HOW *p*-BENZOQUINONE INHIBITS GROWTH OF VARIOUS FRESHWATER PHOTOTROPHS: DIFFERENT SUSCEPTIBILITY AND MODES OF ACTIONS?

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ABSTRACT

Interactions of polyphenols with freshwater phototrophs have been the focus of many studies, since polyphenols of various classes and their oxidation products (quinones) belong to natural organic matter in any freshwater body. Most of these studies lack information regarding the possible physiological modes of action. Therefore, we challenged eukaryotic and prokaryotic phototrophs by exposing them to *p*-benzoquinone (PBQ). Both groups of phototrophs showed clear stress responses that led to inhibition of growth and photosynthetic capacity. Two modes of action were elucidated for the short-term effects in the range of minutes to 24h: PBQ lowered the relative photosynthetic electron transport rate (rETR) and induced oxidative stress in terms of lipid peroxidation. However, inhibition of rETR by PBQ was only transient within minutes and was not detectable after 24 h, whereas growth inhibition continued until the end of culturing. Furthermore, it was shown that the effective concentration (EC) of PBQ to inhibit eukaryotic algal growth was much higher than the threshold for cyanobacterial growth inhibition. *Microcystis aeruginosa* was found to be by far the most susceptible species. Surprisingly, the latter cyanobacterial species showed no inhibitory effect of PBQ on photosynthesis, indicating different physiological target sites governing inhibition of growth and photosynthetic electron transport independently.

Keywords: *p*-benzoquinone, algal growth, chlorococcales, cyanobacteria, oxidative stress, electron transport

1. INTRODUCTION

Dissolved organic carbon (DOC) is a mixture of different organic compounds found in all freshwater bodies and varies from less than 1 to 200 mg L^{-1} [1]. Humic substances (HSs) comprise the majority of organic carbon in freshwater ecosystems and exceed the concentration in living organisms with regards to organic carbon by at least on order of magnitude [2-4]. It is well known that DOC interacts with plankton phototrophs in various direct or indirect mechanisms. It is also well understood that DOC reduces the underwater light climate, thereby indirectly shaping the primary producer community [5]. Surprisingly, little is known about the direct impact of HSs on the performance of eukaryotic and prokaryotic phototrophs. However, since HSs are natural xenobiotics [2], direct interactions with aquatic organisms, including phototrophs, have to be anticipated. The available reports on this issue are contradictory as evidenced particularly with cereal straw leachates. There have been many approaches used over the last decades to find low cost organic compounds for control of nuisance algal growth in freshwater bodies with low environmental risk, e.g. [6-8]. The main target organisms in most studies were cyanobacterial species, especially *Microcystis aeruginosa* and *Anabaena* sp., which have the potential to cause harmful algal blooms [9,10]. Furthermore, several authors have predicted that climate change is a potent catalyst for the further expansion of these blooms [11].

To get detailed information about potential underlying mechanisms, we carried out two studies with two authentic humic preparations [12,13]. Our studies showed that these preparations did not affect different green algal and cyanobacterial species adversely or even have growth stimulating effects. Furthermore, we demonstrated that one of these humic preparations, referred to as HuminFeed® (HF), artificially enriched with quinones, possessed a short-term algicidal potency against cyanobacteria when applied in dosages as high as 4.17 mM DOC [14]. Consequently, we concluded that it was not the HSs themselves that were the inhibitors, but a minor fraction of monomeric quinones which were chemically altered during the experiment and thus lost their algicidal effect. Continued oxidation of quinoid

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and phenolic compounds can result in increasing polymerization to form HS-like substances [15]. Environmental requirements are an alkaline medium and the presence of oxygen, which applies to many freshwater systems.

The widely tested substrate was barley straw, which showed algicidal potential. Besides known algicidal chemicals such as *p*-cresol, phenol, biphenyl-2-ol, hydrocinnamic acid, menadione, hydroxyl-benzaldehyde, and butanoic acid [16,17], 30 unidentified organic compounds were found. The authors related the observed algicidal effects to this mixture of organic compounds and their additive and/or synergistic toxicity. Waybright et al. [18] suggested that polyphenols with molar mass between 1 and 3 kDa were the inhibitory fraction released from aqueous barley straw. In addition, Murray et al. [19] assigned 2-phenyl-phenol, p-cresol, and benzaldehyde the major inhibitory properties, and Ridge et al. [6] assumed that the potential algal inhibitors were oxidized polyphenolics or derived products which originated primarily from decomposing lignin.

Through microbial and photolytic degradation of natural organic matter (NOM), small organic compounds are released from high-molar mass organic matter and interact with phototrophs. Besides various polyphenols, monomeric quinones are also produced by aquatic and terrestrial plants [20]. These quinones are similar to hydroquinone, which is the reduced form of PBQ. From terrestrial plants, precursors of hydroquinone, such as arbutin [21-24], are often released and degraded to hydroquinone in non-sterile soils [25].

Currently, few details are available concerning the underlying modes of action of compounds having algicide effects. Sun et al. [26] described growth inhibition on *Anabaena circulensis* exposed to humic acids (HA) under illumination. A combination of both led to an intracellular accumulation of H_2O_2 and eventually to cell death. In addition Barrington and Ghadouani [10] demonstrated that cyanobacteria are more susceptible to H_2O_2 than green algae and diatoms. Zhang et al. [27] showed that berberine, an alkaloid from goldenthread, led to oxidative damage by reactive oxygen species (ROS). Another possibility is the interaction of organic compounds with enzymes, such as protein precipitation by polyphenols. Indeed, Dedonder and van Sumer [28] described the inhibition of photophosphorylated ATP-synthesis by phenolics in isolated *Spinaciaoleracea* chloroplasts.

Quinones have long been known for their interference with the respiratory and photosynthetic electron transport chain [29]. However, benzo-quinones can also develop highly diverse cytotoxic effects at different sites of cellular metabolism [30]. Furthermore, benzoquinones are often highly chemically reactive under *in vitro* conditions [31,32] and might consequently affect algal growth via the formation of various reaction products. Hence, we tried to elucidate the mode of action of a monomeric quinone, *p*-benzoquinone (PBQ), on growth, photosynthetic performance, gas exchange, and oxidative damage. Furthermore, we wanted to establish whether or not cyanobacterial species are more sensitive to this prominent type of quinone than eukaryotic green algal species.

