abstract** Nutrients derived from hydrothermal gasification of *Acutodesmus obliquus* were tested on its biological compatibility to support growth of the same microalgae. Photosynthetic parameters of photosystems I and II (PS I and PS II) were investigated to study physiological effects on the microalgal cell. The nutrients were collected as liquid residues. Dilutions of 1:500 showed no effect on both photosystems. Lower dilutions affected PS II initially and later also PS I. Cyclic electron flow around PS I compensated for loss of electrons due to partially inhibited PS II. The highest tested concentration of liquid residue erased any photosynthetic activity of PS II after 28 min and onwards. In contrast, PS I remained active. The results suggest that PS I is less susceptible than PS II and that the mixture of chemicals in the liquid residue did not directly affect PS I but PS II. The toxicants in the residues seemed to interfere with linear electron flow of PS II even though light-driven formation of radicals and subsequent damage to one of the photosystems can be excluded as demonstrated in darkness. Lowered photosynthetic activity of PS I during actinic irradiation was caused due to lack of supply of electrons from PS II. The cyclic electron flow might play a key role in delivering the energy needed to restore PS II activity and to biodegrade the toxicants when linear electron flow failed. These negative effects of liquid residue towards microalgal cells require a remediation step for direct

application of the liquid residue to substitute commercial fertilizers in microalgal mass cultures.

**Keywords** *Acutodesmus obliquus* · Chemical stress · Cyclic electron flow · Photoinhibition · Photosystem I · Photosystem II

## Introduction

The use of mass cultivation of microalgae for production of sustainable goods such as biofuel, food, feed, or pharmaceuticals requires the extensive use of fertilizers. To diminish production costs and the use of fertilizers in a life cycle management, the economic principle of “cradle to cradle” should be considered. One possibility is the recycling of nutrients from microalgae mass cultures after energetic use. In this study, we converted *Acutodesmus obliquus* into energetically rich gaseous molecules by hydrothermal gasification and assess the possibility to reuse the nutrients of the liquid residues. The residues were collected in two different fractions, an aqueous phase (AP) and salt brine. Processing microalgae under hydrothermal conversion conditions causes “Maillard” reactions and results in diverse reaction intermediates which remain in the liquid residue. Patzelt et al. (2014) discovered 28 substances, Garcia Alba et al. (2013) 15 classes of organic compounds, and Brown et al. (2010) nearly 90 different compounds after hydrothermal conversion of microalgal biomass. In our case, herbicides occurred in the liquid residues and affected photosystem II (PS II) and growth of *A. obliquus* and *Chlorella vulgaris* measured on a daily basis as demonstrated in a previous study (Patzelt et al. 2014). Microalgae suffered from chemical stress due to AP. Concomitant photoinhibition arose even at low actinic

✉ Dominik J. Patzelt  
dominik.patzelt@uni-hamburg.de

<sup>1</sup> Aquatic Ecophysiology and Phycology, University of Hamburg, Ohnhorststraße 18, 22609 Hamburg, Germany

<sup>2</sup> Strategic Science Consult SSC Ltd., Beim Alten Gaswerk 5, 22761 Hamburg, Germany

light of  $25 \mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$ . Herbicides are known to have approximately 20 modes of action (Duke 2012). Some herbicides such as diuron directly affect PS II (Astier et al. 1984), but others may influence photosynthesis by different feedback reactions when the cell enters unfavorable conditions. The negative effects of AP caused a complete inhibition of PS II and degenerated cell morphology. Eventually, *A. obliquus* was able to restore its photosynthetic activity. For primary production, both photosystems have to work at least within a same rate to establish a continuous linear electron transport. With an additional cyclic electron transport around photosystem I (PS I) the activity of PS I needs to be even higher than that of PS II. If electron flow at PS I is inhibited by AP, negative feedback on PS II due to blocked electron transfer at PS I would occur (Sonoike 2011). Active oxygen produced at the reduction side of PS I is suggested to damage PS II (Tjus et al. 2001). Photoinhibition of PS I is already known and occurred under certain conditions as described for higher plants. PS I is susceptible to chilling temperatures at weak irradiation (Sonoike 1996a). However, it is also discussed whether inhibition of PS II activity may protect PS I from photoinhibition (Sonoike 2011). As diuron blocks the electron flow from PS II, it suppresses photoinhibition of PS I (Sonoike 1996b). In this manner, a strong inhibiting effect of AP on PS II might also cause protection of PS I and thus the effect of stress can yield different photosystem activity patterns. Havaux (1996) observed preferential inactivation of PS II when potato leaves were exposed to heat stress. The same trend was demonstrated in unicellular algae under the effect of nitrogen starvation (Berges et al. 1996) and heavy metal pollution (cadmium) in *Chlorella pyrenoidosa* (Wang et al. 2013). Several other stresses also disturb quantum yield of PS II including hyperosmotic stress in *Chlamydomonas reinhardtii* (Endo et al. 1995), ionic stress in *Dunaliella tertiolecta* (Gilmour et al. 1985) as well as high light conditions that lead to photoinhibition of *C. reinhardtii* (Lidholm et al. 1987), and chronic photoinhibition in *Dunaliella salina* (Neidhardt et al. 1998). In all these cases, PS I-driven cyclic electron flow might play a key role to survive under stress conditions, e.g., as shown for desiccation tolerance (Gao et al. 2011; Gao and Wang 2012) and is potentially the energy supplier for the microalgae cell without PS II activity to biodegrade toxicants (Papazi and Kotzabasis 2013) and eventually restore PS II activity. Yet it is unclear how a mixture of chemicals would affect both photosystems of microalgae. With this study, we enhance the temporal resolution and information of the inhibiting effects of the liquid residues on both photosystems. The photosynthetic activity of PS I and PS II is assessed in vivo as well as several other photosynthetic parameters.

