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Effects of O_2 on N_2 fixation in heterocystous cyanobacteria from the Baltic Sea

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ABSTRACT: The effect of O_2 on nitrogenase activity in natural samples of heterocystous cyanobacteria from the Baltic Sea was studied using on-line laser photo-acoustic trace-gas detection. This technique records nitrogenase activity in near real-time and allows measurements in continuously changing O_2 concentrations. Our results showed that under non-steady state conditions the optimum concentration of O_2 for N_2 fixation differed from that at steady-state O_2 levels. The optimum O_2 concentration depended upon whether the O_2 concentration was increasing or decreasing, with decreasing concentrations yielding higher O_2 optima for dark nitrogenase activity than increasing O_2 concentrations. The cyanobacteria rapidly adapt to changes in O_2, and therefore measurements also reflect the history of O_2 concentrations to which organisms have been exposed. Steady-state and non-steady-state O_2 concentrations both decreased their optimum O_2 concentration for nitrogenase activity rates with increasing irradiance. However, the optimum O_2 concentration was always higher than zero, even at saturating irradiances. Hence, it appears that low levels of O_2 are an obligatory requirement for maximum nitrogenase activity in the field. Low levels of respiration served as a source of additional energy, suggesting that even at light saturation, photosynthetic energy generation in the heterocyst can not saturate the demand of nitrogenase for ATP. The large changes in nitrogenase activity due to the combined effect of variations in O_2 concentration and light emphasize the necessity of including these effects in models that calculate the daily integral of N_2 fixation.

KEY WORDS: Heterocystous cyanobacteria · Nitrogen fixation · Oxygen · Light-response · Laser photo acoustics · Baltic Sea · Cyanobacteria blooms

INTRODUCTION

Cyanobacteria comprise a diverse group of oxygenic photoautotrophic organisms, and many representatives are capable of fixing atmospheric N_2, allowing them to become independent of a supply of combined nitrogen, which often limits the proliferation of algae in natural waters. Diazotrophic heterocystous cyanobacteria form massive algal blooms in lakes and brackish waters. The brackish Baltic Sea is particularly well known for the development of cyanobacterial blooms composed of the filamentous heterocystous species *Nodularia spumigena*, *Aphanizomenon* spp. and, to a lesser extent, *Anabaena* spp. Many heterocystous bloom-forming cyanobacteria occur as aggregates (or colonies) and contain gas vesicles that make them buoyant. On calm days, these cyanobacteria float to the water surface, where they form dense surface aggregations (Walsby et al. 1997).

Nitrogenase, the enzyme-complex responsible for the reduction of N_2 to NH_3, is extremely sensitive to oxygen. In order to provide an anoxic environment for nitrogenase, heterocystous cyanobacteria differentiate special cells, heterocysts, that are the sites of N_2 fixation (Fay 1992). When grown under N_2-fixing conditions, about 1 to 10% of the cells are heterocysts. The heterocysts are distributed at (semi) regular distances along the trichome, usually with 1 heterocyst every 8 to 12 cells.

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Although heterocysts contain light-harvesting pigments, these are present at much lower concentrations than in the vegetative cells, and thus the heterocysts are pale in appearance (Fay 1970, Peterson et al. 1981, Thomas 1972). Heterocysts lack the water-splitting and O_2-evolving Photosystem II, but are capable of Photosystem I-mediated photosynthesis. Hence, heterocysts are able to convert light into biochemical energy (Scherer et al. 1988a). The heterocysts are enveloped by a thick lipopolysaccharide cell wall; this comprises an effective gas-diffusion barrier (Wolk et al. 1994), and limits the diffusion of O_2 into the heterocysts (Walsby 1985). A low O_2 flux into the heterocyst and a high respiratory activity causes a virtually anoxic internal environment, an obligatory requirement for N_2 fixation.

