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Effects of Carbamazepine on Two Microalgae Species Differing in Stress Resistance

Sandra M. Haase · Paweł Panas · Thomas Rath · Bernhard Huchzermeyer

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Abstract Carbamazepine (CBZ) is a representative of a group of compounds found in our rivers that have been classified as upcoming contaminants. Its pharmacological activity to treat mood and neurological disorders is based on its effects on ion channels, but effects on aquatic organisms have not yet been thoroughly investigated.

In our initial analysis, we compared CBZ effects on two microalgae species differing in CBZ sensitivity: *Parachlorella kessleri* and *Neochloris pseudoalveolaris.* While we observed a stimulation in the growth rate in cultures of *P. kessleri* in the presence of 10 μg L^{-1} CBZ, no effect on growth rates of *N. pseudoalveolaris* cultures could be documented at this concentration. Any higher tested CBZ concentration led to growth inhibition.

To gain insight into these effects, biochemical and physiological parameters of these two microalgae species were measured in the presence of CBZ in a concentration-dependent manner.

As the severe inhibition of growth rate correlated with a significant inhibition of most tested parameters in cultures of *N. pseudoalveolaris*, the primary reason for the adverse effect of CBZ on cultures of this microalgae species could not be identified. In cultures of *N. pseudoalveolaris*, experimental data indicate that inhibition of growth rate occurs when the microalgae are no longer able to compensate for adverse CBZ-induced ROS effects.

Analysis of the CBZ response of cultures of *P. kessleri* showed a reduction of growth stimulatory effect if the CBZ concentration exceeds a threshold value. In general, cultures of *P. kessleri* show a great potential to withstand CBZ as an environmental pollutant.

Keywords Carbamazepine · Upcoming contaminants · Microalgae · Photosynthesis

1 Introduction

Upcoming contaminants derive from anthropogenic activity. They are used as cosmetics, fertilizers, pesticides, feed additives, drugs, etc. Typically, they are delivered to river ecosystems via treated wastewater and have the potential to affect river ecosystems (Fent et al. 2006). While the acute toxicity of pharmaceuticals is often documented at concentrations higher than those found in the aquatic environment, chronic toxicity can already occur at significantly lower concentrations (Ferrari et al. 2004, Fent et al. 2006). Nevertheless, the database for

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chronic toxicity is deficient (Webb 2001). In most cases, the risk caused by an individual compound, as well as its mechanism of action, is poorly understood due to the cumulative effect of interaction with other compounds. However, considering that antibiotics and other compounds of pharmaceutical relevance are found in the group of upcoming contaminants, their accumulation in the ecosystem and entry into the food chain have to be prevented (Halling-Sørensen et al. 1998, Hirsch et al. 1999, Kümmerer 2003, Kümmerer 2009, Grote et al. 2006, Grote et al. 2007).

Generally, it has been agreed that a more detailed classification system is needed regarding these upcoming contaminants. Therefore, their mode of action as well as the capacity of river ecosystems to degrade these compounds has to be examined.

It is impossible to monitor the specific effects of each of these compounds in the environment. One feasible approach is to discriminate upcoming contaminants by physical and chemical features, as well as their risk potential for river ecosystems. This approach allows for the identification of model substances representing separate classes of contaminants. It appears to be feasible to analyze the behavior of individual model substances in place of a high and ever increasing number of upcoming contaminants (Stahlschmidt-Allner et al. 2014).

For our investigation, we have chosen carbamazepine (CBZ) as our model contaminant and have identified two microalgae species from the local river Leine, differing in their CBZ tolerance. This allows us to experimentally distinguish between general responses of algae to changes in the culture medium and toxic effects on algae metabolism and physiology. Limits of CBZ concentrations are not specified in surface waters. Ferrari et al. (2003) documented a predicted no-effect concentration (PNEC) of 0.42 μg L^{-1}.

It is convincingly documented in the literature that pre-adaptive mutations are occurring in microalgae populations ensuring survival of moderate stresses, provided the population size is sufficiently large (Baos et al. 2002, López-Rodas et al. 2006). Resistant phenotypes are maintained in populations even in the absence of stressors, such as environmental pollutants. In contrast, genetic plasticity (Lürling 2003, Peña-Castro et al. 2004) and epigenetic effects (López-Rodas et al. 2006) are not based on mutations. In populations, the physiological phenotype of plasticity, such as stress resistance, is strictly associated to the respective adverse environment, while a stress-induced epigenetic phenotype will persist.

Based on these facts, we can conclude that in laboratory experiments using algae species from the collection in Göttingen, pre-adaptive mutations resulted in improved stress tolerance. Algae collected from the river Leine had been exposed to CBZ but had to be cultivated in the absence of the contaminant to produce stock cultures of sufficient cell density. Thus, genetic plasticity may be excluded, while improved tolerance may be based on both, pre-adaptive mutations as well as epigenetic effects.