2. MATERIALS AND METHODS

2.1. Algal Species

To compare the susceptibility of prokaryotes and eukaryotes to PBQ, three cyanobacterial species and three green algal species were chosen for this study. All tested species are common in freshwater ecosystems. The green algal species were obtained from the Algal Culture Collection, Göttingen, Germany. The coccal green alga *Pseudokirchneriella subcapitata* (strain no. 61.81) (Koršikov) Hindák NIVA-CHL was originally isolated from the River Nitelva at Åkershus, Norway, by Skulberg in 1959 [33]. *Monoraphidium braunii* (strain no. 2006) was isolated from Große Fuchskuhle, Germany, and *Desmodesmus armatus* (syn. *Scenedesmus armatus, Scenedesmus quadricauda*) from Marburg, Germany (strain no. 276-4d).

Synechocystis sp. PCC 6803, *Nostoc* sp. PCC 7803, and *Microcystis aeruginosa* PCC 7806 were obtained from the Institut Pasteur (Paris) Cyano-bacteria Collection. All phototrophs were grown in a modified BG11 Medium [34]. The amount of nitrogen was reduced to 20% of the original recipe, but was still high enough for non-N-limited growth and for a realistic ratio to phosphorus (Redfield ratio: N:P = 16:1). Furthermore, 1.2 mM of $NaHCO_3$ was added as an internal carbon source. The modification was applied to achieve an appropriate medium that allowed runs of the experiments under the same growth conditions for all tested phototrophs. The experiments were conducted in duplicate and each treatment was run in triplicate.

2.2. Cultivation, Sampling, and Pulping of Phototrophs

To determine the effective concentration inhibiting 50% of growth (EC50), the algae were exposed to various concentrations of PBQ (dissolved in 10% DMSO) ranging from 0.127 to 127 µM PBQ (0.01 –

10 mg L^{-1} DOC). Only algae growing in the log-phase were used in the experiments. 50 mL of modified BG11-medium in 100 mL flasks were inoculated with algae growing in the log phase up to a starting concentration of approximately $2x10^5$ and $5x10^6$ cells mL^{-1}. The calculated starting concentration was dependent on the cell size so that every experiment started with approximately the same biovolume ((cells/mL) x mean cell volume), which varied between 0.03 and 0.035 µL per mL medium. For an optimal CO_2 supply, closed polyethylene bags with $KHCO_3$, which continuously released CO_2 to the algae medium were used [35]. Illumination was cool white light set to 150 µmol m^{-2} s^{-1} photons with a light/dark rhythm of 14:10 h. Only *Microcystis* and *Nostoc* were exposed to a lower light intensity of 100 µmol photons m^{-2} s^{-1}, due to its poor growth performance at 150 µmol m^{-2} s^{-1}. To determine growth rates, algae grew for 4 days to reach a high density. With respect to their lower growth rate, the cyanobacterial species grew for 5 days.

For the determination of thiobarbituric acid reactive species (TBArS), a biovolume of approx. 0.2 µL•mL^{-1} medium was harvested out of 50 mL of algal suspension and exposed to EC 10/50/90-concentrations for 24 h. The experimental setting was identical to that described above.

For analyzing the acute inhibition of the electron transport rate (ETR), 2 mL with a biovolume of 0.2 µL•mL^{-1} were used in the Phyto-PAM fluorometer. Exposure to PBQ took place directly in the cuvette.

To exclude any adverse effects on algal growth by the solvent DMSO, equal amounts were added to the controls resulting in the highest final DMSO concentration. Final DMSO concentrations in the PBQ exposures varied between 0.005 and 0.1% (v/v) and showed no negative effects on controls.

2.3. Cell Numbers, Growth Rates and, Cell Size

Cell numbers and cell size were recorded with a Z2 Coulter Particle Counter and Size Analyzer (Beckman Coulter, Krefeld, Germany). This method is based on measurable changes in electrical resistance produced by cells suspended in an electrolyte. The growth rate µ was calculated on the basis of the biovolume ((cells/mL) x mean cell volume) with the formula:

$$\mu\ [d^{-1}] = (\ln(Nt_2) - \ln(Nt_1))/t_2 - t_1,$$

where Nt = biovolume at time t; t_1 = the start of the experiment; t_2 = the end of the experiment.

Biovolume was chosen instead of the absolute cell number because of its higher precision as a biomass variable. In fact, the cell volume was variable and would be misleading in calculations of biomass using only absolute cell counts.

2.4. Calculation of Effective Concentrations (ECs)

Final DMSO concentrations varied between 0.005 and 0.1% in PBQ exposures. At least 5 different concentrations ranging from 0.127 to 127 µM PBQ (0.01 – 10 mg L^{-1} DOC) were used for the growth inhibition test. Algal growth was analyzed after 48 h and after 96 h for the green algal species and 48 h and 120 h for the cyanobacterial species, respectively. Both green algal and cyanobacterial growth were in the logarithmic growth phase during the whole exposition.

Based on the observed growth inhibition by PBQ of algal and cyanobacterial growth, the effective concentrations (EC) were calculated with the probability unit model (probit-model) [36]. Therefore, the probit value, which depends on percentage of growth inhibition, was plotted against the log of the molarity of PBQ. The PBQ concentrations inhibiting 10, 50 and 90% of algal growth were calculated by a linear regression function $Y = ax + b$, with y meaning the probit value, a and b being constants and x being the variable of the linear regression. The effective concentration is the antilogarithm 10^x.

The three different effective concentrations (EC10; 50; 90) derived from growth experiments were chosen to get a better understanding of the kinetic physiological behavior under different toxicity levels.

2.5. Chlorophyll Fluorescence Parameters

All fluorescence parameters were determined using a Phyto-PAM fluorometer (Phyto-PAM, Emitter Detector Phyto-US, Software PhytoWin v 2.0, Walz, Effeltrich, Germany). The Phyto-PAM is a four-wave-length chlorophyll fluorometer for the assessment of chlorophyll content and photosynthetic activity of plankton algae. Fluorescence is alternatingly excited at high repetition rates by µs pulses of 470, 520, 645, and 665 nm light, originating from light emitting diodes (LED). Actinic light and light saturation pulses were delivered by red LEDs (655 nm).