Heterocysts depend on oxygenic phototrophic, CO_2-fixing vegetative cells for a supply of the reducing equivalents necessary for N_2 fixation and respiration. Reducing equivalents are imported as carbohydrate, most probably sucrose (Böhme 1998, Curatti et al. 2002). N_2 fixation is an extremly energy-demanding process. The reduction of 1 N_2 molecule to 2 NH_3 molecules involves 8 low-potential electrons, mostly in the form of reduced ferredoxin, and at least 16 molecules of ATP (Miller et al. 1993). Nitrogenase diverts 2 electrons to the reduction of 2 protons to H_2, an obligatory side-product of nitrogenase activity. In addition to these direct energetic costs, the assimilation of NH_3 into amino acids also requires energy. Moreover, when NH_3 is not immediately assimilated, it may diffuse out of the cells and must then be taken up by a high-affinity transporter. This futile cycle may also represent a considerable loss of energy (Kleiner 1985).

Respiration and Photosystem I share a number of enzymes of the respiratory electron transport chain in the thylakoid membranes of the heterocyst, and Photosystem I is able to reduce nitrogenase via ferredoxin (Scherer et al. 1988a). Hence, there is competition for reducing equivalents between nitrogenase and the cytochrome oxidase. Some of the available reducing equivalents in the heterocyst are used to scavenge O_2 by respiration, and therefore provide a source of energy. At air-saturation levels, O_2 can support up to 50% of the maximum rate of nitrogenase by aerobic respiration (Staal et al. 2002). Photosystem I-mediated energy generation supplies the rest of the ATP necessary to achieve maximum nitrogenase activity, and this explains the light-dependence of N_2 fixation in heterocystous cyanobacteria (Staal et al. 2002).

The vegetative cells of cyanobacteria store glycogen as a storage carbohydrate that is used to sustain growth and metabolism in the dark. The mobilization of glycogen in the vegetative cells also allows N_2 fixation for prolonged periods in the dark.

To enable N_2 fixation, carbohydrate has to be transported from the vegetative cells to the heterocyst. Carbohydrate transport proceeds at a constant rate, until the supply is exhausted (Fredriksson et al. 1998, Evans et al. 2000). It is not known whether carbohydrates transport occurs throughout the whole 24 h period or only during the light period. In the latter case, heterocysts should be capable of maintaining their own store of carbohydrate.

The heterocyst provides an optimal environment for nitrogenase and protects it efficiently from O_2 inactivation. Nevertheless, O_2 has pronounced effects on N_2 fixation in heterocystous cyanobacteria. In the natural environment, heterocystous cyanobacteria may experience strong fluctuations in O_2 concentrations. For instance, in the light, surface accumulations of bloom-forming cyanobacteria aggregates and their surroundings may become supersaturated with O_2 (Paerl & Bebout 1988, Ibelings & Mur 1992), while in the dark respiration can deplete O_2 (Ploug et al. 1997). Moreover, fluctuating irradiances will cause rapid changes in O_2 concentration. It is not known how N_2-fixing cyanobacteria react to such situations and how rapidly changing O_2 concentrations affect the amount of N_2 fixed. Until recently, such experiments could not be performed successfully because suitable methodology was lacking. We have developed an on-line and near real-time method for the measurement of nitrogenase activity based on the acetylene reduction assay (ARA; Staal et al. 2002). Using this method, rapid changes in the gas atmosphere can be effected through automated mass-flow controllers. The set-up can be installed in a field laboratory or on board a research vessel. We have applied this approach to studying the effect of O_2 and fluctuations in pO_2 on nitrogenase activity in natural samples of a cyanobacterial bloom in the Baltic Sea dominated by 2 heterocystous cyanobacteria, *Nodularia spumigena* and *Aphanizomenon* sp.

MATERIALS AND METHODS

Sampling locations. The experiments were carried out during a research cruise in the Baltic Sea, in June 1999, on board the RV 'Valdivia'. Samples were taken in the Baltic Proper at the stations shown in Fig. 1. During the sampling period, the weather was calm and the water column was stratified, with a thermocline at 9 m depth. At each station, the ship followed a drifting buoy connected to a water anchor that was put in place at 06:00 h, assuring sampling in the same water body during that day. Based on microscopic observations, we estimated that *Nodularia spumigena* and *Aphanizomenon* sp. accounted for 5 to 15 and >80% of the phytoplankton biomass (>100 µm) in the sample,