## Material and methods

### Generation of microalgal biomass and hydrothermal gasification

The green microalgae *A. obliquus* was isolated from open waters in Hamburg (strain No. 10169, Microalgae and *Zygnematophyceae* Collection Hamburg) and cultivated at the façade of a residential building (BIQ- Das Algenhaus) in Hamburg-Wilhelmsburg (Germany) in flat panel bioreactors in natural sunlight. For further cultivation details, refer to Patzelt et al. (2014). The hydrothermal conversion device is described by Boukis et al. (2005). For the conversion of algal biomass, the gasification unit was operated with a temperature of  $600 \text{ }^\circ\text{C}$  within the reactor and the pressure was set to 280 bar. The algal biomass was fed to the reactor ( $1.2 \text{ g min}^{-1}$ ), along with an additional stream of pure water ( $3.6 \text{ g min}^{-1}$ ). This achieved a final biomass concentration of 2.5 % cell dry weight (CDW) inside the reactor. An average gas production (mainly  $\text{H}_2$ ,  $\text{CH}_4$ ,  $\text{CO}_2$ , and  $\text{C}_2\text{H}_6$ ) of  $10 \text{ L h}^{-1}$  was measured with continuous process operation of 50 h. The liquid residues were collected in an aqueous phase (AP) and salt brine. The main nutrient in the aqueous phase was  $\text{N-NH}_4^+$  at concentration between 2230 and 2630  $\text{mg L}^{-1}$ . Effects of the untreated AP as nutrient source for further cultivation of microalgae was tested using a Dual-PAM-100 instrument (Walz, Effeltrich, Germany) to study changes in activity of photosystem I and II (PS I, PS II). Microalgae under study were cultivated at laboratory scale of 600 mL in an Erlenmeyer flask containing 2  $\text{g L}^{-1}$  Flory Basic Fertilizer 1 (Euflor, Germany) and 3.22  $\text{g L}^{-1}$   $\text{KNO}_3$  (Carl Roth, Karlsruhe, Germany). Cultivation parameters included  $20 \text{ }^\circ\text{C}$  and a light/dark cycle of 16:8 at  $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and a constant bubbling with  $\text{CO}_2$ -enriched air (4 %v/v). During linear growth phase, samples were withdrawn and the optical density (OD) at 750 nm was determined. CDW was calculated via a CDW-OD<sub>750</sub> correlation curve.

### Assessment of photosynthetic parameters

For measurements of the photosynthetic activity, cell density of the samples was adjusted to  $1 \text{ g L}^{-1}$  by dilution with centrifuged supernatant of the preculture or by centrifugation for 5 min at 4000 rpm and discharge of the excessive medium. Afterwards, 2 mL of the samples was transferred into a glass cuvette and a small amount of  $\text{NaHCO}_3$  (Merck, Darmstadt, Germany) was added to supply microalgae with sufficient carbon during the experimental trials. The cuvette was then placed into the Dual-PAM-100 device. Samples within the glass cuvette were stirred with a magnetic stirrer during the experiment to avoid settling of microalgae. Ten seconds prior to each measurement of P700-dependent absorption change and chlorophyll fluorescence detection, the magnetic stirrer

was switched off to minimize the signal noise of the P700 signal due to a fast oscillation of algal cells within cuvette.

Prior to the measurements with the Dual-PAM, a 5-min dark adaption period was applied. Subsequently, minimal fluorescence yield ( $F_0$ ) and maximal fluorescence yield ( $F_m$ ) was determined using the saturation pulse method. A saturation pulse (SP) of 10,000  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  for 600 ms was applied to close all reaction centers of PS II. The ratio of variable to maximal fluorescence gives the quantum yield of photochemical energy conversion  $F_v/F_m=(F_m-F_0)/F_m$  and enables the estimation of the maximal photosynthetic efficiency of PS II in vivo (Hanelt and Nultsch 1995) of dark adapted samples (Kitajima and Butler 1975). Effective quantum yield of irradiated samples is described as  $Y(\text{II})=(F_m'-F_0)/F_m'$  (Genty et al. 1989) and is determined by the fluorescence yield which depends on the redox state of the reaction center in light. The fluorescence measurement of PS II enables to detect not only the quantum yield of photochemical energy conversion ( $Y(\text{II})$ ) but also allows to detect the fate of absorbed excitation energy that does not contribute to photochemistry. The heat dissipation which competes with  $Y(\text{II})$  is divided in two different pathways: The quantum yield of regulated light-induced non-photochemical ( $Y(\text{NPQ})$ ) and quantum yield of non-regulated non-photochemical ( $Y(\text{NO})$ ) fluorescence quenching. The distribution of absorbed excitation energy in PS II can be described as  $Y(\text{II})+Y(\text{NO})+Y(\text{NPQ})=1$  (Klughammer and Schreiber 2008a).