For determination of the potential inhibition of Photosytem II (PSII) activity by PBQ, a quartz cuvette was filled with 2 mL of the algal suspension, with a biovolume of approximately 0.2 µL•mL^{-1} and put in the light channel of the Phyto-PAM. The actinic light was set to 192 µmol photons m^{-2} s^{-1} and a saturation pulse (SP) with 2,000 µmol m^{-2} s^{-1} was added every 30 s. When the variable fluorescence was stable the *p*-benzoquinone was added directly to the cuvette and

the change in the fluorescence was recorded.
The activity of the photosystem II (PS II) was determined using the so-called ΔF mode, which measures the variable Chl-*a* fluorescence $F_v' = F_m' - F$, with F_v' = variable fluorescence, and F_m' = the maximal fluorescence under actinic light. Based on the variable fluorescence, the relative electron transport rate (rETR) was calculated as follows:

rETR [μmol electrons m^{-2} s^{-1}] =
(F_v'/F_m') x PAR x 0.5 x 0.84

where PAR is the photosynthetic active radiation. More detailed information about the use of chlorophyll fluorescence is provided by Maxwell and Johnson [37] and Jakob et al. [38].

2.6. Thermoluminescence Measurements

A thermoluminometer set up [39] was used to measure glow curves. Samples with a total amount of 50 μg Chl-*a* (same PBQ to biovolume ratio as in rETR measurements) were incubated with PBQ for 10 min at growth light intensities. Following this they were filtered on a glass fiber filter (GF 6; Whatman, Düren, Germany) with a diameter of 20 mm. A total of 4 discs (8 mm in diameter, 8 μg Chl-*a* each) were punched out and transferred to the filter holders in the TL set-up. The samples were then fixed with steel clamps. A computer-controlled program was applied including the following steps: Samples were excited by two single-turn-over flashes to activate S-state cycling followed by a dark period of 5 min at 20°C. Samples were then cooled to 2°C followed by one single-turn-over flash. They heating of the samples was performed at 20°C min^{-1} to the final temperature of 70°C paralleled by recording of thermo-induced light emission [39,40]. For further investigations one species from each group, the green alga *Desmodesmus armatus* and the cyanobacterium *Synechocystis* sp., was chosen in order to obtain detailed insight into the mechanistic background of the impact of PBQ.

2.7. Oxygen Evolution

The measurements were taken with the Light-pipette (Illuminova, Sweden). The oxygen evolution rates were determined using a Clark type electrode (MI 730; Microelectrodes Inc., New Hampshire, USA). The Light-pipette also provided an actinic light source (OsramXenophot HLX 64625), which enabled the measurement of P-I-curves. A final concentration of 10 mM $NaHCO_3$ was added to the samples (~2.5 mg Chl-*a* L^{-1}) in order to avoid carbon limitation during the 50 min of measurements at 25°C. A computer-controlled program with increasing irradiances was used to measure P-I-curves. Each irradiance had a duration of 3 min and alternated with a subsequent dark period of the same length. The program always started with a dark phase. The samples were incubated with PBQ for 10 min before starting the first light period. Gross oxygen rates were calculated by adding the net oxygen production to the absolute values of the corresponding respiration rates. The curves were fitted in accordance with ref. [41]. The resulting fitting parameters were used to calculate α-slope, P_{max} and I_k. In case of *Synechocystis* sp., a more concentrated sample had to be used in oxygen measurements compared to Phyto PAM analysis for the following reason: For oxygen measurements, a Chl-*a* concentration of approximately 2.5 μg mL^{-1} was needed for a reliable signal to noise ratio. Therefore, the biovolume for the rather small *Synechocystis* cells had to be increased to 0.51 μL mL^{-1}, yielding a Chl-a concentration of 2.50 μg mL^{-1}. To keep the biovolume to PBQ ratio also constant in this sample preparation, the PBQ concentration for *Synechocystis* was increased by a factor of 2.5.

2.8. Lipid Peroxidation

Lipid peroxidation was measured in terms of thiobarbituric acid reactive substance (TBArS) according to ref. [42]. After 24 h of exposure to EC10/50/90 values of PBQ, 10 mL of the algal suspension were centrifuged for 5 min at 12,000 rpm, the supernatant was discarded and the pellet was re-suspended in 10 mL of doubly-distilled water. After a second centrifugation step the pellet was dissolved in 1 mL of 0.25% thiobarbituric acid (dissolved in 20% trichloroacetic acid, TCA) and transferred to a heating block. After 30 min at 95°C with continuous shaking the samples were centrifuged for 5 min at 12,000 rpm and the absorbance of the supernatant was measured at 532 nm in a Shimadzu UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). Turbidity was measured at 600 nm and subtracted from the absorbance at 532 nm. An absorptivity of 155 M^{-1} cm^{-1} was used. TBArS concentrations were standardized to protein content which was measured in accordance with Bradford [43].

2.9. Statistics

All data were statistically analyzed using SigmaStat 3.5 (Systat Software, CA, USA). Means were compared using the t-test. Levels of significance were: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

3. RESULTS

3.1. Effective Concentrations of *p*-Benzoquinone

As shown in Table 1, the three tested cyanobacterial species were generally much more sensitive to PBQ than the green algal species. Within the cyanobacteria, *M. aeruginosa* (0.49 µM) was approximately 10 to 20 times more susceptible than *Nostoc* (5.7 µM) and *Synechocystis* (8.54 µM) and approximately 40 to 60 times more sensitive than the green algal species with *D. armatus* (22.5 µM) being the most susceptible compared to *M. braunii* (27.5 µM) and *P. subcapitata* (33.6 µM). The EC-values after 48 h of exposition were, in general, lower than after 4 to 5 days of exposure, but the relative proportion between eukaryotic and prokaryotic susceptibility was the same (data not shown).

Table 1 Effective concentrations of p-benzoquinone inhibiting 10, 50 and 90% of growth after 96 h (green algae) and 120 h (cyanobacteria) with R^2 meaning the stability index of the linear regression

	[µM][1]			R^2
green algae	EC10	EC50	EC90	
P. subcapitata	12.6	33.6	89.5	0.951
M. braunii	6.79	27.5	112	0.991
D. armatus	9.24	22.5	54.9	0.999
cyanobacteria	EC10	EC50	EC90	
Synechocystis sp.	2.12	8.54	34.4	0.989
Nostoc sp.	2.06	5.7	15.8	0.946
M. aeruginosa	0.12	0.49	1.97	0.912

[1] Effective concentration of *p*-benzoquinone

3.2. Relative Photosynthetic Electron Transport Rates (rETR)

In contrast to *M. aeruginosa*, the other tested phototrophs were clearly affected in their photosynthetic performance. The short-term exposure to PBQ induced a clear decrease in rETR in all tested green algal species (Fig. 1). The inhibition of *D. armatus* continuously increased over a period of 18 minutes. By contrast, *M. braunii* and *P. subcapitata* showed the highest decrease of rETR after three to four minutes and quickly recovered fully or partially afterwards. The maximum inhibition of rETR was approximately 35% in *M. braunii* and *P. subcapitata* after exposure to PBQ in the effective concentration inhibiting 90% of growth (EC90).