CBZ qualifies as a model compound due to (1) being one of the most frequently found upcoming contaminants in wastewater and (2) its poor degradability in common wastewater treatment plants (Ternes 1998, Zhang et al. 2008, Leclercq et al. 2009). It is an antiepileptic drug (Albani et al. 1995) but its high and continuously increasing market potential is based on its use for the treatment of (i) several central nervous system (CNS) disorders typically linked to age-related malfunctions such as bipolar disorder and neuralgia (Taylor et al. 1981), as well as (ii) attention-deficit hyperactivity disorder in children (Swanson et al. 1998). These disorders require prescriptions on a regular basis throughout the year. Thus, we may expect to find a constant level of CBZ contamination in river water. The analysis of such continuous data will be easier than the analysis of more seasonally dependent contaminations by products such as herbicides or antibiotics.

Eighty-seven tons of CBZ were administered in Germany in 1999 (Rohweder 2003). Thereof, 29 % is secreted unchanged via feces and urine, while the largest share (71 %) enters the water cycle as metabolites (Zhang et al. 2008). The most important metabolites are CBZ-diol and, to a lesser extent, CBZ-epoxide. The latter compound has been shown to possess similar anti-epileptic properties as CBZ and may cause neurotoxic effects (Semah et al. 1994, Rambeck et al. 1993) The CBZ-diol metabolite with the largest proportion of 30 % is not pharmaceutically active (Miao et al. 2005, Zhang et al. 2008).

This manuscript reports on a study investigating the physiological response of two microalgae species (*Parachlorella kessleri* and *Neochloris pseudoalveolaris*) to the pharmaceutical Carbamazepine. The aim was to investigate the effects of CBZ by measuring inhibition of reactions directly linked to the growth rate. At low concentrations typically found in most river ecosystems, CBZ

does not cause significant changes as it is summarized in Ferrari et al. 2003 and Schwaiger et al. 2004.

Chronic effects are very important to evaluate the toxicity of pharmaceuticals and their impact on the river ecosystem. Therefore, biochemical and physiological mechanisms chronic CBZ toxicity is based on have to be identified. We have tested higher CBZ concentrations far beyond those found in the local river water (0–1000 μg L^{-1}) to surmount non-specific effects and identify and evaluate possible targets of CBZ. Moreover, in view of basic research application of extremely high CBZ concentrations became necessary in order to identify toxic effects, the observed "CBZ threshold concentration" was based on in cultures of *P. kessleri*. This study may contribute to current understanding of the environmental impact of contaminants.

2 Materials and Methods

If not mentioned otherwise, all chemicals were purchased from Sigma Aldrich, Munich, and Roth, Karlsruhe, and were of highest purity available.

2.1 Collection of Microalgae

Samples of river water have been regularly collected at three defined locations: (a) at a pedestrian bridge in the recreation area upstream of the wastewater treatment plant of the City of Hanover; (b) at the discharge point of the wastewater treatment plant; (c) at a bridge about half a kilometer downstream of the discharge point. The water quality of the river Leine in the region of Hanover is ranked as quality grade II (Anonymous 2012). The river is moderately polluted due to nutrient input and a low flow rate. However, drug impacts on water bodies are not included in the official assessments of water quality. Such additional pollutions can be expected downstream of the wastewater treatment effluent. Microalgae species abundant in the samples were identified by direct phenotyping and subsequently cultured on agar containing tetracycline (0.01 mg L^{-1}) and Previcur (0.15 %) to reduce bacterial and fungal contaminations. Cultures were purified by repetitively picking isolated subcultures, which were used for individual stock cultures kept in 100 mL flasks, as well as on agar plates for continuous cultivation.

2.2 DNA Sequencing

Before starting our experiments, stock cultures were genotyped. The genomic DNA was extracted from algae pellets by a chloroform/isopropanol method. The cells were broken by shaking with ceramic beads. 500 μL DNA extraction buffer (200 mM TRIS HCL, pH 8.0; 240 mM NaCl; 25 mM EDTA, pH 8.0; 1 % SDS) was added and the samples were shortly vortexed before adding 75 μL chloroform. The mixture was shaken on a rotating shaker (IKA®, type VIBRAX VXR basic, Germany) for 10 min at 1000 rpm and chloroform and water phases were separated by centrifugation at 13,000 g for 10 min at room temperature. 75 μL of the supernatant were transferred to a 2 mL tube containing 75 μL cold isopropanol. The samples were again mixed and centrifuged at 13,000 g for 20 min at 4 °C. Afterwards, the supernatant was completely removed and the pellet was washed in 70 % ethanol. After drying, the pellet was resuspended in 75 μL distilled water.

Subsequently, a Phusion PCR reaction was performed using two primers for green microalgae (Sherwood and Presting 2007). The PCR samples (20 μl) included 1 μL of each primer, 2 μL DNA template (10–20 ng), 4 μL Phusion PCR buffer (5×), 0.4 μL dNTP, and 0.2 μL Phusion Hot Start Polymerase II. The PCR reaction was performed by a standard three-step program. Shortly, the samples were initial denatured at 98 °C for 30 s before a 35-fold cycle with a denaturation step at 98 °C for 10 s, an annealing step at 58 °C for 30 s, and an extension step at 72 °C 30 s was started. The final extension was performed at 72 °C for 10 min following retention at 4 °C.