The PS I fluorescence yield is independent of the redox state of the PS I reaction center; hence, the transmission or remission difference signal 875–830 nm is assessed. For PS I activity measurements, the maximal P700 signal ( $P_m$ ), i.e., the P700-dependent absorption changes, was determined after far-red preillumination and following SP. Comparable to the fluorescence yield, signal changes occur between the zero P700 signal  $P_0$  for fully reduced P700 as well as  $P_m$  for fully oxidized P700 thus allowing to assess the redox state of PS I with P700 signal changes of  $P$ . The zero P700 signal is recorded before SP and  $P_m'$  after onset of a SP under experimental

conditions. Three different complementary quantum yields can be derived from the P700 signals and can be defined as  $Y(\text{I})$  the quantum yield of photochemical energy conversion,  $Y(\text{ND})$  the quantum yield of non-photochemical energy dissipation due to donor side limitation, and  $Y(\text{NA})$  the quantum yield of non-photochemical energy dissipation due to acceptor side limitation where  $Y(\text{I})+Y(\text{ND})+Y(\text{NA})=1$  (Klughammer and Schreiber 1994, 2008b).

To elucidate the effect of AP on the physiological status of PS I and PS II, two different activity patterns of the photosynthetic apparatus were studied. Firstly, microalgae were continuously irradiated with 277  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  causing an active electron transport. In preliminary trials without AP, higher light intensities were tested and caused photoinhibition during the experiment and thus were not considered to be appropriate for studying the effect of AP to the active photosystems. Secondly, the yields were measured with microalgae exposed to darkness to observe the effects of the AP on the photosystems under dark-acclimated conditions. It is hypothesized that toxicants within the AP unfold their destructive nature to the photosynthetic apparatus only in light due to the light-dependent formation of radicals (Fuerst and Norman 1991). Since the SP method does not induce photochemical quenching, microalgae are presumably not harmed in the dark. All samples were measured in triplicates and the mean of the three measurements as well as the standard deviation was calculated. To perform the triplicate measurements, the cuvette was washed prior to the next measurement and fresh algae material was transferred into the cuvette as well as new AP at different concentrations was added.

The photosynthetic parameters of PS I and PS II of *A. obliquus* were assessed either at a constant irradiation of 277  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  or in darkness over a time period of 2 h. For the first 12 min, yields of PS I and PS II were determined every 2 min to observe adaption of the undisturbed microalgae to the new environmental conditions (light/darkness). Then, 500  $\mu\text{l}$  of one of the solutions as displayed in Table 1 was added. To assess short-term effects of the added

**Table 1** Added medium composition to study effects of different AP concentrations on *A. obliquus*

| AP dilution factor | Light                      |                                     | Dark                       |                                     |
|--------------------|----------------------------|-------------------------------------|----------------------------|-------------------------------------|
|                    | Added AP ( $\mu\text{l}$ ) | Added supernatant ( $\mu\text{l}$ ) | Added AP ( $\mu\text{l}$ ) | Added supernatant ( $\mu\text{l}$ ) |
| 1:X                |                            |                                     |                            |                                     |
| Control            | 0                          | 500                                 | 0                          | 500                                 |
| 5                  | 500                        | 0                                   | 500                        | 0                                   |
| 10                 | 250                        | 250                                 | n.m.                       | n.m.                                |
| 50                 | 50                         | 450                                 | n.m.                       | n.m.                                |
| 500                | 5                          | 495                                 | n.m.                       | n.m.                                |

Two activity patterns of the photosystems were studied. (Light) four different total AP dilution factors (5, 10, 50, and 500) were adjusted by adding 500  $\mu\text{l}$  of the mixture described above to 2 mL algal solution in comparison to a control group at constant irradiation of 277  $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ . AP dilution factor 5 was compared to a control group in darkness (dark). Supernatant=culture medium

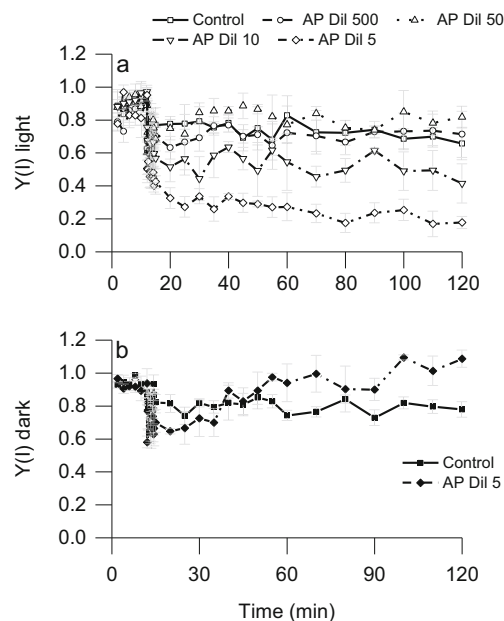
n. m. not measured, AP aqueous phase from hydrothermal conversion

media combination, the activity was measured subsequently every 15 s for 3 min. Long-term effects were assessed by obtaining PS I and PS II yields every 5 min for the next 45 min and later every 10 min for 60 min. The maximum of the respective yield of photosystem I and II ( $Y(I)$ ,  $Y(II)$ ) of each replicate within the first 12 min of adaption was standardized to a value of 1 and afterwards mean values were calculated. Standardization was necessary for a better demonstration of AP effects on *A. obliquus* under the assumption that within the first 12 min without addition of AP the cultures are undisturbed (values of 1 correspond to a 100 % yield of  $Y(I)$  and  $Y(II)$ ). Thus, yields of photosystem I and II of the regular functioning photosynthetic apparatus are at an optimal level. Original values of measurements of the redox specific limitations of photosystem I ( $Y(I)$ ,  $Y(ND)$  and  $Y(NA)$ ), and II ( $Y(II)$ ,  $Y(NO)$ , and  $Y(NPQ)$ ) were used to determine quenching parameters. To assess which photosystem is more active, a subtraction of  $Y(II) - Y(I)$  was done, and values higher than zero demonstrating PS II to be more active and values lower than zero presenting PS I as the more active photosystem.