As shown in Fig. 1, *Synechocystis* and *Nostoc* were also significantly affected by PBQ treatment. The effects were stronger than the observed effects in the tested green algal species, but, again, the kinetics of response were different. Whereas in *Synechocystis* rETR was reduced strongly immediately after exposure to PBQ, in *Nostoc* the reduction in photosynthetic performance was lowered continuously over 18 min. In *Synechocystis,* rETR was reduced by 72% and by 58% in *Nostoc.*

With the exception of *Microcystis*, the reduction of photosynthetic performance was clearly dose-dependent in all tested species. Surprisingly, *Microcystis* did not show any reduction in rETR although this species was the most sensitive with respect to growth inhibition. Only in *Nostoc* did EC50 and EC90 provoke a similar response, while the impact of EC10 and EC50 on the growth of *P. subcapitata* and *D. armatus* was also similar.

To establish whether or not this short-term effect of exposure remained or vanished over time, the rETRwas also checked after 24 h. Interestingly, the inhibition of rETR disappeared after 24 h in all tested species with the exception of *Synechocystis*. In contrast to *Synechocystis*, which was still negatively affected by the highest dosage of PBQ after 24 h, the rETR in *P. subcapitata* and *D. armatus* was slightly stimulated (Fig. 2).

3.3. Thermoluminescence Glow Curves

The measurement of thermoluminescence (TL) glow curves allows a focused view on the donor and acceptor side of the highly stress-sensitive PSII reaction center. TL represents thermal activated charge recombination under light emission of radical pairs prior-generated in light. Positive charges of the donor side (water splitting apparatus) recombine with negative charges on the acceptor side of PSII [40]. The thermally-activated B band peaking around 20°C originates from the radical pair $S_{2/3}Q_B^-$. Here, the positively charged S-states (S_2/S_3) in the water splitting apparatus recombine with the negatively charged secondary plastoquinone Q_B^-.

After exposure to PBQ, *D. armatus* (Fig. 3A) showed no significant effects on the B band, whereas in *Synechocystis* (Fig. 3B), the B band decreased significantly only at a PBQ concentrations of EC90. Whereas rETR measurements were performed at steady state actinic illumination of 192 µmol quanta m^{-2} s^{-1} and represent also the effective PSII quantum yield (Fv'/Fm') at this light intensity, TL was measured after 5 min dark adaption. Therefore, TL will be more related to the maximum PS II fluorescence quantum yield (Fv/Fm) also measured after dark adaptation. After inhibitory treatments,

effects can be more easily observed in steady state rETR and effective quantum yield Fv'/Fm', where energization of the thylakoid membrane and whole chain electron transport is fully established, because this state increases the probability to detect inhibitor-induced restrictions at different sites of the photosynthetic apparatus. Instead, the dark adapted quantum yield Fv/Fm represents the variable closing of PSII reaction centers and electron filling of the PQ pool within a short saturation pulse, lacking the restrictions of membrane energization and whole chain electron transport including the capacity of the Calvin Cycle. These differences in the dark and light adapted state might explain why no effects are observed for the TL B band in *D. armatus* and only at the highest PBQ concentration in *Synechocystis* sp.

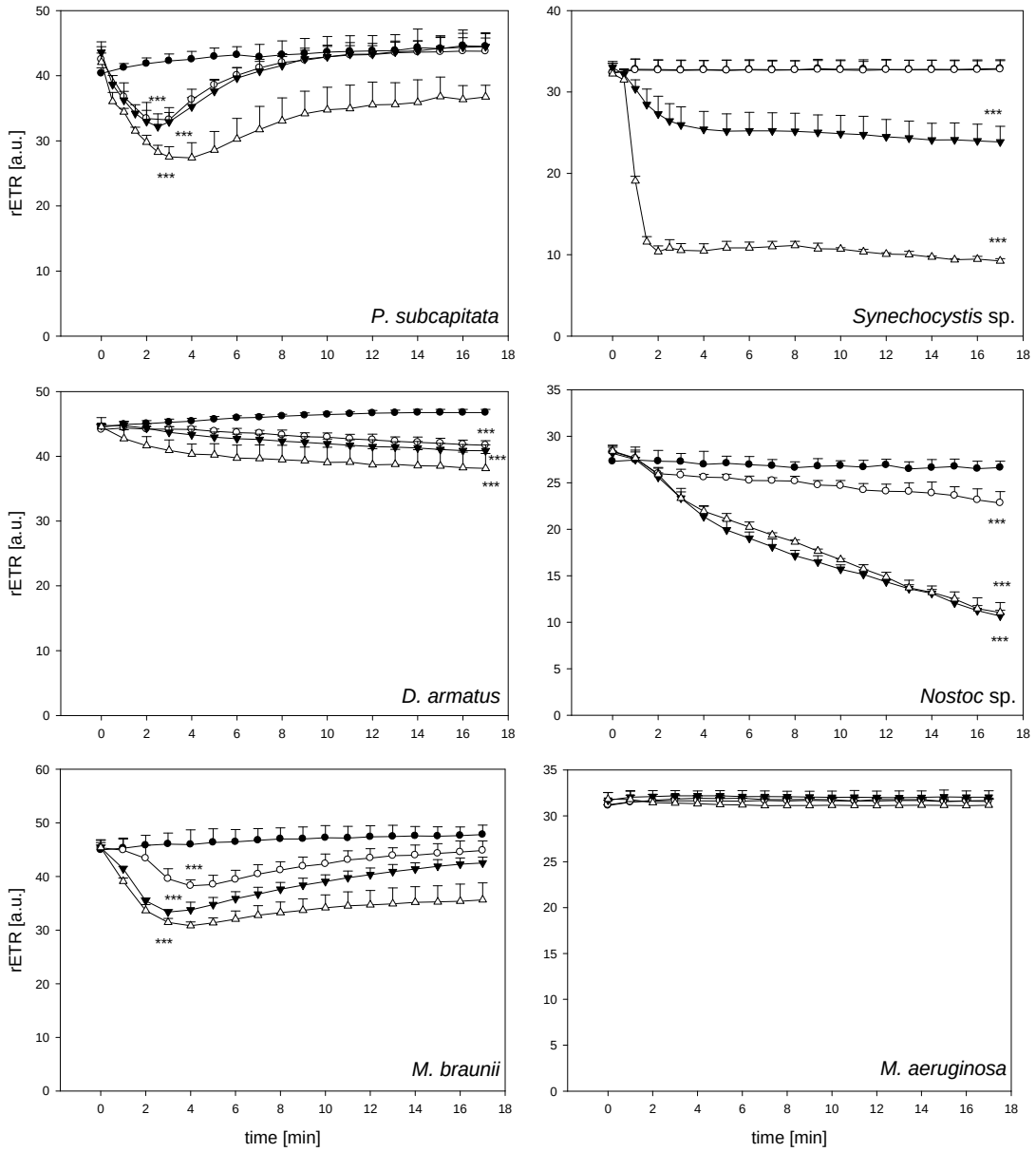

Figure 1 Relative photosynthetic electron transport rates (rETR) of the tested green algal and cyanobacterial species at different effective concentrations of PBQ: black circles = Control; white circles = EC10; black triangle = EC50; white triangle = EC90.