After replication of the selected DNA sequences, the products were purified using MSB® Spin PCRapace Kit (Invitek, Germany) and pretreated in order to perform a TOPO-TA cloning (TOPO-TA Cloning Kit (Invitrogen, Germany)). The TOPO-TA cloning was based on incorporation of the PCR products into a vector, which is integrated into TOP10 cells (*E. coli*) by heat-shock transformation. By plating on 2 % LB agar medium (1 % tryptone; 0.5 % yeast; 0.5 % NaCl; pH 7.0) containing kanamycin (50 μg mL^{-1}), successfully transformed cells could be identified by their kanamycin resistance gene and reproduced by Colony-PCR. The Colony-PCR samples included 3 μL DNA, 0.5 μL of two M13 primer (M13f: 5′-GTA AAA CGA CGG CCA G-3′, M13r: 5′-CAG GAA ACA GCT ATG AC-3′), 0.5 μL dNTP, 2.5 μL Dream Taq buffer, and 0.15 μL Dream Taq Polymerase. The mixture was filled up with

distilled water to 25 μL. The thermal program included an initial denaturation step at 95 °C for 5 min and a 35-fold cycle with a denaturation step at 95 °C for 30 s, an annealing step at 58 °C for 30 s, and an extension step at 72 °C for 90 s, followed by a final extension at 72 °C for 5 min. Afterwards, the cells were cultivated on LB medium containing kanamycin (50 μg mL^{-1}) at 37 °C overnight.

A purification step was performed by a GeneJET Plasmid Miniprep Kit (Thermo Scientific, Germany) and the plasmid DNA was verified by gel electrophoresis (2 % (*w*/*v*) agarose). Finally, the sequence was determined (Seqlab, Germany) and compared to homologous sequences by the Basic Local Alignment Search Tool (BLAST).

The samples taken from these cultures were compared to reference cultures purchased from the Experimental Phycology and Culture Collection of Algae at the University of Göttingen (EPSAG). In this study, two microalgae species *P. kessleri* and *N. pseudoalveolaris* differing in stress resistance have been chosen.

2.3 Growth Conditions of the Algae

The agar medium for permanent cultures, as well as purification of algae cultures by dilution plating, contained 2 % agar dissolved in distilled water supplied with 2 % fertilizer solution (Max Bahr, universal fertilizer containing 0.2 % guano), 0.01 mg L^{-1} tetracycline, and 0.15 % Previcur. The fertilizer contained: 0.2 % guano, 7 % total nitrogen, 5 % phosphate, 6 % potassium oxide, 0.01 % boron, 0.02 % ferric, 0.01 % manganese, and 0.001 % molybdenum.

Suspension cultures were grown at 20 °C in a medium containing the aforementioned fertilizer solution dissolved in distilled water. All cultures were illuminated with white actinic light (120 μmol m^{-2} s^{-1}), as measured with a PAR meter (LI-COR Biosciences, Germany). Algae growth was monitored by measuring light scattering at 850 nm using a microplate spectrophotometer (BioTek, type EPOCH, Germany). The cell density of cultures was calculated from this data, using calibration curves individually established for each algae species by cell counting in a Thoma chamber.

In order to characterize CBZ-induced effects related to the photosynthesis, results from experiments performed in the presence of 0, 10, 100, and 1000 μg L^{-1} CBZ are shown.

2.4 Cell Extracts and Protein Content

Pellets from algae cultures were extracted by 4 min of shaking with ceramic beads at 30 s^{-1} in a Cell Mill (Retsch, type MM400, Germany). A 1.8 mL of 0.1 M sodium phosphate buffer (pH 7) containing 1 mM EDTA was added and the shaking step was repeated. Samples were centrifuged for 20 min at 15,000 g at 4 °C. Using the decomposition method with ceramic beads ensures that intracellular proteins were completely released (López et al. 2010). The protein content of the supernatant was measured as described by Lowry et al. (1951) using BSA as a reference.

2.5 Sugar Content of Cells

Sugar was measured according to Dubois et al. (1956) using glucose as a reference. A 2 mL algae pellet was treated successively with ethanol of different concentrations (80 %; 10 %). Two-hundred fifty microliters of the supernatant were mixed with the same amount of 5 % phenol and incubated for 2 min at room temperature. After incubation, 1.25 mL of concentrated sulfuric acid was added and mixed again. The samples were incubated at 80 °C for 10 min. The absorbance of samples then was read at 490 nm.

2.6 Pigment Content

For pigment analysis, algae cultures were centrifuged for 5 min at 8,700 g. After treatment with ceramic beads, the pellet was re-suspended in 2 mL 80 % acetone and the sample was mixed and kept in the dark at room temperature for a period of at least 1 h. Cell debris and denaturated proteins then were removed by 5 min centrifugation at 8700 g. The absorbance of the clear supernatant was measured at 663, 644, and 452.5 nm, respectively. Total chlorophyll content and total carotenoid content were calculated as described by Röbbelen (1957).

