Growth, toxicity and oxidative stress of a cultured cyanobacterium (*Dolichospermum* sp.) under different CO$_2$/pH and temperature conditions

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SUMMARY

Cyanobacteria blooms are a worldwide nuisance in fresh, brackish and marine waters. Changing environmental conditions due to upwelling, changed mixing conditions or climate change are likely to influence cyanobacteria growth and toxicity. In this study, the response of the toxic cyanobacterium *Dolichospermum* sp. to lowered pH (−0.4 units by adding CO$_2$) and elevated temperature (+4°C) in an experimental set-up was examined. Growth rate, microcystin concentration and oxidative stress were measured. The growth rate and intracellular toxin concentration increased significantly as a response to temperature. When *Dolichospermum* was exposed to the combination of elevated temperature and high CO$_2$/low pH, lipid peroxidation increased and antioxidant levels decreased. Microcystin concentrations were significantly correlated with growth rates. Our results show, although oxidative stress increases when exposed to a combination of high CO$_2$/low pH and high temperature, that growth and toxicity increase at high temperature, suggesting that the cyanobacterium in general seems to be fairly tolerant to changes in pH and temperature. Further progress in identifying biological responses and predicting climate change consequences in estuaries experiencing cyanobacteria blooms requires a better understanding of the interplay between stressors such as pH and temperature.

Key words: cyanobacteria, growth rates, microcystin, oxidative status, pH levels, warming.

INTRODUCTION

The ongoing global climate change caused by combustion of fossil fuels and the following carbon dioxide (CO$_2$) emissions result in warming of the atmosphere and seawater. If emissions continue at the current speed, a 2–4.5°C rise is most likely to occur (IPCC 2013). In addition, up-welling events and changed mixing conditions can expose plankton to high CO$_2$/low pH water (Feely *et al.* 2008; Barry *et al.* 2011). Estuaries and coastal areas in particular have a unique status in the future: they will be important natural laboratories concerning ecosystem changes caused by increased upwelling, fuelled by ocean acidification (Feely *et al.* 2010; Wootton & Pfister 2012). The present pH of the ocean surface has already declined by 0.1 pH units compared with pre-industrial levels, and is predicted to continue decreasing (Caldeira & Wickett 2003; IPCC 2007). Therefore, the combined dynamics of CO$_2$/pH and temperature is more relevant than changes in a single factor.

In this context, of immediate interest are the responses of nuisance species, such as toxic cyanobacteria that today pose environmental problems in many aquatic ecosystems, including the Baltic Sea (O'Neil *et al.* 2012). For example, warming and low pH (31°C/900 µatm *p*CO$_2$) caused a significant increase in growth rates of the oceanic cyanobacterium *Trichodesmium* spp. (Levitan *et al.* 2010), consistent with results in previous studies (Barcelos e Ramos *et al.* 2007; Hutchins *et al.* 2007; Levitan *et al.* 2007). Similar responses have been measured for the toxic cyanobacterium *Nodularia spumigena* Mertens (Wannicke *et al.* 2012; Endres *et al.* 2013; Unger *et al.* 2013).

Although genetic and/or phenotypic adaptations to low pH alone, or in combination with other factors, have been investigated for most algal groups including chlorophytes (Collins & Bell 2004), coccolithophores (Feng *et al.* 2008), diatoms (Sun *et al.* 2011; Kremp *et al.* 2012), dinoflagellates (Fu *et al.* 2008, 2010), raphidophytes (Fu *et al.* 2007), and cyanobacteria (Fu *et al.* 2007; Levitan *et al.* 2010), little is known about oxidative stress responses in algae during warming and low pH. Reactive oxygen species (ROS) are produced as by-products during photosynthetic electron transport and metabolism in photosynthetic organisms (Apel & Hirt 2004). Primary producers, such as higher plants and algae, are constantly exposed to oxidative stress from changes in light and other environmental factors. If the cells exposed to oxidative stress are not able to handle it, a loss of protein function, membrane integrity and eventually cell death can result (Monaghan *et al.* 2009). To counteract or prevent damage by ROS, microalgae, including cyanobacteria, need an anti-oxidative mechanism. The knowledge of the ecological role of oxidative stress and antioxidants in cyanobacteria is limited (Latifi *et al.* 2009; Zilliges *et al.* 2011), and there is

a need to include these measurements when studying cyanobacteria responses to environmental factors.