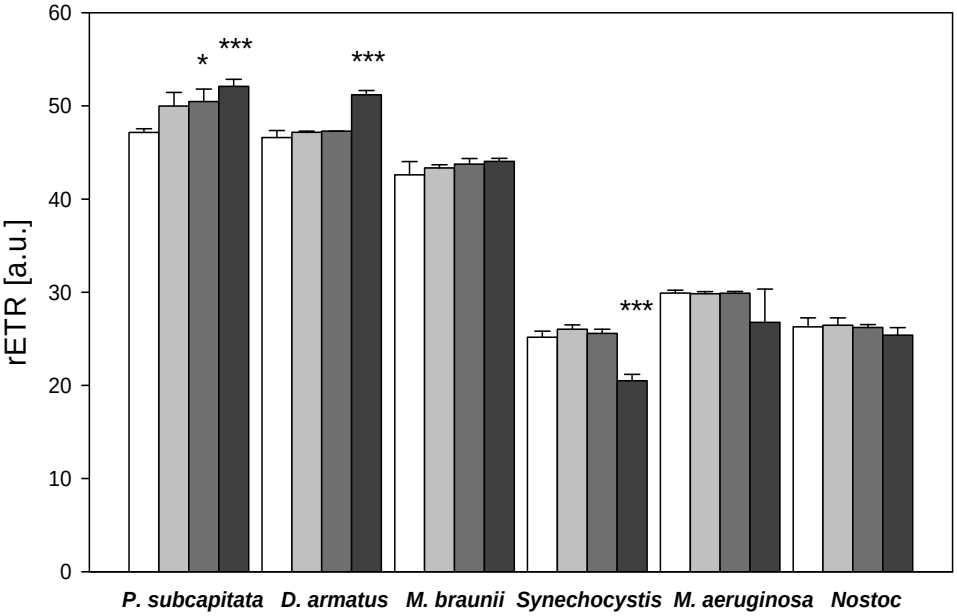

Figure 2 Relative photosynthetic electron transport rates (rETR) of the tested green algal and cyanobacterial species at different effective concentrations of PBQ and 24 h of exposure [white = Control; light grey = EC10; grey = EC50; dark grey = EC90].

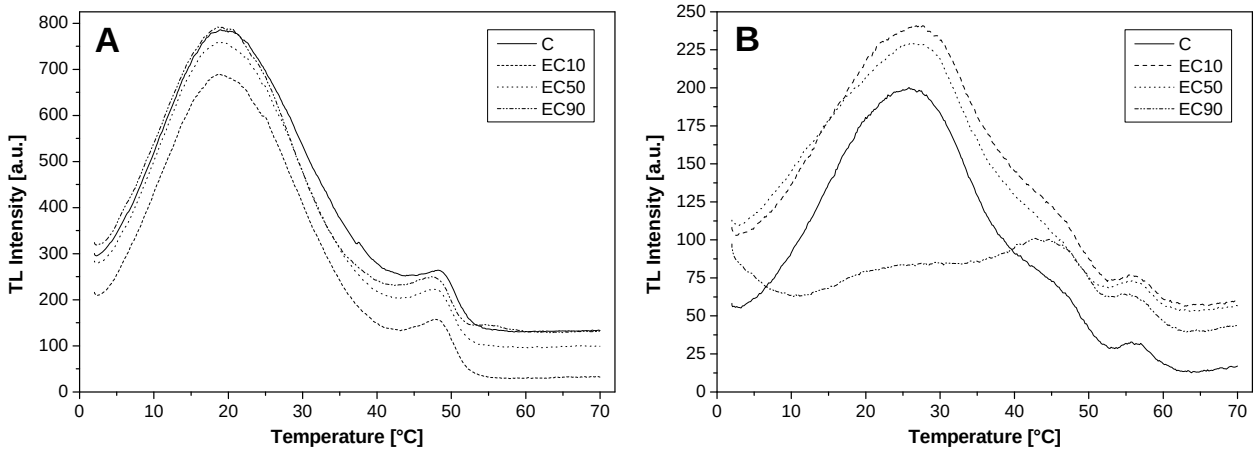

Figure 3 Thermoluminescence glow curves of *Desmodesmus armatus* (**A**) and *Synechocystis* sp. (**B**) cells after 10 min exposure to increasing PBQ concentrations.

3.4. Oxygen Evolution

For the gas exchange measurements, the highest concentrations of PBQ (EC90) were used to get an adequate response in these short-tirm experiments.

The gross oxygen production of *D. armatus* cells (Fig. 4) followed a typical P-I curve with the initial linear α-slope at low light intensities, whereas at high intensities the maximum photosynthetic capacity (P_{max}) was reached. At higher light intensities (above 500 µmol photons m^{-2} s^{-1}), the PBQ-treated cells showed a significant decrease in gross oxygen production. At 192 µmol photons m^{-2} s^{-1} (light intensity of Phyto-PAM measurements), a similar reduction in oxygen evolution was observed as in rETR measurements but the difference was not significant. This is probably due to the lower signal to noise ratio for oxygen measurements, especially at lower oxygen yields.

In *Synechocystis*, gross oxygen evolution at this light intensity was strongly reduced by 85% after PBQ addition (Fig. 5). This is a similar reduction of photosynthetic activity as observed in rETR (70% reduction) measurements (Fig. 1). The gross oxygen production in *M. aeruginosa* was not affected by exposure to the EC90 concentration of PBQ (Fig. 6). These results agree with the measurements of rETR, where no effects occurred as well (Fig. 1).