2.7 Photosynthetic Activity

Photosynthetic activity of algae suspensions was measured by monitoring light-induced oxygen release in a Clark type electrode (Hansatech, type Oxygraph 10002, Great Britain). Concentration of algae cells in standard culture medium was adjusted to 10^6 cells per mL. For illumination with red actinic light (120 μmol m^{-2} s^{-1}), a slide projector equipped with a RG-630 filter (Schott)

was used. Temperature of the electrode's reaction chamber was controlled with an accuracy of +/−0.2 °C by a water jacket supplied by a circulation water bath (Julabo, type F10, Germany).

2.8 Chlorophyll Fluorescence

Chlorophyll fluorescence was measured in 96-well plates using a Walz Imaging-Pam (pulse-amplitude modulation) fluorometer. Fluorescence parameters of dark adapted algae suspensions were used to analyze non-photochemical quenching (NPQ) using the formula (Fm-Fm`) $(Fm')^{-1}$ described by Bilger and Björkman (1990).

2.9 Lipid Peroxidation

Lipid peroxidation was measured as an indicator of ROS-mediated damage. The level of lipid peroxidation was estimated by measuring the concentration of malondialdehyde (MDA), a product of lipid peroxidation, by modifying the method from Heath and Packer (1968). Algae cells were harvested by centrifugation (5 min at 8700 g). The final pellet was suspended in a buffer medium (pH 7.4) consisting of 25 mM TRIS and 150 mM KCl and incubated at room temperature for 2 min. Then, an equal volume of 30 % trichloroacetic acid (TCA) was added and the suspension was centrifuged for 10 min at 2000 g. A sample of the supernatant was mixed with an equal volume of 0.75 % thiobarbituric acid dissolved in 20 % TCA and incubated for 20 min in a boiling water bath. After cooling, the absorbance of the samples was read at 532 nm. If the samples were turbid, the absorbance at 532 nm was corrected by subtracting the absorbance measured at 600 nm. For calculation, the millimolar absorbance coefficient ε_{532nm}=155 L $mmol^{-1}$ cm^{-1} was used.

2.10 Statistical Methods

All tests were performed with 3 biological and 5 technical replications. To investigate the cell density, 3 technical measurements were carried out.

The growth rate and its inflection point were calculated by fitting a 4 sigmoidal function with Sigma plot (Systat, Germany). Statistical analyses were performed using the STATISTICA program (StatSoft, Germany). Specifically, an analysis of variance (ANOVA) was used in conjunction with a Dunnett test after HSD calculation (α=0.05). Statistical information is always given with respect to the control (marked with an asterisk).

In order to achieve comparability of results, all data were compared at the inflection point or at the first day of measurement after a cultivation period of 3 days. This way, stress-related changes of growth rate were taken into consideration. The inflection point represents a suitable measurement point because the algae populations are homogeneous and exhibit their respective maximum growth rates under the chosen experimental conditions.

3 Results

The influence of CBZ on reactions directly linked to growth rate of the two microalgae species, *P. kessleri* and *N. pseudoalveolaris*, has been analyzed. The results are summarized in Tables 1 and 2.

3.1 Cell Density

The growth rates of the microalgae cultures treated with CBZ are shown in Tables 1 and 2. While the growth rate of *P. kessleri* was stimulated, growth rate of *N. pseudoalveolaris* was inhibited with increasing CBZ concentration. Growth curves were fitted 4-sigmoidal using Sigma plot (Systat, Germany).

3.2 Protein Content

CBZ affected the protein content of both algae cultures. In cultures of *N. pseudoalveolaris*, protein content decreased with increasing CBZ concentration (Fig. 1). No such clear response was found in cultures of *P. kessleri*. Obviously other factors than CBZ had a stronger effect on cellular protein content. Illumination time prior to harvesting of cells and light intensity (influenced by cell density) are known to have such effects (Reith and Cattolico 1985).

3.3 Sugar Content

Exposure to CBZ resulted in a significant reduction of sugar content in both algae species at most tested concentrations as compared to a control (Tables 1 and 2). A significant dip in sugar concentration was found in cultures of *P. kessleri* in the presence of 10 μg L^{-1} CBZ, i.e., the CBZ concentration inducing maximal growth rate. From comparison of all available data, it may be postulated that there is an inverse correlation of

Table 1 Carbamazepine effect on reactions directly linked to the growth rate of *Parachlorella kessleri*. Microalgae cultures have been cultivated under standard culture conditions using 4 CBZ concentrations in the range of 0–1000 μg L^{-1}

CBZ concentration μg L^{-1}	Growth rate 1 d^{-1}	Sugar content μg (mean ± SD)	Pigment content μg (mean ± SD)			O_2 production μmol O_2 m^{-2} s^{-1}	Malondialdehyde content mmol MDA L^{-1} (mean ± SD)
			chl_a	chl_b	Carotenoid		
0	0.45	9.21±0.65	0.19±0.03	0.06±0.02	0.05±0.01	3.38	11.0±1.92
10	0.76	5.87*±0.34	0.18±0.05	0.06±0.02	0.05±0.01	3.23	9.80±2.02
100	0.60	7.21*±0.54	0.38*±0.09	0.14*±0.05	0.09*±0.01	2.78	11.0±1.53
1000	0.69	7.82*±0.59	0.26*±0.03	0.08±0.02	0.07*±0.01	2.46	8.45*±1.82

Shown values are based on the respective inflection point and are indicated as average value of 10^6 cells (3 biological replications)
*Significances indicate comparisons to control treatment, Dunnett test, α=0.05

sugar concentration and growth rate in cultures of *P. kessleri*. No such relation was found in cultures of *N. pseudoalveolaris*.