The aim of the current study is to measure growth, toxicity and oxidative stress of a cultured cyanobacterium under different CO_2/pH and temperature conditions. The study area of the Baltic Sea is a shallow semi-enclosed brackish water basin, with a restricted connection to the North Sea. Due to high anthropogenic nutrient load from the land, the Baltic Sea is at present highly eutrophicated (Fleming-Lehtinen *et al.* 2008). The pollution is manifested for instance as annual cyanobacteria mass-occurrences covering large areas consisting mainly of three taxa: *Aphanizomenon* sp., *Dolichospermum* spp. and *Nodularia spumigena*. *N. spumigena* is a well-known toxin producer that affects aquatic and terrestrial organisms, and may pose a health hazard for humans (Sellner 1997; Mazur-Marzec & Pliński 2009). *Aphanizomenon* sp. and *Dolichospermum* spp. are known to produce toxins in freshwaters (Sivonen & Jones 1999) and more recently, *Dolichospermum* has proven to be hepatotoxic in the Baltic Sea, producing microcystins (Halinen *et al.* 2007; Fewer *et al.* 2009). In this experimental study, we mimicked a cyanobacteria bloom and monitored how increased CO_2/lowered pH and elevated temperature, as single factors and combined, affect growth, intracellular toxin concentrations and the oxidative status of the cyanobacterium *Dolichospermum* sp.

MATERIALS AND METHODS

Cultivation of the strain

The *Dolichospermum* sp. BIR257 strain (previously *Anabaena* sp., Wacklin *et al.* 2009) originates from the Gulf of Finland (Baltic Sea) and is a microcystin-producer (Halinen *et al.* 2007). The culture was grown as batch monocultures in Z8 medium (Kótai 1972) without nitrogen at 18°C in 13.7 µmol photons $m^{-2} s^{-1}$ in a 16:8 h light: dark cycle. Even though the strain was not axenic, aseptic techniques were always used when handling the culture.

Sampling and experimental set-up

We used a factorial (2 × 2) experimental design with four treatments: (i) Control: 17°C and normal pH, (ii) Low pH: low pH (−0.4 units) and 17°C, (iii) High temp: 21°C and normal pH, and (iv) Low pH + high temp: low pH (−0.4 units) and 21°C (Table 1). Normal pH is defined as pH in filtered seawater at 17°C. Water from 5 m depth was collected from a nearby pelagic sea area, located at the south-western coast of Finland, in the vicinity of Tvärminne Zoological Station (59°50′ N, 23°15′ E, University of Helsinki). The treatments were prepared by first filtering the seawater (salinity 5.5) using Sartobran 300 filters (<0.2 µm; Sartorius Stedim Biotech GmbH, Göttingen, Germany). As filtration can shift the carbonate chemistry of the seawater, CO_2 bubbling should take place after filtration (Riebesell *et al.* 2010). Then the filtered seawater was pre-conditioned to reach the experimental temperatures (17 and 21°C). Subsequently, the treatments with low pH were supplemented with CO_2 gas until pH had decreased by ~0.4 units (TUNZE pH controller 7070/2, Penzberg, Germany) according to predictions for 2100 (Caldeira & Wickett 2003; IPCC 2007). The low pH used corresponds to the approximate expected pH change given as a general prediction for up-welling water (Feely *et al.* 2008). Finally, filtered seawater was mixed with *Dolichospermum* sp. culture (target concentration: 100 µg C L^{-1}). To avoid nutrient limitation during incubations, phosphate, nitrate (Table 2), vitamins and minerals were added in surplus, following the Guillard (1975) recipe. pH and temperature were measured from each bottle prior to closing the lid (9 replicates treatment^{-1}). 1.2 L Duran clear airtight glass bottles with Melamin resin screw caps with polytetrafluoroethylene seals were used and attached to plankton wheels (1 rpm) in climate chambers with respective temperatures. Light intensity was 18 µmol photons $m^{-2} s^{-1}$ at the top of the plankton wheels. Samples for dissolved nutrients, particulate organic carbon (POC), oxidative stress biomarkers and intracellular toxins were collected at the start. Three replicate samples per treatment (Control, high CO_2/low pH, high temp, high CO_2/low pH + high temp) for each variable were collected directly after mixing. These start samples were taken before the treatment water was filled into bottles. In order to evaluate potential differences between treatments, one set of replicates was sacrificed and samples were collected after 8 days of incubation (data not shown). Since no difference was detected, the experiment was prolonged. After 11 days of incubation clear differences in biomass were observed between treatments and the experiment was terminated. End samples were taken on