3.5. Thiobarbituric Acid Reactive Substance (TBArS)

As shown in Fig. 7, the concentration of TBArS increased significantly after 24 h exposure to PBQ. Again, this stress parameter surprisingly was not activated in *M. aeruginosa*, which showed the highest sensitivity to PBQ in terms of growth.

In the tested green algal species, the concentration of TBArS was twice as high after exposure to EC90 compared to the controls. This increase of lipid peroxidation was clearly dose dependent. In the cyanobacterial species, exposure to PBQ also led to increased levels of TBArS: the effect was strongest in *Nostoc* and was again dose-dependent. The increase of TBArS in *Synechocystis* was significant but only about 50% higher than in the controls. In addition, there was no dose-dependency in *Synechocystis* and no effects at all in *Microcystis.* However, when comparing the induction of growth inhibition (Fig. 1) and that of TBArS formation (Fig. 7) in the different phototrophs, no significant correlation could be found.

4. DISCUSSION

There has been intensive research addressing the impact of different DOC types on freshwater phototrophs. The majority of these studies were, more or less, descriptive. They showed the various effects on growth performance of algal and cyanobacterial species, from total inhibition to growth promotion. Most of these studies focused on nuisance bloom-forming cyanobacteria like *M. aeruginosa* [6,8,15,27, 44,45] and *Anabaena* sp. [26,45].

The fact that cyanobacterial species are more sensitive to algicidal organic compounds than eukaryotes has been demonstrated by several authors: Islami and Filizadeh [46] observed a shift in algal composition from Cyanophyceae to Bacillariophyceae after exposure to decomposing barley straw. A reduction in the cyanobacterial dominance after exposure to rotting barley straw was also reported by Everall and Lees [44]. Martin and Ridge [47] showed that four strains of *M. aeruginosa* were most susceptible to decomposing barley straw, but also disproved theories regarding the general susceptibility of Cyanophyceae. As shown in Table 1, growth of the three tested cyanobacterial species was much more sensitive to PBQ than growth of the green algal species. Within the cyanobacteria, *M. aeruginosa* was approximately one order of magnitude more susceptible than *Synechocystis* sp. and *Nostoc* sp. and nearly 60 times more sensitive than the green algal species.

Besides the PBQ-induced growth inhibition, a short-term reduction of the photosynthetic rETR (Fig. 1) was also observed, which correlated well with a reduction in oxygen evolution (Fig. 4 and 5). However, whereas growth inhibition continued, rETR recovered in all tested species with exception of *Synechocystis* sp. after a minimum of 24h (Fig. 2). Hence, the long-term growth inhibition cannot be caused by the short-term reduction of photosynthetic capacity. This fact is convincingly demonstrated for *M. aeruginosa*, which is the most sensitive species concerning growth inhibition by PBQ, but lacks any detrimental effect on rETR. Furthermore, the recovery of rETR showed that the cells were still viable, even when growth inhibition was retained. These principal findings indicate a rather complex reactivity of PBQ and not a single mode of action with the various phototrophs.

Benzoquinones have been described as interacting with the photosynthetic electron transport chain at the level of the secondary acceptor quinone Q_B of the PS II reaction center or the level of the plastoquinone pool [29]. The lipid-soluble PBQ in particular has been used formerly as an artificial Hill electron acceptor for PSII, applying final concentrations of 0.5 to 2 mM [48]. In our experiments, far lower concentrations (0.1 to 112 µM) were applied. Our results did not indicate a Hill type reaction of PBQ in the studied cyanobacteria and green algae. That is, no accelerated rETR or oxygen evolution rate was observed. Benzoquinones might also act as redox cycling agents. The oxidized benzoquinone could be reduced to the semiquinone-anion-radical or to hydroquinone by the photosynthetic electron transport chain and then reoxidized by molecular oxygen. The resulting superoxide radical and hydrogen peroxide are detoxified via superoxide dismutase and ascorbate peroxidase in the Asada-Halliwell cycle [49]. Similar to the pseudocyclic or so-called Mehler reaction [50], no net oxygen exchange would result. In case of redox cycling by PBQ, treated samples would show a decreased oxygen evolution rate compared to the control but an unchanged or even accelerated rETR.

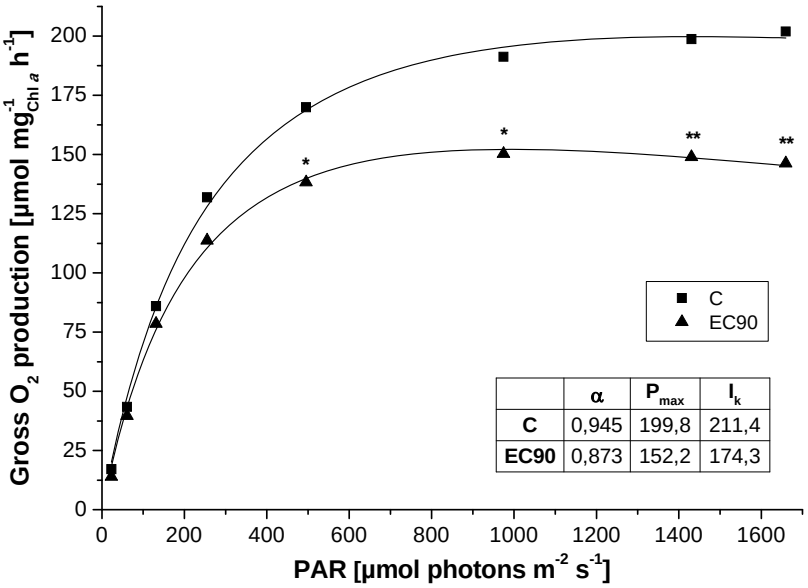

Figure 4 Gross O_2 production of *Desmodesmus armatus* cells after exposure to PBQ EC90 concentrations relative to photosynthetic active radiation (PAR). Curves were fitted according to Eilers and Peeters [41]. The resulting fitting parameters were used to calculate α-slope [(µmol O_2 mg Chla^{-1} h^{-1}) (µmol photons m^{-2} s^{-1})$^{-1}$], P_{max} [µmol O_2 mg Chla^{-1} h^{-1}] and I_k [µmol photons m^{-2} s^{-1}].