3.4 Pigment Content

In cultures of *P. kessleri,* the addition of 100 and 1000 μg L^{-1} CBZ resulted in an increase in pigment concentration (chlorophyll a, carotenoids). For chlorophyll b, a significant increase was only found at 100 μg L^{-1} (Table 1). Conversely, a decrease in the average pigment content of chlorophyll a and b were found to be statistically significant in the presence of 1000 μg L^{-1} CBZ in cultures of *N. pseudoalveolaris* (Table 2).

3.5 Photosynthetic Activity

The effect of CBZ on the photosynthetic activity of both algae cultures is summarized in Tables 1 and 2. In both cultures, the photosynthetic activity decreased with increasing CBZ concentrations. Significant differences were not calculated because only 3 biological replicates were measured.

3.6 Chlorophyll Fluorescence

Chlorophyll fluorescence measurement was performed to assess CBZ effects on non-photochemical quenching. It was observed that in cultures of *P. kessleri*, there was no significant change in NPQ, while in cultures of *N. pseudoalveolaris*, NPQ decreased with increasing CBZ concentrations (Fig. 2).

3.7 Lipid Peroxidation

MDA was measured as an indicator of lipid peroxidation. A significant reduction in MDA was measured in both cultures at the highest CBZ concentration of 1000 μg L^{-1} (Tables 1 and 2).

Table 2 Carbamazepine effect on reactions directly linked to the growth rate of *Neochloris pseudoalveolaris*. Microalgae cultures have been cultivated under standard culture conditions using 4 CBZ concentrations in the range of 0–1000 μg L^{-1}

CBZ concentration μg L^{-1}	Growth rate 1 d^{-1}	Sugar content μg (mean ± SD)	Pigment content μg (mean ± SD)			O_2 production μmol O_2 m^{-2} s^{-1}	Malondialdehyde content mmol MDA L^{-1} (mean ± SD)
			chl_a	chl_b	Carotenoid		
0	0.31	5.45±0.23	0.66±0.03	0.17±0.02	0.24±0.02	3.50	27.5±6.44
10	0.31	4.40*±0.66	0.60±0.18	0.18±0.07	0.24±0.04	3.22	20.6*±3.88
100	0.23	7.87*±0.51	0.57±0.06	0.15±0.02	0.22*±0.02	3.59	27.2±4.86
1000	0.21	1.89*±0.91	0.53*±0.03	0.15±0.02	0.19*±0.01	2.98	20.6*±5.79

Shown values are based on the respective inflection point and are indicated as average value of 10^6 cells (3 biological replications)
*Significances indicate comparisons to control treatment, Dunnett test, α=0.05