Table 1. Changes in pH and dissolved inorganic carbon (DIC) in the units with and without *Dolichospermum* sp. pH is reported as minimum to maximum range

	Without cyanobacteria		With cyanobacteria	
	Start	End	Start	End
pH				
Control	7.8–7.9	7.7–7.8	7.6–7.8	8.3–8.8
High CO_2/low pH	7.5–7.5	7.5–7.5	7.4–7.5	8.0–8.7
High temp	7.8–7.9	7.8–7.8	7.8–7.9	9.0–9.3
High CO_2/low pH + high temp	7.5–7.5	7.5–7.5	7.5–7.6	8.9–9.2
DIC				
Control	1.8 ± 0.0	1.8 ± 0.0	1.9 ± 0.0	1.7 ± 0.1
High CO_2/low pH	1.9 ± 0.0	1.9 ± 0.0	1.9 ± 0.0	1.7 ± 0.1
High temp	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.5 ± 0.1
High CO_2/low pH + high temp	1.9 ± 0.0	1.9 ± 0.0	1.8 ± 0.0	1.5 ± 0.0

DIC (mmol L^{-1}) values are mean ± SD.

Table 2. Dissolved inorganic phosphate (PO_4^{3-}), nitrite + nitrate ($NO_2^- + NO_3^-$) and ammonium (NH_4^+) from each of the four pH and temperature conditions for units with and without *Dolichospermum* sp.

	PO_4^{3-}	$NO_2^- + NO_3^-$	NH_4^+
Without cyanobacteria			
Start	n.m.	n.m.	n.m.
Control	9.2 ± 0.0	51.3 ± 2.1	0.9 ± 0.2
High CO_2/low pH	9.0 ± 0.0	50.9 ± 3.3	0.9 ± 0.5
High temp	9.1 ± 0.0	49.8 ± 0.8	1.2 ± 0.2
High CO_2/low pH + high temp	9.0 ± 0.1	51.0 ± 1.1	1.2 ± 0.1
With cyanobacteria			
Start	12.7 ± 0.2	50.1 ± 1.7	0.4 ± 0.1
Control	10.5 ± 0.5	52.4 ± 2.0	0.3 ± 0.1
High CO_2/low pH	10.6 ± 0.4	51.6 ± 1.4	0.2 ± 0.1
High temp	8.9 ± 0.3	53.1 ± 2.2	0.2 ± 0.1
High CO_2/low pH + high temp	8.9 ± 0.3	52.2 ± 1.6	0.2 ± 0.2

Start values are based on 4 replicates. All *Dolichospermum* sp. end samples are an average of 9 replicates ± SD, whereas controls are an average of 3 replicates ± SD. All concentrations are expressed as µM. n.m., not measured.

day 11 from all remaining nine replicate bottles of each treatment.

Biomass and growth

Cyanobacterial biomass was assayed using POC, by filtering cells onto pre-combusted filters (Whatman GF/C, 4 h, 450°C, Little Chalfont, United Kingdom). The filters were dried for at least 24 h at 60°C and stored dry in a desiccator until analysis. POC was determined with a mass spectrometer (Elemental Combustion System CHNS-O 4010; Valencia, CA, United States), as described in Grasshoff (1976). Growth was calculated as $(\ln POC_2 - \ln POC_1)/(t_2 - t_1)$ where POC_2 and POC_1 are the measured POC at time t_2 and t_1 (between day 0 and day 11).