## Results

### Photosynthetic activity of photosystem I

The liquid phase after the hydrothermal conversion of algal biomass contains not only nutrients but also organic substances which can inhibit algal photosynthesis. As was shown in Fig. 1, AP inhibited PS I determined by  $Y(I)$  of *A. obliquus* at constant irradiation of  $277 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  and the negative effect increased with increasing AP concentrations. To allow a better comparison of the results of different experiments, the maximal yields of each replicate during the first 12 min were standardized to a value of 1. After addition of 500  $\mu\text{L}$  of culture medium or increasing AP concentrations,  $Y(I)$  dropped immediately. The control, AP dilution 500 and 50, followed the same trend over time displaying a stable yield. Only at lower AP dilutions of 10 and 5 a decrease of  $Y(I)$  compared to control was visible. A fivefold AP dilution caused the strongest decrease of the photosynthetic activity of PS I. After 80 min, the  $Y(I)$  declined to its lowest value of 0.18 which was still the same after 120 min. However, a certain quantum yield of the photochemical energy conversion of PS I in *A. obliquus* during irradiation was detectable in all treatments. The effect of fivefold dilution of AP on the  $Y(I)$  of *A. obliquus* in darkness is demonstrated in Fig. 1b. Immediately after addition of AP or supernatant (control) to the 2 mL microalgae suspension, the  $Y(I)$  dropped as could be observed in light as well. However,  $Y(I)$  of the control stabilized over time and reached a value of 0.78 after 120 min. In contrast to the control and also in contrast to curve progression

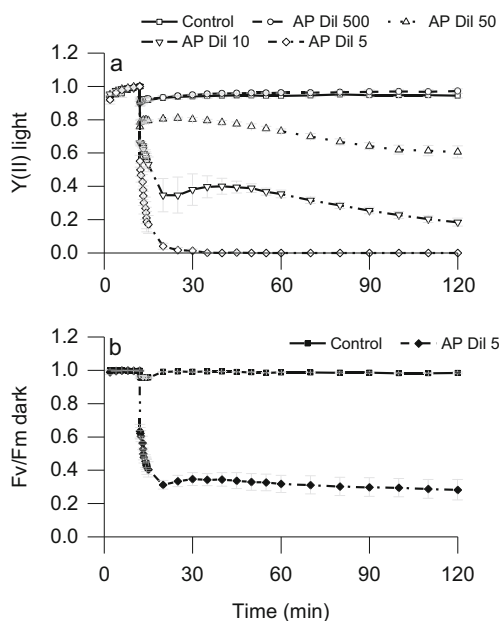


**Fig. 1** Effects of different concentrations of AP on photosynthetic activity of photosystem I ( $Y(I)$ ) of *A. obliquus* at **a** constant actinic irradiation of  $277 \mu\text{mol m}^{-2}\cdot\text{s}^{-1}$  or **b** in darkness compared to the control. Total AP dilution factors 5 (*AP Dil 5*), 10 (*AP Dil 10*), 50 (*AP Dil 50*), and 500 (*AP Dil 500*) were applied (2 mL algal solution + 500  $\mu\text{L}$  of different AP concentrations). The highest original values of  $Y(I)$  during the 12 min of adaption in light: control 0.448, *AP Dil 5* 0.450, *AP Dil 10* 0.412, *AP Dil 50* 0.417, *AP Dil 500* 0.434; and in darkness: control 0.664, *AP Dil 4* 0.607

of  $Y(I)$  during constant irradiation,  $Y(I)$  of the fivefold dilution recovered continuously and reached a final value of about 1.08, which is higher than at the start of the experiment. There is a clear difference between the results when AP was applied in light or during darkness.

### Photosynthetic activity of photosystem II

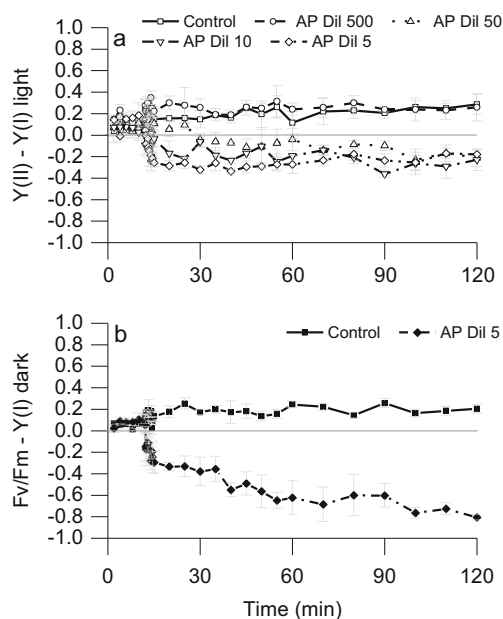
The effect of AP on  $Y(II)$  differs from that on  $Y(I)$  under constant irradiation (Fig. 2a). The addition of 500  $\mu\text{L}$  of each of the different treatments caused a decline in  $Y(II)$ . The decline was lowest in the control and the 500-fold dilution. Dilution treatments with higher amounts of AP (dilution factors 50, 10, and 5) caused a stronger decrease in  $Y(II)$ . The extent of the decrease is clearly correlated to the AP concentration. By adding 500  $\mu\text{L}$  of undiluted AP to the microalgae,  $Y(II)$  declined strongly until minute 40 and displayed no photosynthetic activity in light onwards. After the addition of 500  $\mu\text{L}$  supernatant in darkness (Fig. 2b), the values decreased marginally. From minute 20 onwards,  $F_v/F_m$  values remained as high as  $>0.98$ . In contrast, after addition of 500  $\mu\text{L}$  AP, the  $F_v/F_m$  values decreased strongly until minute 20 to a value of 0.31. From this point onwards,  $F_v/F_m$  remained relatively constant.