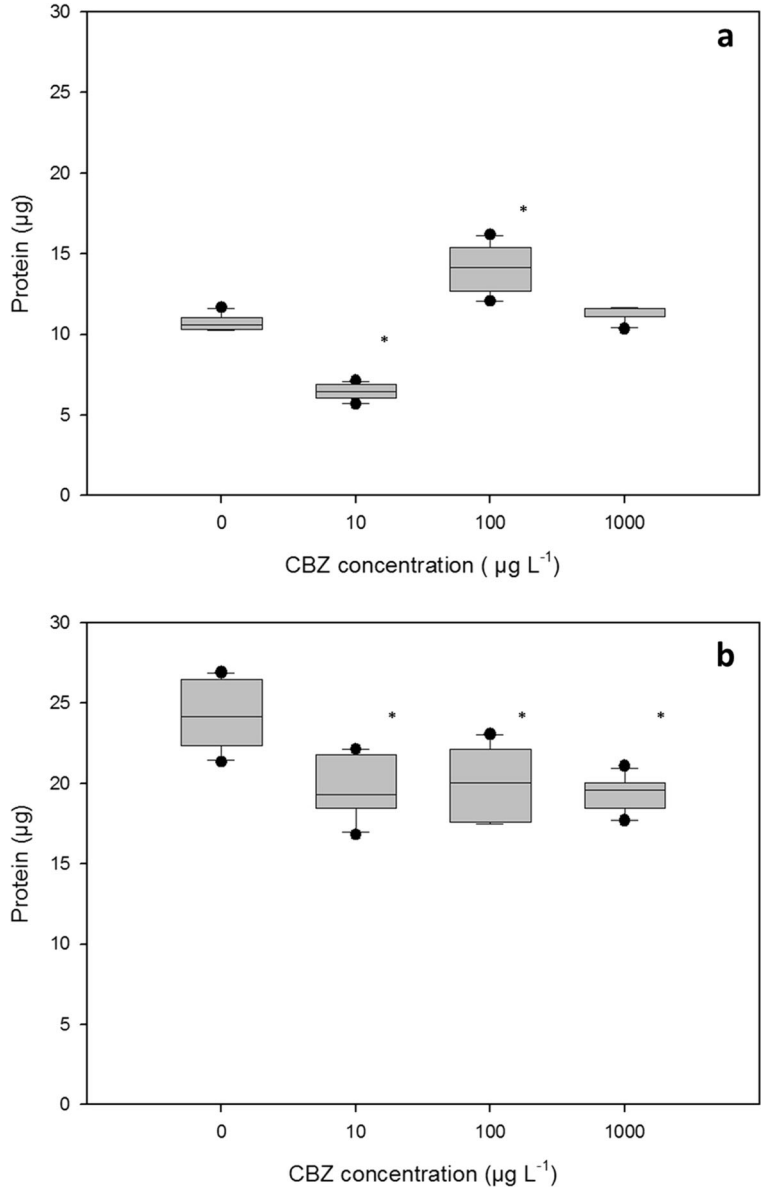

Fig. 1 Carbamazepine effect on the protein content of the microalgae *Parachlorella kessleri* (**a**) and *Neochloris pseudoalveolaris* (**b**) measured at the respective inflection point as average value of 10^6 cells (3 biological replications). (*Significances indicate comparisons to control treatment, Dunnett test, α=0.05)

4 Discussion

A high number of pharmaceutically effective substances are annually detected in biologically active concentrations in the water cycle, giving rise to public concern about possible effects on the environment and the quality of drinking water. In various studies, it has already been noted that pharmaceuticals and their metabolites may occur in wastewater in the range of ng L^{-1} to µg L^{-1} because of low removal capacity of wastewater treatment plants (Heberer 2002). It can be assumed that the desired effect in humans can also occur in aquatic organisms.

Due to its properties (a ubiquitous pharmaceutical compound, characterized by low acute toxicity and long solids retention time), CBZ can be considered as a model substance for persistent compounds, as CBZ has already been described as an anthropogenic marker in the aquatic environment (Clara et al. 2004). Therefore, results achieved may also be transferable to other persistent compounds of the same drug class. CBZ concentrations found in German surface waters are in

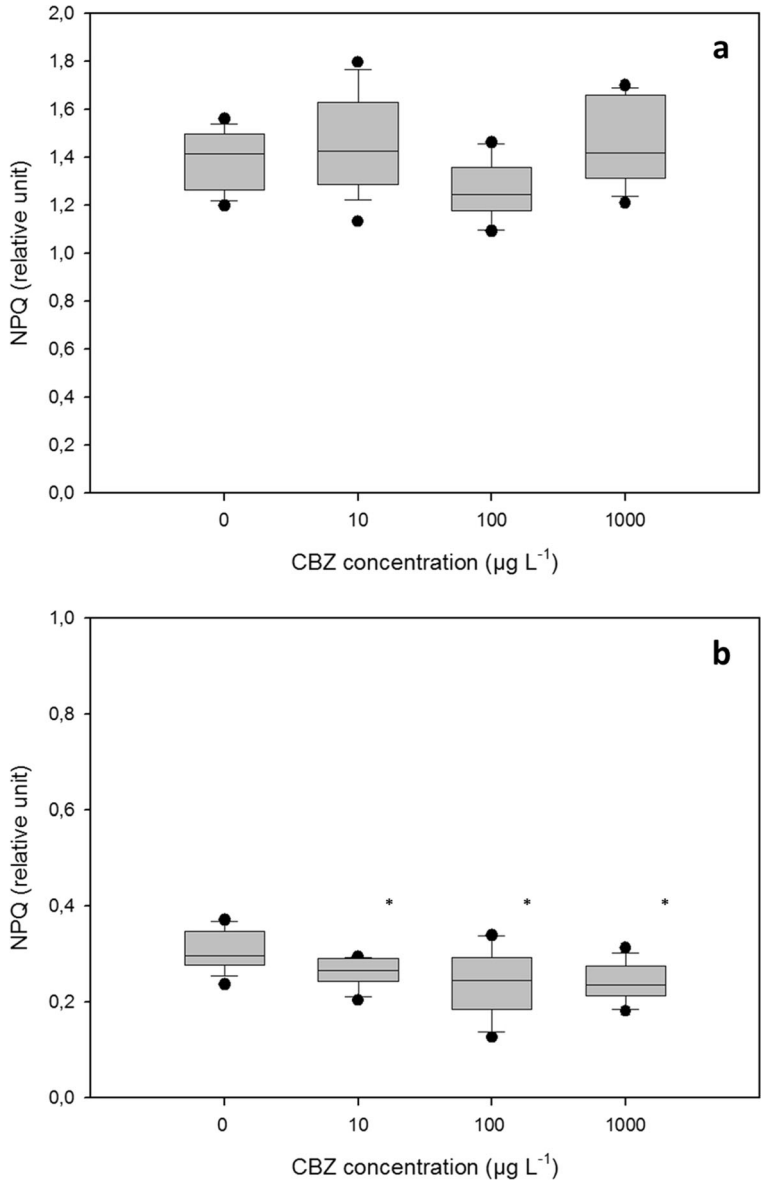

Fig. 2 Carbamazepine effect on chlorophyll fluorescence of the two microalgae species *Parachlorella kessleri* (**a**) and *Neochloris pseudoalveolaris* (**b**) exposed to indicated CBZ concentrations. The shown NPQ values have been determined at the respective inflection point of algae cultures as average value of 10^6 cells (3 biological replications). (*Significances indicate comparisons to control treatment, Dunnett test, α=0.05)