Toxin analysis

Intracellular microcystin levels were sampled by filtering the cultures onto a GF/F filter. All samples were frozen in −20°C until further processing, according to instructions in Engström-Öst *et al.* (2011). Intracellular microcystin concentrations (µg L^{-1}) were analyzed with enzyme-linked immunoabsorbant assay, using a microcystin plate kit (EnviroLogix; Portland, ME, USA), according to kit instructions. A negative control and a standard curve were measured accordingly. The toxin concentration was determined by dual reading from the absorbance at 450 nm and as reference 655 nm, measured with a photometer (Tecan infinite M200, Tecan Group Ltd, Männedorf, Switzerland), using Magellan software. Data are reported as microcystin-LR equivalents.

Dissolved nutrients, inorganic carbon and pH

pH (pH-controller 7070/2, TUNZE) and temperature measurements were performed from each bottle at the start and at the end of the experiment (Table 1). After the dissolved inorganic carbon (DIC) measurement, pH was measured once more with pH meter Jenway (3510, calibrated with three buffers, Staffordshire, United Kingdom), when samples had

reached room temperature. DIC was measured according to Salonen (1981) from samples preserved in airtight bottles on ice (+3°C) and stored in darkness max 24 h until analysis (Table 3). Based on the measured DIC and the dissociation constant equations provided by Lueker *et al.* (2000), the concentrations of the three DIC forms (CO_2, HCO_3^-, CO_3^{2-}) were calculated according to Zeebe and Wolf-Gladrow (2001) (Table 3). Samples for dissolved inorganic phosphate, ammonium and nitrite + nitrate were analyzed according to Grasshoff (1976) (Table 2).

Oxidative status biomarkers

Oxidative status of the cyanobacteria was determined as intracellular soluble antioxidant capacity assayed by oxygen radical absorption capacity (ORAC) and lipid peroxidation, assayed as thiobarbituric acid reactive substances (TBARS). Samples were collected onto GF/C filters and were stored frozen in −80°C until analysis. Each filter with cyanobacteria was homogenized in 1 mL of phosphate-buffered saline (PBS) for 4 min using FastPrep (Santa Ana, CA, USA) with cooling function and 100 µm ceramic beads, centrifuged at 10 000 *g* for 5 min at 4°C and supernatant was used to measure ORAC, TBARS and protein concentration. Then, supernatant were used for ORAC assay, 200 µL for the lipid peroxidation assay and 50 µL for protein determination.

Oxygen radical absorption capacity was measured in 200 µL of the supernatant using oxidation of a fluorescent probe by peroxyl radicals by way of a hydrogen atom transfer process (Cao & Prior 1999; Prior *et al.* 2003) and a microplate reader (FLUOROstar OPTIMA, BMG Labtech, Germany) set to 485 nm excitation/520 nm emission. The procedures were based on the modified $ORAC_{FL}$ method (Prior *et al.* 2003) using fluorescein (FL) as a fluorescent probe, 2,2′-Azobis (2-amidinopropane) dihydrochloride (AAPH) as a peroxyl radical generator, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water soluble vitamin E) as a standard (6 to 50 µM range). The final ORAC values were

Table 3. Contribution of different dissolved inorganic carbon forms; carbonate (CO_3^{2-}), bicarbonate (HCO_3^-), and carbon dioxide (CO_2) given as $mmol\ L^{-1}$