**Fig. 2** Effects of different concentrations of AP on photosynthetic activity of photosystem II ( $Y(II)$ ) of *A. obliquus* at **a** constant actinic irradiation of  $277 \mu\text{mol m}^{-2}\text{s}^{-1}$  or **b** in darkness ( $F_v/F_m$ ) compared to the control. Total AP dilution factors 5 (*AP Dil 5*), 10 (*AP Dil 10*), 50 (*AP Dil 50*), and 500 (*AP Dil 500*) were applied (2 mL algal solution + 500  $\mu\text{L}$  of different AP concentrations). The highest original values of  $Y(II)$  during the 12 min of adaption in light: control 0.484, *AP Dil 5* 0.487, *AP Dil 10* 0.442, *AP Dil 50* 0.431, *AP Dil 500* 0.466; and  $F_v/F_m$  in darkness: control 0.661, *AP Dil 4* 0.662

### The relative more active photosystem under chemical stress

It is assumed that  $Y(I)$  and  $Y(II)$  reach their maximal relative value in a fully operative photosynthetic apparatus during the 12-min period of adaption. The subtraction of the standardized yields ( $Y(II) - Y(I)$ ) shows which of both photosystems was more active under the respective stress conditions (Fig. 3). It seems that during the first 12 min of the adaptation period, the yields of PS II were in general slightly higher than of PS I, especially under the actinic light condition (Fig. 3a). The addition of the different amounts of AP caused in dependence on its concentration a different activity level of the photosystems. In the control and the lowest AP concentration (500-fold dilution), the yields of photosystem II were always higher compared to photosystem I. The differences show that at the end of the experiments, the  $Y(II)$  of the control was 29 % higher than  $Y(I)$  and microalgae incubated with the 500-fold diluted AP had a 26 % higher activity of PS II. In contrast, the dilution factors 50, 10, and 5 displayed an opposite pattern. PS I was clearly less affected by the AP application so that the differences became negative directly after addition of the dilution factor 10 and 5 or after 30 min at dilution 50. At the end,  $Y(I)$  of the 50-, 10-, and fivefold AP dilution was 21, 23, and 18 % higher than  $Y(II)$ , respectively. In darkness, the same pattern occurred as in the light (Fig. 3b). The optimal quantum yield



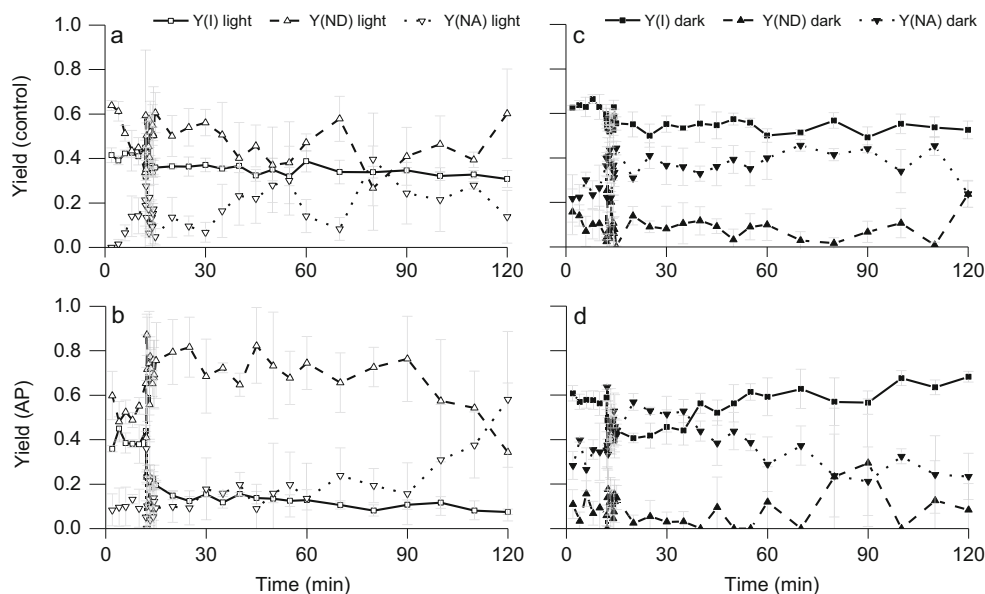
**Fig. 3** Effects of different concentrations of AP on the performance of both photosystems of *A. obliquus* at **a** constant actinic irradiation of  $277 \mu\text{mol m}^{-2}\text{s}^{-1}$  or **b** in darkness compared to the control. Total AP dilution factors 5 (*AP Dil 5*), 10 (*AP Dil 10*), 50 (*AP Dil 50*), and 500 (*AP Dil 500*) were applied (2 mL algal solution + 500  $\mu\text{L}$  of different AP concentrations). Positive values mean a higher activity of PS II whereas negative values mean a higher activity of PS I

of PS II in the control treatment was higher than of PS I, and it further increased to about 20 % after the addition of 500  $\mu\text{L}$  supernatant. The addition of 500  $\mu\text{L}$  AP immediately inhibited PS II in a much higher extent than PS I, so that the difference became negative. In darkness, this shift was stronger than under actinic irradiation (Fig. 3a). The inhibition increases gradually so that PS I was about 80 % more active than PS II at the end of the experiment.