the range of 0.25 μg L^{-1} (Ternes 1998). These concentrations are definitively lower than the calculated PNEC value of 0.42 μg L^{-1} (Ferrari et al. 2003). In order to evaluate the spectrum of possible CBZ effects, microalgae from the river Leine were investigated after their exposure to CBZ concentrations higher than those found in the environment. In initial analysis of CBZ effects on growth, we observed that the population of *N. pseudoalveolaris* is more sensitive as compared to the one of *P. kessleri.* In the current investigation, we wanted to find out differences in CBZ response of the two microalgae species; i.e., we wanted to characterize the strategy allowing *P. kessleri* to be resistant to CBZ pollution of river water.

4.1 CBZ Effects on Algae Growth

In general, primary and secondary effects of contaminants can be discriminated. In our approach, we wanted to focus on inhibition of reactions directly linked to growth rate. In order to exclude from data analysis effects on cell development and differentiation, such as adaptation of algae cells to a new environment (the lag phase subsequent to inoculation of a culture medium), we have measured all

tested parameters at the inflection point of the respective growth curve. At this period of time, most of the algae cells of a population are growing at maximal rate.

In the present study, we have found that inhibition of growth rate of *N. pseudoalveolaris* definitively brought about by CBZ could not be detected up to CBZ concentrations which are 25 times higher than the PNEC value. But with respect to the tested concentration range, inhibition of growth rate continuously increased with increasing CBZ concentration (Table 2). On the other hand, a biphasic response of growth rate was found in experiments with *P. kessleri*. Up to a threshold CBZ concentration of 10 μg L^{-1} growth rate of this species was stimulated, while higher CBZ concentrations resulted in a reduction of this stimulating effect (Table 1). Such a biphasic response can be explained by the occurrence of two opposite effects differing in substrate (CBZ) affinity. While the high affinity response is stimulating growth rate, the low affinity effect results in irreversible inhibition of growth. Thus, CBZ-induced stimulation of growth rate is dominant at low CBZ concentrations, while at high CBZ concentrations, the stimulatory effect can no longer compensate for losses.

In subsequent experiments, we have investigated in more detail the different responses to CBZ contamination of the two algae species. We wanted to find out (i) the differences in CBZ response of the two algae species in the presence of the CBZ concentration resulting in maximal stimulation of the growth rate of *P. kessleri* and (ii) changes in the pattern of CBZ responses of *P. kessleri* cultures in the presence of higher CBZ concentrations, which caused a reduction in the stimulatory effect.

Most tested parameters were significantly inhibited in cultures of *N. pseudoalveolaris*, and inhibition increased with increasing concentrations of added CBZ. Our experimental approach did not allow sufficient time resolution of inhibitory events. Therefore, we could not identify a primary target of CBZ toxicity in experiments with this algae species. On the other hand, the biphasic responses of *P. kessleri* cultures prompted us to try and distinguish responses related to the stimulatory effect at low CBZ concentration and those related to inhibitory effects in presence of high CBZ concentrations, respectively.

4.2 CBZ Effects on Photosynthetic Sugar and Protein Synthesis

Two possible targets of CBZ could be the (i) sugar and (ii) protein synthesis. It is known from the literature that both parameters undergo circadian changes (Bläsing et al. 2005, Haydon et al. 2011). Most likely these changes are light-mediated, but changes in metabolite concentration during the cell cycle have been observed as well (Claustre and Gostan 1987). Due to the fact that different factors can interfere with sugar and protein analysis, significant experimental error may occur. Despite such experimental problems, a significant decrease in sugar and protein content with increasing CBZ concentrations was found in cultures of *N. pseudoalveolaris* (Table 2, Fig. 1). Observed effects correlate well with the respective degree of CBZ inhibition of growth rate of this algae species. In contrast, protein concentration of cultures of *P. kessleri* is apparently not affected by CBZ and, in concentration effect plots, a dip in total sugar content was found at 10 μg L^{-1} CBZ (Table 1, Fig. 1). The results indicate that sugar concentration is lowest when the highest rate of cell division is reached, and the internal sugar concentration of cells increases with a reduction in growth rate. From this observation, it may be concluded that in *P. kessleri*, (i) cell division is driven at the expense of consumption of free cytosolic sugars, (ii) in the presence of maximal growth rate, the rate of sugar being fed into the cytosolic pool is lower compared to the rate of sugar consumption, and (iii) at high concentrations CBZ affects the mechanism of cell division, rather than the one of sugar production. Thus, internal concentration of free sugar is increasing with decreasing growth rate in presence of extremely high CBZ concentrations.