| | CO_3^{2-} | | HCO_3^- | | CO_2 | |
	Start	End	Start	End	Start	End
Without cyanobacteria						
Control	0.04 ± 0.00	0.03 ± 0.00	1.74 ± 0.00	1.75 ± 0.02	0.03 ± 0.00	0.04 ± 0.00
High CO_2/low pH	0.02 ± 0.00	0.02 ± 0.00	1.78 ± 0.02	1.77 ± 0.02	0.07 ± 0.00	0.07 ± 0.00
High temp	0.05 ± 0.01	0.04 ± 0.00	1.72 ± 0.01	1.73 ± 0.01	0.03 ± 0.00	0.03 ± 0.00
High CO_2/low pH + high temp	0.02 ± 0.00	0.02 ± 0.00	1.80 ± 0.01	1.82 ± 0.01	0.07 ± 0.00	0.07 ± 0.00
With cyanobacteria						
Control	0.03 ± 0.00	0.24 ± 0.06	1.78 ± 0.02	1.41 ± 0.12	0.04 ± 0.00	0.00 ± 0.00
High CO_2/low pH	0.02 ± 0.00	0.16 ± 0.06	1.79 ± 0.02	1.56 ± 0.11	0.09 ± 0.00	0.01 ± 0.01
High temp	0.05 ± 0.00	0.50 ± 0.06	1.69 ± 0.01	0.97 ± 0.09	0.03 ± 0.00	0.00 ± 0.00
High CO_2/low pH + high temp	0.02 ± 0.00	0.47 ± 0.06	1.74 ± 0.02	1.05 ± 0.09	0.06 ± 0.00	0.00 ± 0.00

Values are shown units with and without *Dolichospermum* sp.

calculated using a linear regression between the concentration of Trolox standards and the net area under the FL decay curve for the standards. $ORAC_{FL}$ values were expressed as µM trolox equivalent.

The lipid peroxidation assay was carried out using QuantiChrom TBARS Assay Kit (DTBA-100; BioAssay Systems, Hayward, CA, USA) as specified by the manufacturer with minor modifications. Standards were prepared by mixing 15 µL of the 1.5 mM malondialdehyde (MDA) with 735 µL dH_2O and diluting to set a range 0 to 30 µM MDA. To the 200 µL of the supernatant, 400 µL of trichloroacetic acid (TCA, 10%) were added, incubated on ice for 15 min and centrifuged at 14 000 *g* for 15 min at 4°C. Two 200 µL aliquots, one for sample and one for individual blank, were transferred to microcentrifuge tubes, and 200 µL of the TBA Reagent were added to all tubes designated for samples and standards. Individual blanks were prepared by replacing the TBA Reagent with 3 mM HCl (pH 1.6). All tubes were then incubated at 100°C for 60 min. After cooling down to room temperature, samples and standards were loaded in duplicate to wells of a black flat bottom microplate (100 µL well^{-1}) and fluorescence was measured with the FLUOstar Optima microplate reader at excitation/emission wavelengths of 544/590 nm. The TBARS levels were expressed as nmol MDA per mg protein.

Water soluble protein concentration (µg mL^{-1}) was measured with the bicinchoninic acid assay (Pierce, Rockford, IL, USA) with bovine serum albumin as standard following directions of the manufacturer for microtiter assays. In brief, 50 µL of the supernatant were mixed with 250 µL test kit reagents and incubated for 1 h at room temperature. The microplates were read at 550 nm using FLUOstar Optima microplate reader with absorbance configuration.

Statistical analyses

A two-way analysis of variance (ANOVA) was used to test for differences between pH (normal and low) and temperature (normal and elevated) treatments and their interaction. Levene's test was used to assess homogeneity of variance. Spearman's rank correlation analysis was used to assess the relationship between toxin concentrations and growth rates and partial correlation analysis to assess the relationship between ORAC and toxin concentrations. These statistical analyses were performed using SPSS 15.0 (SPSS, Chicago, IL, USA).

RESULTS

The growth rates of the cyanobacterium varied between 0.06 g POC day^{-1} in high CO_2/low pH and 0.11 g POC day^{-1} in high CO_2/low pH + high temperature (Fig. 1a). Intracellular toxin concentrations varied between 3.6 mg g^{-1} in high CO_2/low pH and 6.7 mg g^{-1} in high temperature (Fig. 1b). ORAC was lowest in the control (105.2 µM trolox equivalents), and highest in the high temp (132.2 µM trolox equivalents) (Fig. 1c). Lipid peroxidation (measured as TBARS) varied between 0.64 mmol MDA mg protein^{-1} in the high temperature and 0.78 mmol MDA mg protein^{-1} in high CO_2/low pH + high temperature (Fig. 1d).