### Acceptor and donor-side limitations of photosystem I

The measurements of change of absorbance of PS I offer the possibility to elucidate if the electron transport at the donor side ( $Y(ND)$ ) or at the acceptor side ( $Y(NA)$ ) of the reaction center becomes inhibited (Fig. 4). The control group under constant actinic light conditions (Fig. 4a) displayed a stronger donor than acceptor side limitation during the time course of experiment.  $Y(ND)$  was almost always higher at all measuring points than  $Y(NA)$ . Microalgae treated with 500  $\mu\text{L}$  AP (Fig. 4b) displayed a strong increase of donor-side limitation.  $Y(NA)$  showed an opposite trend and remained relatively constant at low values until minute 100 but increased afterwards. During darkness, the control values of  $Y(NA)$  and  $Y(ND)$  displayed contrary results (Fig. 4c) compared to actinic irradiation. The microalgae were rather subjected to acceptor side limitations. The addition of 500  $\mu\text{L}$  of AP (Fig. 4d) generally displayed the same trend as the control group, that is,

**Fig. 4** Effects of different concentrations of AP on photosynthetic activity of photosystem I (Y(I)), acceptor-side limitation (Y(NA)), and donor-side limitation (Y(ND)) of *A. obliquus* at **a** constant actinic irradiation of  $277 \mu\text{mol m}^{-2}\text{s}^{-1}$  or **b** in darkness compared to the control. Total AP dilution factors 5 (AP Dil 5) was applied (2 mL algal solution + 500  $\mu\text{L}$  AP)



$Y(\text{NA}) > Y(\text{ND})$ . However, after the addition of AP,  $Y(\text{NA})$  primarily increased and later seems to decrease after 20 min until the end of experiment whereas  $Y(\text{ND})$  remained constantly at low values.

#### Non-photochemical quenching of excess energy at photosystem II

The fluorescence measurement of PS II enables to detect not only the quantum yield of photochemical energy conversion but also the non-photochemical quenching parameters of regulated thermal energy dissipation ( $Y(\text{NPQ})$ ) and non-regulated heat dissipation ( $Y(\text{NO})$ ) (higher values mean more heat dissipation). The control group under constant irradiation (Fig. 5a) displayed a constant  $Y(\text{II})$ .  $Y(\text{NO})$  decreased slightly and  $Y(\text{NPQ})$  increased slightly. The addition of 500  $\mu\text{L}$  AP (Fig. 5b) caused a rapid decrease of  $Y(\text{II})$  to 0 and a strong increase of  $Y(\text{NPQ})$  whereas  $Y(\text{NO})$  changed to a lesser extent. Fluorescence parameters of the control group (Fig. 5c) showed constant values during the time course of experiment in darkness. The addition of 500  $\mu\text{L}$  AP during dark conditions displayed the same pattern as in light but with less pronounced effects (Fig. 5d).

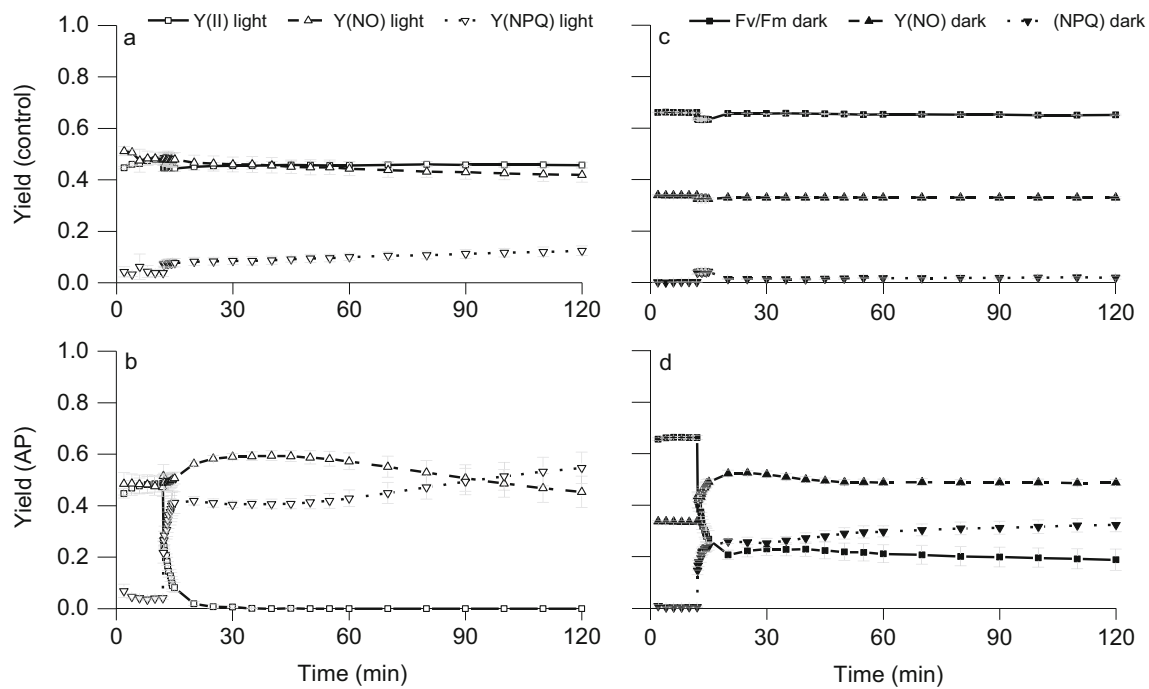
#### Discussion

The effects of different concentrations of the aqueous phase (AP) of hydrothermally converted *A. obliquus* on the photosystems (PS) I and II were tested in short-term experiments. Fluorescence yields of PS II and transmission or remission difference signals of PS I at wavelength of 875 and 830 nm were assessed. Two activity patterns were studied to elucidate

the effect of AP on the photosystems with light-driven electron flow at  $277 \mu\text{mol photons m}^{-2}\text{s}^{-1}$  and in darkness.