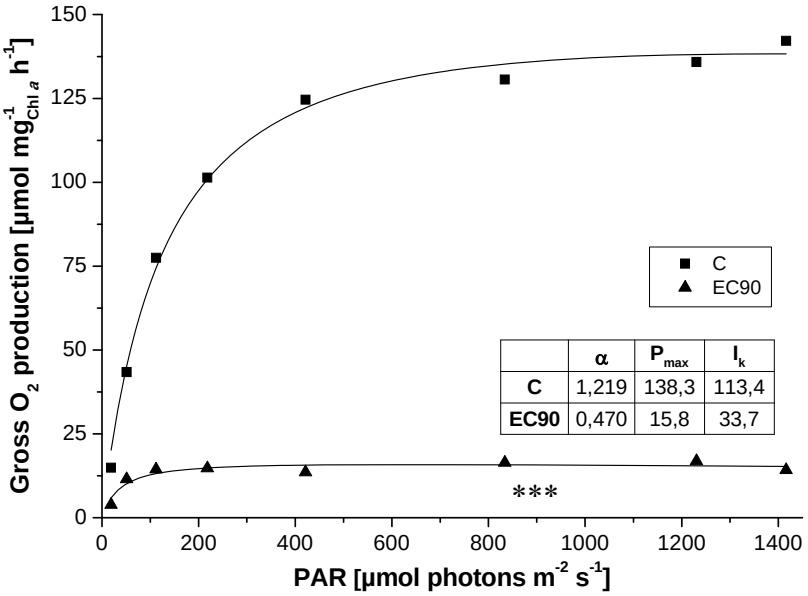

Figure 5 Gross O_2 production of *Synechocystis* sp. cells after exposure to PBQ EC90 concentrations in reference to photosynthetic active radiation (PAR). Curves were fitted according to Eilers and Peeters [41]. The resulting fitting parameters were used to calculate α-slope [(µmol O_2 mg Chla^{-1} h^{-1}) (µmol photons m^{-2} s^{-1})$^{-1}$], P_{max} [µmol O_2 mg Chla^{-1} h^{-1}] and I_k [µmol photons m^{-2} s^{-1}].

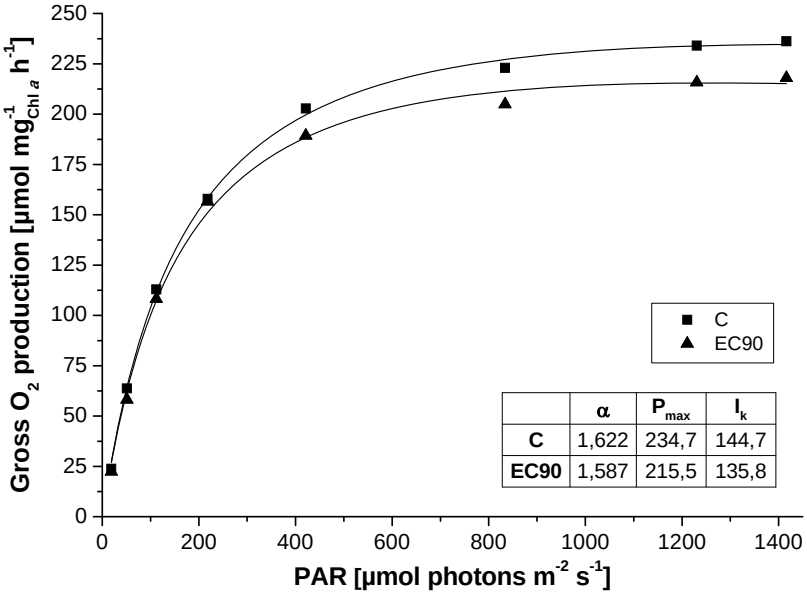

Figure 6 Gross O_2 production of *Microcystis aeruginosa* cells after exposure to PBQ EC90 concentrations in reference to photosynthetic active radiation (PAR). Curves were fitted according to Eilers and Peeters [41]. The resulting fitting parameters were used to calculate α-slope [(µmol O_2 mg Chl*a*$^{-1}$ h^{-1}) (µmol photons m^{-2} s^{-1})$^{-1}$], P_{max} [µmol O_2 mg Chl*a*$^{-1}$ h^{-1}] and I_k [µmol photons m^{-2} s^{-1}].

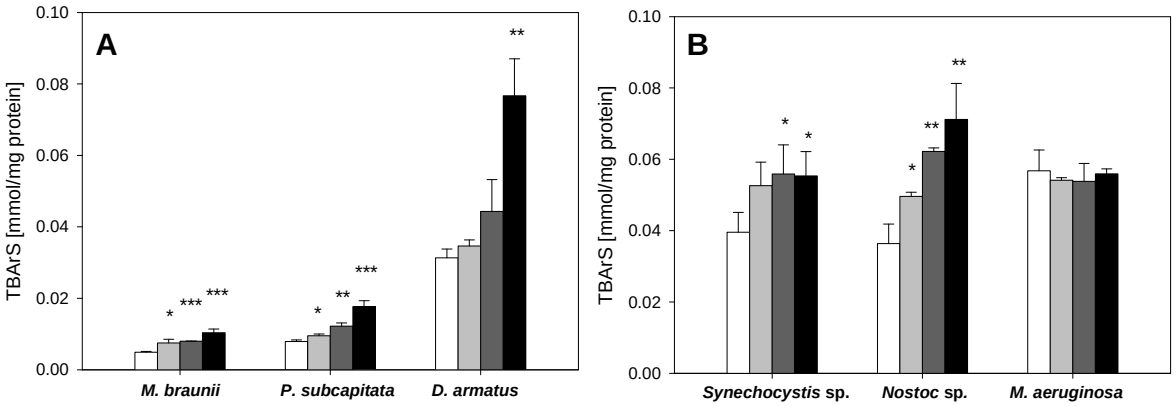

Figure 7 Thiobarbituric acid reactive substance (TBArS) of three tested green algal species **(A)** and 3 cyanobacterial species **(B)** after 24 h exposure and at different effective concentrations of PBQ: white = Control; light grey = EC10; dark grey = EC50; black = EC90.

However, the measurements of photosynthesis in *D. armatus* and *Synechocystis* sp. clearly showed that inhibition by PBQ was of similar magnitude to rETR and oxygen evolution. The slightly stronger reduction of the oxygen evolution in *Synechocystis* (Fig. 5) compared to the fluorescence-based rETR (Fig. 1) points to some portion of Mehler reaction or redox cycling by PBQ. The two latter indistinguishable processes are still detected as electron flow in rETR, but are not visible as net oxygen exchange. Hence, the rETR rate remains higher than the oxygen evolution rate. By contrast, *M. aeruginosa*, which was the most susceptible to PBQ by far, did not show a negative response in its photosynthetic performance, neither in

terms of rETR (Fig. 1) nor oxygen production (Fig. 6).