Growth rate was significantly positively affected by temperature, but not by CO_2/pH (Fig. 1a, Table 4). Intracellular microcystin concentrations per unit biomass were significantly positively affected by temperature (Fig. 1b, Table 4), but not by CO_2/pH. There was no interaction effect of CO_2/pH and temperature on microcystin concentrations (Fig. 1b, Table 4). Toxin concentrations were also significantly positively correlated with growth rates (Spearman's, R = 0.601, n = 36, P < 0.001). There were some initial extracellular microcystin present, 3.1 ± 0.5 µg L^{-1}, but as the experiment progressed, the extracellular microcystin disappeared and was close to detection (data not shown).

Temperature, CO_2/pH and their interaction affected the oxidative status of the cells. ORAC responded positively to elevated temperature and CO_2/pH (Fig. 1c, Table 4). There was also an interaction effect of temperature and CO_2/pH on ORAC. In addition, there was a non-significant correlation between ORAC and toxin concentrations, with POC as a fixed variable (Partial correlation, R = 0.173, n = 33, P > 0.05). TBARS was higher in high CO_2/low pH treatments (Fig. 1d). Moreover, there was a significant CO_2/pH × temperature interaction (Table 4), suggesting increased lipid peroxidation in cells exposed to both increased CO_2/lowered pH and elevated temperature.
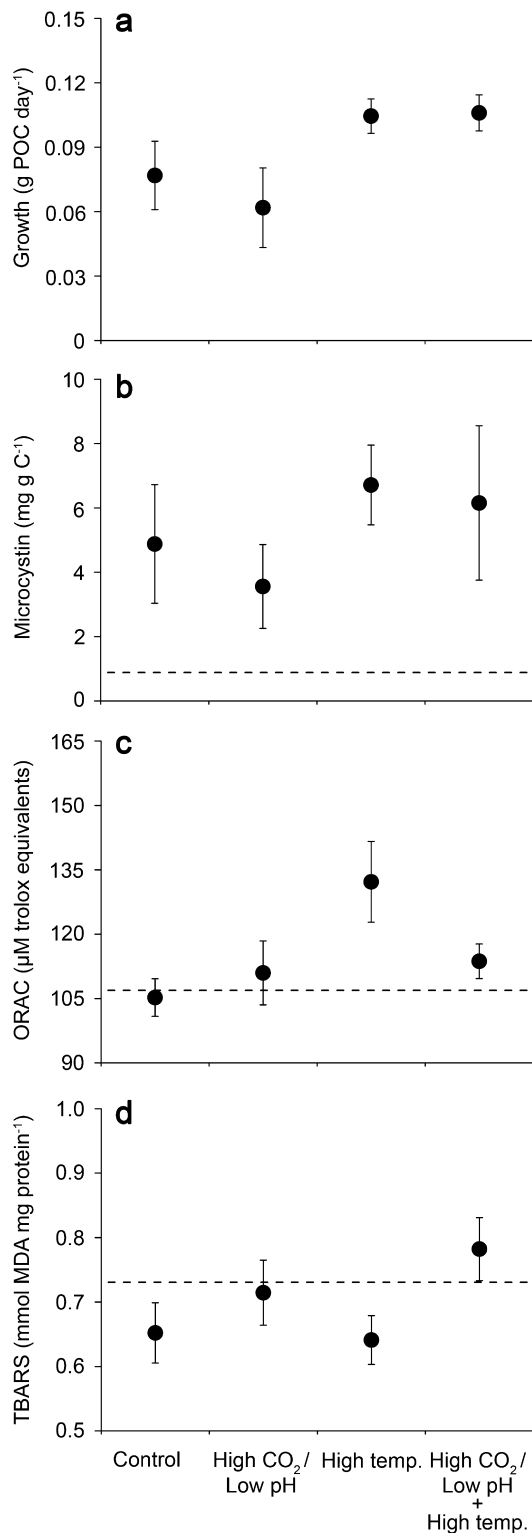
Fig. 1. Response of the cyanobacterium *Dolichospermum* sp. to high CO_2/lowered pH (CO_2/pH) and elevated temperature. (a) growth, (b) carbon normalized intracellular microcystin levels, (c) oxygen radical absorbance capacity (ORAC), and (d) lipid peroxidation, which was measured as thiobarbituric acid reactive substances (TBARS), at the end of the 11-day experiment. Mean ± SD, $n = 9$. Dashed lines represent average initial levels.