4.3 Pigment Concentration, Photosynthetic Electron Transport Rate, and Energy Use Efficiency

Photosynthetic capacity correlates with absorption of light energy by chlorophylls. Therefore, the analysis of pigment patterns may provide some further insights. Again, both tested algae species reacted to CBZ addition in opposite ways (Tables 1 and 2); while chlorophyll a and carotenoid concentrations decreased with increasing CBZ concentrations in cultures of *N. pseudoalveolaris*, an increase was found in cultures of *P. kessleri*. As chlorophyll a is found in reaction centers and primary light harvesting complexes, a CBZ-induced inhibition of the maximal photosynthetic capacity of *N. pseudoalveolaris* can be postulated. On the other hand, an increase in absorption of photosynthetic light quanta can be expected for *P. kessleri*. Both expectations go in line with the aforementioned observations. Moreover, this interpretation was supported

when measuring CBZ effects on the light-induced oxygen evolution, i.e., photosynthetic electron transport rate of cultures of *N. pseudoalveolaris*. The observed inhibition nicely correlates with a reduction of chlorophyll a concentration (Tables 1 and 2). However, the result of the same experiment with cultures of *P. kessleri* was a surprise: an addition of any CBZ concentration resulted in the inhibition of photosynthetic electron transport rate. This indicates that all CBZ concentrations resulted in a reduction of light energy use efficiency, despite the stimulating effects of low CBZ concentrations on pigment content in this algae species.

We decided to investigate this observation in more detail. The expectation was that an inhibition of the electron transport rate will result in overreduction of the reaction center of photosystem II. It is well documented in the literature that light-activated chlorophyll can transfer an electron to molecular oxygen to form an oxygen radical (Dubois et al. 1956, Vranova et al. 2002). Therefore, if absorbed light energy will not be consumed by electron transport activity, it can be expected that CBZ-mediated inhibition of electron transport will stimulate alternative reactions, such as ROS production (Melis 1999). This assumption agrees with current literature; it is known that CBZ induces oxidative stress in algae (Tsiaka et al. 2013). But in algae, just like in higher plants, various energy-consuming pathways exist. Photosynthetic electron transport, chlorophyll fluorescence, and the release of heat energy are competing for energy from activated chlorophyll. Moreover, heat release can be regulated, as futile reaction sequences exist in addition to direct heat release from activated chlorophyll (Melis 2009, Johnson et al. 2009, Johnson et al. 2012). Any of these reactions will lead to a reduction in chlorophyll fluorescence intensity. Activity of these energy-consuming reactions can be estimated from chlorophyll fluorescence measurements in terms of chlorophyll fluorescence quenching. In order to better understand the observed CBZ effects, we have analyzed non-photochemical quenching (NPQ). As NPQ consists of 3 components (qE, qI, and qT), qE as energy-dependent quenching constitutes the largest proportion (Müller et al. 2001, Krause and Jahns 2010). In higher plants, qE correlates with the activity of the xanthophyll cycle (Havaux et al. 2007, Dall'Osto et al. 2012). In Fig. 2, results of NPQ calculations in the presence of different CBZ concentrations are shown. A continuous decrease in NPQ with increasing CBZ concentrations was observed in cultures of *N. pseudoalveolaris.* On the other hand, no significant effects of CBZ could be found in cultures of *P. kessleri*.

It is likely that ROS production and control mechanisms regulating cellular ROS content, as well as ROS functions and cellular responses to high ROS concentrations, are similar in higher plants and algae. ROS are continuously produced as metabolic by-products and they function as key regulators of pathways localized in chloroplasts, mitochondria, and peroxisomes (Apel and Hirt 2004, Liu et al. 2007, Kim et al. 2009, Gill and Tuteja 2010). The equilibrium, at a cellular level, can be influenced by different stress factors (Gill and Tuteja 2010). If ROS production rate exceeds the intracellular detoxification capacity, toxicity will eventually result in cell death (Ledford and Niyogi 2005). At an early state of ROS toxicity, damage of the photosynthetic apparatus (Dewez et al. 2005), lipid peroxidation (Gill and Tuteja 2010), and DNA damage (Valko et al. 2006) can be observed. We took advantage of previous experience and measured malondialdehyde concentration as an indicator of lipid peroxidation. However, we found that no meaningful results could be achieved in experiments with both algae species (Tables 1 and 2). In the presence of the highest tested CBZ concentration a significant reduction in malondialdehyde content was found. As it is known that glutathione peroxidase (GPX) is active in detoxifying the lipid peroxidation product (Vera-Estrella et al. 1992, Eshdat et al. 1997), this result may indicate that CBZ is inducing ROS scavenging activity. Compared to the growth rate at highest CBZ concentration, it can be concluded that cultures of *P. kessleri* are able to compensate for ROS stress in contrast to *N. pseudoalveolaris.* In cultures of *N. pseudoalveolaris*, a correlation between ROS production and inhibition of growth can be suggested.

Cultures of *P. kessleri* show great potential to withstand CBZ as an environmental pollutant. As microalgae represent the basis of the food chain of aquatic organisms, changes in populations will have a major impact on ecosystems (Carvalho et al. 2004). Since it is assumed that pollution of the environment will continue to increase due to pharmaceutical residues, algae species with the ability to tolerate oxidative stress by controlling intracellular ROS levels will have an important role in the environment and ecosystem in the future (Gill and Tuteja 2010).

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