Differences in the reactive mechanism depending on the applied concentration have also been described for other benzoquinones such as 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). At concentrations below 10 µM, DBMIB inhibits electron transport at the $Cytb_6/f$ complex but at concentrations between 10-20 µM it acts as an efficient PS II Hill acceptor [32]. Hence, we conclude that in our experiments, PBQ acted at concentrations between 0.1 and 112 µM as a principal inhibitor in the photosynthetic electron transport chain, but its effect was reduced slowly within 24 h, probably due to detoxification. The exact mode of action for PBQ on photosynthetic rETR has been viewed critically by various research groups according to its chemical instability and anomalous behavior in cells and in vitro experiments [31,32]. Hence, PBQ was replaced by more stable *p*-benzoquinones such as 2,6-dimethyl-*p*-benzoquinone as a favored PSII Hill acceptor [31].

Various effects of PBQ on eukaryotic algae have been described. In *Scenedesmus quadricauda* (probably identical with *D. armatus* in the present study), PBQ began to be toxic above 55 µM after 96 h of exposure [51]. This was consistent with the EC90 value for PBQ for *D. armatus* (Table 1). After 48 h, PBQ at 30 µM induced no significant changes in oxygen evolution and CO_2 fixation, but there was a small depression in cell number in *Chlorella pyrenoidosa* [52]. These authors also found that PBQ stopped cell growth in *C. pyrenoidosa* which but did not result in cell destruction. Therefore, Zweig et al. [52] classified PBQ as "algistatic". In *P. subcapitata*, 90 µM of PBQ decreased ^{14}C-fixation by 90% [53]. These data agree well with our EC90 data. With an EC50 value of 83 µM (72h) for PBQ induced growth inhibition, the dinophyte *Prorocentrummicans* [54] appeared to be far less sensitive than the chlorococcal algae tested in the present study. Deleterious effects of PBQ on the plasmalemma, the thylakoid membrane and an uncoupling effect on rETR has also been described for unicellular algae [55,56]. Interestingly, the motility of some eukaryotic algae, such as *Dunaliellasalina*, *Euglena gracilis,* and *Chlamydomonas reinhardii,* was completely inhibited within 15 min by PBQ at concentrations of 30 µM, 20 µM and 10µM, respectively [57]. These authors also observed that the cytoplasmic stream of *Nitella* sp. was completely inhibited when 200 µM of PBQ were applied for 15 min. The rather fast immobilization of the motile algae described above indicates a direct effect of PBQ on energy metabolism and the cellular ATP resources.

PBQ and substituted benzoquinones can act either as arylating agents or as redox cycling compounds [58]. Some of the benzoquinones even show a mixed reactive behavior. The arylating activity is caused by covalent binding to macromolecules (protein, DNA) and enzymes via Michael addition [30]. Redox cycling of benzoquinones leads to the formation of ROS and to oxidative stress that can induce the oxidation of lipids, proteins, and DNA. Our results showed a PBQ-induced increase in TBArS, indicative of oxidative stress (Fig. 7). However, the oxidative mechanism for PBQ might be different from that of redox cycling: PBQ can react in a non-enzymatic reaction with glutathione (GSH) leading to a depletion of the cellular thiol pools [59,60]. GSH can be depleted either by conjugation or by oxidation to GSSG. Arylating benzoquinones which were covalently bound to GSH were more cytotoxic in rat hepatocytes than those inducing only oxidation to GSSG [61]. Most authors regard PBQ as a purely arylating benzoquinone and its redox cycling activity as negligible [58,60,62]. However, Bramble et al. [63] detected a slightly enhanced oxygen consumption by PBQ in mitochondrial and microsomal preparations, which they ascribed to redox cycling and ROS production, but not enhanced respiration. From the high activity of PBQ in GSH pool depletion, we conclude that oxidative stress is induced in our PBQ treatments most likely by the depletion of the antioxidant pool.

It is hard to identify a principal reaction mechanism of PBQ because it will most probably have multiple target sites in the cellular metabolism. Different cytotoxic and genotoxic effects for PBQ have been described [64]. PBQ inhibits not only photosynthetic electron transport, but also mitochondrial oxidative phosphorylation [58]. In the case of PBQ and other arylating benzoquinones, this effect is associated with a depletion of the ATP pool but not with a depolarization of the membrane potential as observed for redox cycling benzoquinones [58]. Furthermore, PBQ can also inhibit mitochondrial DNA replication [65]. Some authors have also described a PBQ-induced inhibition of trans-plasma electron transport [62]. Due to its high reactivity with SH-groups, PBQ also inactivates many enzymes [66,67]. In summary of the results presented here and the experimental data from the literature, the conclusion can be drawn that the cytotoxicity of PBQ is not due to a single mode of action but to a complex reactivity at different target sites in the cellular metabolism.

5. CONCLUSIONS

The evident inhibitory potential of *p*-benzoquinone results from various modes of action: one is direct interaction with photosynthesis - the acute short-term inhibition of relative electron transport in PSII, while the other is inhibition of growth and probably to a small extent oxidative stress. Furthermore, we proved the higher susceptibility of cyanobacterial species to PBQ: the concentration inhibiting 50% of growth was clearly lower in all tested prokaryotic phototrophs with *M. aeruginosa* being the most sensitive. Surprisingly, *M. aeruginosa* was the only species of the six tested phototrophs that did not adversely respond to PBQ in terms of photosynthetic electron transport rate, oxygen production and oxidative stress. Further investigation is necessary in order to show which kind of stress mechanism leads to growth inhibition in *Microcystis*. Whereas inhibition of photosynthetic electron transport is a transient short-term event, growth inhibition is retained over the whole time period of culture. Hence, different target sites are involved. In case of permanent growth inhibition, the depletion of the respiratory and cellular ATP pool by PBQ interaction is the most likely mechanism. Strong indications for this type of mechanism are the immobilization of motile algae by PBQ as described by [57] as well as the findings of Henry and Wallace [58]. Further investigation is required to thoroughly analyze the complex reaction pattern of PBQ with the respective phototrophs.

In the context of humic substances and related compounds and how they control the phototroph community in fresh waters, our present and former results [12-14] clearly show that monomeric quinones, formed by biodegradation and photolysis from higher molecular DOC, might play the key role in their distinctive reaction with eukaryotic and prokaryotic phototrophs. Future studies in humic waters should therefore aim to decipher the precise underlying mechanism that controls the phototroph community rather than extending the existing catalog on the bulk DOC phenomena.

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