Both photosystems generally cooperate to transfer electrons from the water molecule oxidized by PS II to  $\text{NADP}^+$  ( $\text{H}_2\text{O} \rightarrow \text{PS II} \rightarrow \text{Plastoquinone} \rightarrow \text{Cytochrom } b_6/f \rightarrow \text{Plastocyanin} \rightarrow \text{PS I} \rightarrow \text{NADP}^+$ ) (Tikhonov 2013). However, the toxic nature of the AP affected both photosystems differently. If changes occurred, they were immediate, showing the ability of this chemical mixture to penetrate microalgae cells without delay. A steep decrease or increase of yields of PS I and PS II parameters in the first minutes after addition of AP was the consequence.

Fluorescence parameters of *A. obliquus* changed slightly upon the addition of supernatant. This observation can be attributed to dilution effects of the microalgae suspension within the cuvette. After the addition of the supernatant, the algae concentration got diluted to  $0.8 \text{ g L}^{-1}$  and hence the photosynthetically active radiation per cell increased to which the microalgae needed to adapt. The microalgae coped with the per cell more energy by increase of  $Y(\text{NPQ})$ . However, AP had a greater influence on changes of fluorescence parameters than the supernatant. The AP was able to alter the activity of PS II and thus diminish the ability of microalgae to convert light energy into chemical energy via the linear electron flow. Microalgae treated with AP in light had overall more pronounced changes in PS II activity compared to darkness.  $Y(\text{II})$  gradually decreased under actinic light conditions with higher amounts of AP added to the microalgae, except for AP dilution 1:500. Here, no effects were visible compared to the control. A sufficient dilution of the toxic compounds was achieved, which did not harm PS II. This was also demonstrated in long-term experiments at a lower dilution of 1:355 over a period of 9 days of cultivation (Patzelt et al. 2014). Dilutions of 1:5, 1:10, and 1:50 induced chemical stress with



**Fig. 5** Effects of different concentrations of AP on photosynthetic activity of photosystem II (Y(II)), non-regulated non-photochemical quenching (Y(NO)), and regulated photochemical quenching (Y(NPQ))

of *A. obliquus* at **a** constant actinic irradiation of  $277 \mu\text{mol m}^{-2}\text{s}^{-1}$  or **b** in darkness ( $F_v/F_m$ ) compared to the control. Total AP dilution factors 5 (*AP Dil 5*) was applied (2 mL algal solution + 500  $\mu\text{L}$  AP)

concomitant occurrence of photoinhibition of PS II. Addition of the highest amount of AP completely erased Y(II). Electron flow of PS II was inhibited by AP (which can be considered as an herbicide) and according to the results of Fuerst and Norman (1991) resulted in an overreduction of PS II by binding of the toxicants to the D1 protein (Gardner 1989) of PS II. In darkness, the reaction intermediates in AP only partly interrupted photochemistry of PS II as can be seen by lowered but not erased Y(II). The concentration of AP was potentially not high enough to bind to all reaction center proteins in the given microalgal biomass concentration of  $1 \text{ g L}^{-1}$ . Though AP only in combination with light achieved maximal phytotoxicity not only inhibiting electron flow but also possibly generating radicals which degenerated the protein structure of PS II. When AP was added, heat dissipation of excess energy (Y(NPQ) and Y(NO)) increased in darkness and in light. Similarly, an increase of non-photochemical quenching under environmental stress conditions at a comparable photon flux density was also described by Klughammer and Schreiber (2008a) for heat-pretreated rose leaf. The chemical mixture did not affect the short-term photoprotective capacity of *A. obliquus*. Both mechanisms of excess energy dissipation were still active. Photoinhibition is a broad assemblage of stress factors towards the photosystems and can be summarized as light-induced loss of PS II activity (Baker and Bowyer 1994; Tyystjärvi 2013; Adams et al. 2013). Oxygenic photosynthetic organisms evolved efficient systems for repairing PS II (Aro et al. 1993) to cope with the consequences of photo-

oxidative damage (Niyogi 1999). However, if the rate constant of damage exceeded repair mechanisms of PS II, it is followed by irreversible inactivation of PS II reaction center activity (Tyystjärvi and Aro 1996) and leads proximately to photoprotective non-photochemical quenching and loss of photochemical energy conversion. In our case, AP at maximum tested concentrations inhibited Y(II) after 28 min of addition for a minimum of 1 day as detected in a previous study until photochemistry was reconstituted. Slightly more toxic AP from different batch runs resulted in a Y(II) inhibition of 2 days and thus the photosynthetic proteins must be affected after a first-order damage (Patzelt et al. 2014). Photoprotective mechanisms include continual new synthesis of chloroplast-encoded proteins (Niyogi 1999) indicating that in our case either the synthesis was disturbed or renewed proteins got destroyed immediately after synthesis. Linear electron flow failed for at least 24 h to deliver electrons for eventually generating NADPH.