Table 4. Statistical results of two-way ANOVA, including F-values, P-values and degrees of freedom, assessing effects of temperature and CO_2/pH

	$F_{1,32}$	P
Growth		
Temp	74.768	<0.001
CO_2/pH	0.25	0.876
Temp × CO_2/pH	1.651	0.208
IC		
Temp	46.615	0.001
CO_2/pH	7.308	0.132
Temp × CO_2/pH	1.576	0.478
ORAC		
Temp	44.195	<0.001
CO_2/pH	8.186	0.007
Temp × CO_2/pH	29.526	<0.001
TBARS		
Temp	3.369	0.076
CO_2/pH	43.380	<0.001
Temp × CO_2/pH	6.530	0.016

IC, intracellular microcystin concentration; ORAC, oxygen radical absorption capacity; TBARS, lipid peroxidation.

DISCUSSION

Both growth rates and microcystin concentrations were significantly positively affected by temperature, regardless of CO_2/pH. These results are supported by previous studies; Fu *et al.* (2007) reported higher cell division rates of picocyanobacteria *Synechococcus* in higher temperatures (+4°C). Similar results are shown also for other cyanobacteria: *Microcystis aeruginosa* Kützing (Imai *et al.* 2009; Bouchard & Purdie 2011; Dziallas & Grossart 2011), *Nodularia spumigena* (Lehtimäki *et al.* 1997) and *Anabaena* sp. currently *Dolichospermum*; Wagner & Adrian 2009; O'Neil *et al.* 2012). Increased growth due to warming is common among cyanobacteria (reviewed by O'Neil *et al.* 2012). Paerl and Scott (2010) suggest that higher temperatures will be a catalyst for future expanding cyanobacteria blooms in a warmer climate. Cyanobacteria generally exhibit optimal growth rates at relatively high temperatures and they also compete most effectively with eukaryotic primary producers at elevated temperatures (reviewed by Paerl & Huisman 2009).

Microcystin concentrations were significantly correlated with growth and this is supported in the literature (Orr & Jones 1998; Long *et al.* 2001). In the current study, microcystin concentrations and growth both increased significantly as a response to elevated temperature. Higher temperature may enhance cyanobacterial toxicity by stimulating toxin biosynthesis (El-Shehawy *et al.* 2011), but this process seems to be associated with growth. Dziallas and Grossart (2011) showed that the toxicity and also the toxic potential (i.e., the ratio of toxic vs. non-toxic cells) of *Microcystis aeruginosa* increased considerably in higher temperature. Further, a toxin-producing strain is more tolerant to oxidative stress than a non-toxic one (Dziallas & Grossart 2011), and it has been suggested that toxins protect the cell against stressors (Zilliges *et al.* 2011).

Effects of pH on cyanobacteria are also studied intensively and results commonly show increasing growth in systems with

continuous input of CO_2 (Hutchins *et al*. 2007; Levitan *et al*. 2010; Wannicke *et al*. 2012), with some exceptions (Czerny *et al*. 2009). In the current study, a single initial CO_2 addition and resulting change in pH did not change cyanobacterial growth significantly. Working with live, actively growing and CO_2 consuming phytoplankton in batch cultures, such as the *Dolichospermum* sp. culture in the current study, the initial CO_2 treatment is prone to changes, which was an expected outcome. We consider such a change realistic during a typical cyanobacteria bloom in the Baltic since the high production may quickly result in exhaustion of free CO_2 whereby pH may reach well beyond 9 (Hansen 2002; Brutemark *et al*. 2011). Thus, the difference between initial and end pH values reflect the growth of the cyanobacterium, similar to conditions prevailing during a cyanobacteria bloom in the Baltic Sea. Consequently, the high CO_2 demand of an algal bloom may result in carbon limitation. In our experiment, CO_2 depletion was evident in all treatments; and initial addition of CO_2 had no effect on growth, suggesting that *Dolichospermum* sp. was not carbon limited. Although not measured in the present study, it appears that *Dolichospermum* sp. is able to utilize HCO_3^- and does not become carbon limited when CO_2 is diminished, a feature common among cyanobacteria (Kaplan *et al*. 1991; So & Espie 2005). In addition, dissolved nutrients (primarily nitrate and phosphate) were available in ample amounts throughout the experiment in all treatments, and the cyanobacterium was therefore not nutrient limited. CO_2/pH did not significantly affect toxin concentrations in the current study, and only a few studies show the rise of toxin concentrations by increasing external CO_2 concentration (dinoflagellates, Fu *et al*. 2010; diatoms, Tatters *et al*. 2012).

Apart from influencing the water chemistry, pH can also have a direct effect on the cells by affecting metabolism and membrane transport, and thereby impairing cellular function or increasing cellular energy demand (Hinga 2002). Although CO_2/pH in our study did not have an effect on growth, it did influence both antioxidant levels and lipid peroxidation. High temperature alone was beneficial as evidenced by high antioxidant levels. In contrast, when high temperature was combined with high CO_2/low pH, the anti-oxidative level was most likely weakened by increased ROS production, which resulted in significant 20% lipid peroxidation increase (measured as TBARS). In cyanobacteria, respiration and photosynthesis are considered to be sources of ROS production (Latifi *et al*. 2009). When the balance between oxidant level and antioxidant production is lost, the organism faces oxidative stress that can generate damage on cell level (Latifi *et al*. 2009). Cell death due to oxidative stress is shown in *Microcystis aeruginosa* (Ross *et al*. 2006), and is suggested to be a major cause of bloom ending, shown in *Trichodesmium* (Berman-Frank *et al*. 2004). In our experiment *Dolichospermum* growth did not seem to be limited by carbon and we therefore suggest that pH, in combination with warming, have an oxidative effect on the cyanobacteria cells (Fig. 1). Both CO_2/pH and temperature are stressors that can cause changes in the oxidative status of a photosynthetic organism (Bouchard & Purdie 2011). Bouchard and Purdie (2011) and references cited therein (Jochem 1999; Wu *et al*. 2008) suggest that cyanobacteria in general are tolerant of stress conditions as they are able to reduce their metabolic activity.

The role of microcystin has been debated, and recently it was suggested microcystin may act as defense against oxidative stress (Alexova *et al*. 2011; Dziallas & Grossart 2011; Phelan & Downing 2011). Consequently, we analyzed potential relationships between intracellular microcystin concentrations and ORAC, but as both were correlated to biomass, there was a risk of false relationship, and partial correlation analysis revealed that this was the case. Thus, the intracellular microcystin levels and ORAC are not directly associated, and there is, seemingly no relationship between cellular microcystin content and the oxidative status of the cells shown in the current study. However, to evaluate the response of *Dolichospermum* sp. to changes in CO_2/pH and temperature we subjected the cyanobacterium to two CO_2/pH levels (normal and high CO_2/lowered pH) and two temperatures (normal and high) corresponding to CO_2/pH changes that can occur in upwelling areas (Feely *et al*. 2008) as well as corresponding to temperature changes that are predicted for 2100 (Caldeira & Wickett 2003; IPCC 2007). The biological responses highlighted in our study may certainly differ in the presence of additional stressors and other cyanobacteria species/strains, but nonetheless, warming seems to benefit growth, toxicity, and antioxidant levels of *Dolichospermum* sp. When the cyanobacterium is exposed to the combination of elevated temperature and high CO_2/lowered pH, it creates an imbalance between antioxidant levels and lipid peroxidation, indicating a shift of oxidative changes at cell level, while no simultaneous changes occurred in growth or toxicity. To conclude, it is suggested that escalating growth and toxicity of bloom-forming cyanobacteria as a response to warming have serious socio-economic implications (Paerl & Otten 2013), especially in heavily polluted basins such as the Baltic Sea.

ACKNOWLEDGMENTS

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