For delivering energy in the form of ATP to decompose toxicants and restore PS II activity, we suggest cyclic electron transport which avoids the need for electrons from inhibited PS II. It also plays a key role in several other stressful environmental conditions. Gao et al. (2011) demonstrated that Y(II) of the macroalgae *Ulva* sp. decreased sharply after onset of desiccation whereas Y(I) increased. Even at an absolute water content of 20 % where no Y(II) was observed, Y(I) remained detectable. Similar effects were observed by Gao and Wang (2012) for *Porphyra yezoensis* exposed to

desiccation during early stages of water loss of the blades and after rehydration. This trend was also true for *C. pyrenoidosa* cells treated with cadmium (Cd) (Wang et al. 2013). Y(II) decreased with increasing Cd concentrations whereas Y(I) was only negatively affected at the highest tested concentrations of 200  $\mu\text{M}$  Cd, but cyclic electron flow at this concentration was much higher than that of the control.

In our case, all applied concentrations of AP were not able to erase P700 signals in light and darkness verifying an active PS I throughout the time course of experiment. Under actinic light conditions, the highest AP dilutions of 1:500 and 1:50 did not affect Y(I) whereas Y(II) was already negatively affected by an AP dilution of 1:50. Here, cyclic electron flow compensated for the loss of electron flow through PS II. Higher concentrations of AP decreased Y(I). The lowest yield of PS I was obtained at the highest AP concentrations with a Y(I) of 0.18. Thus, only cyclic electron flow occurred because Y(II) was already non-existent after 40 min. Comparable to the AP, the herbicide diuron diminishes or abolishes linear electron flow (Joët et al. 2002). Donor-side limitation exceeded acceptor side limitation of PS I in the control and AP treatment under constant irradiation. This effect, which “may be considered typical for a well-regulated, healthy leaf that is able to protect itself against damage by excess radiation by non-photochemical energy dissipation” at light intensities above 200  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  (Klughammer and Schreiber 2008b), was also observed in Fig. 4 for microalgae irradiated at 277  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ . However, AP increased the amount of closed PS I reaction centers by donor-side limitation in analogy to PS II as described above for Y(NPQ). The Y(ND) increased as a result of lacking electron supply from PS II due to inhibitory effects of AP and thus more oxidized P700.

Y(I) of microalgae which were kept in the dark showed no negative effect of AP. On the contrary, AP increased Y(I) after 120 min above Y(I) during adaption due to a decrease of acceptor-side limitation and was also higher compared to control (+0.15 of the original Y(I) value/+0.3 of normalized Y(I) values). Thus, AP even seemed to enhance activity of Y(I). Comparing both photosystems in light and darkness (Fig. 3), photosystem I was the relatively more active photosystem when AP was added in critical concentrations. Cyclic electron flow was also stimulated in cyanobacteria, lichen, and higher plants under various stimuli (Jeanjean et al. 1993; Hagemann et al. 1999; Joët et al. 2002; Golding and Johnson 2003; Bukhov and Carpentier 2004).

PS I parameters Y(NA) and Y(ND) displayed the opposed trend in darkness compared to light. PS I was rather acceptor-side limited in darkness. The ferredoxin pool as acceptor of electrons from PS I might be overreduced. Acceptor-side limitations during darkness have been previously observed even for transition from darkness to light at 750  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  for the first 10–20 s of irradiation (Foyer et al. 1992). Possibly ferredoxin-NADP<sup>+</sup> reductase (FNR) for the sink of

electrons to NADPH or transfer of electrons from ferredoxin to cytochrome b6/f for cyclic electron transport needs light activation (Satoh 1981). Additionally, during the light induction phase, the Calvin cycle is inactive. PS I becomes overreduced due to limited consumption of NADPH and thus reductants accumulate at the acceptor side. The excess of reductants on the acceptor side of PS I diverts the outflow of electrons under actinic light from ferredoxin to thioredoxin which in its reduced state activates other photosynthetic enzymes, including those of the Calvin cycle (Tikhonov 2013) leading to decreased acceptor-side limitations.

Concerning the repair mechanisms of PSI, the turnover rates of PS I subunits are slower compared to PS II and hence recovery rates are suspected to be lower for PS I than for PS II (Sonoike 2011). Damaged PS I would be more fatal for the microalgae than damaged PS II. It is difficult to estimate whether the AP directly affected PS I or only due to feedback reactions from PS II. However, if Y(I) would decrease further and eventually vanish, the cell would rely solely on internal energy storage. We assume that PS I is active during prolonged times of PS II inhibition because it is less susceptible to stress than PS II (Canaani et al. 1989). We resolved the temporal modes of action of the AP on PS I and PS II showing that only PS II was negatively affected by AP during darkness. The complex chemical mixture seemed to interfere with linear electron flow even without the light-induced formation of radicals and following destruction of key enzymes of photosynthesis. In contrast, AP seemed not to affect PS I in darkness and rather increased the photochemical energy conversion. Main cause of reduction of photochemical energy conversion and increase of donor-side limitations during light is suggested to result from lacking electrons from PS II. PS I compensated for PS II activity losses arising due to the toxic mixture. Cyclic electron flow around PS I might deliver the energy needed for reactivation of PS II and remediation of the toxic liquid residue. Under stressful conditions, cyclic electron flow around PS I serves as a protective mechanism to supply the cell with energy as long as possible when PS II is harmed. For industrial scale application, direct usage of liquid residues of hydrothermally converted microalgae would be optimal. In this case, both photosystems I and II were undisturbed at dilutions of 1:500 and thus thinning out the nitrogen concentration 100-fold resulting in 22.3–26.3  $\text{mg L}^{-1}$ . This concentration can support high microalgal growth as desired for industrial scale applications when a chemostat cultivation is applied. However, to replace cost intensive fertilizers with nutrients from hydrothermally converted microalgae, a remediation step is necessary (Patzelt et al. 2014) to remove any possible health risks. The activated carbon treatment



eventually enabled the cultivation of microalgae on the undiluted liquid residues resulting in high biomass productivities.

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