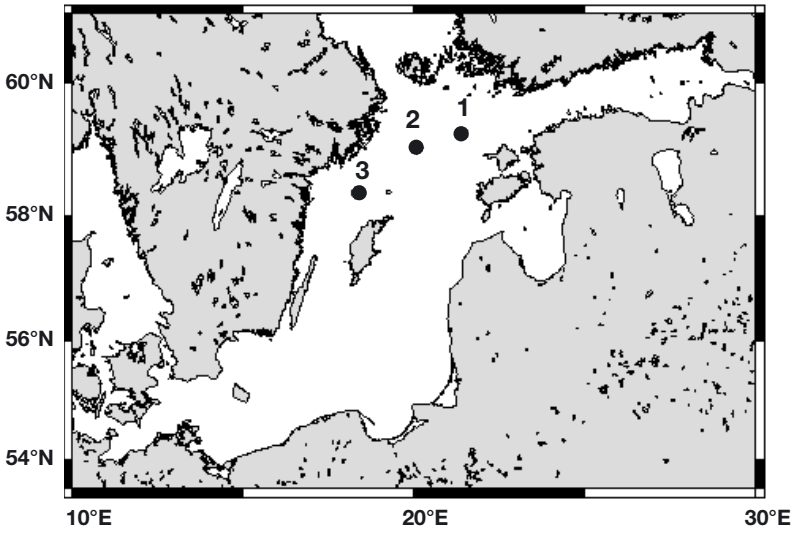

Fig. 1. Sampling Stations 1 to 3 in Baltic Sea at which nitrogenase activity, O_2 concentrations and irradiance were recorded from natural heterocystous cyanobacteria bloom (mainly *Nodularia spumigena* and *Amphizomeno* spp.)

respectively; *Anabaena* sp. was present in small concentrations (<2%). Few other phytoplankton organisms were present. Most of the material occurred as aggregates.

Sampling techniques. Samples were taken with a 100 µm plankton net during several vertical hauls through the water column above the thermocline (9 m). Depending on the biomass present and the method used for detection (laser-based trace-gas detection or gas chromatography; see later subsection), 2.5 to 20 ml of each sample was filtered on GF-F glass-fibre filters (Whatmann, ∅ 47 mm). The filters were immediately transferred to the on-line incubation cell, and measurements were typically started within 10 min of the samples being filtered. Care was taken to maintain the sample at the same temperature — the incubator was kept at the ambient seawater temperature (12 to 13°C).

Cultures. A non-axenic culture of *Nodularia spumigena* was obtained from P. K. Hayes (Bristol University, UK). The organism was isolated from the Baltic Sea and grown in a mixture of 1 part of artificial seawater medium (ASN3°) and 2 parts of freshwater medium (BG11°) (Rippka et al. 1979). The salinity of this mixture was ~9‰, corresponding to the salinity of the water from which the organism was isolated. The medium did not contain a source of combined nitrogen. Cultures were grown in 100 ml Erlenmeyer flasks under continuous light (60 µmol photons m^{-2} s^{-1}) at 20°C in a shaking incubator (Sanyo-Gallenkamp).

On-line, near real-time measurement of acetylene reduction. Nitrogenase activity was measured using the acetylene reduction assay (ARA). Ethylene was detected either with laser-based trace-gas detection (LPA) or with conventional gas chromatography (GC). The laser-based trace-gas detection system has been described in detail by te Lintel Hekkert et al. (1998). In the present study we used a mobile version of the LPA that can be taken on board a research vessel. LPA was used when a high sampling frequency was required, e.g. for experiments studying the effect of dynamic O_2 gradients on nitrogenase activity. The detection limit of ethylene using the mobile LPA was 0.2–0.5 ppb during calm weather decreasing to 0.5–2 ppb in rough weather (>8 m s^{-1}).

Gas chromatography detection of ethylene was used for measurements at steady-state O_2 concentrations. The gas chromatograph was a Shimadzu GC14A equipped with an in-line sample loop (1 ml) for automatic injection. The detector was a flame-ionization detector (FID). The conditions for the gas chromatograph were as follows. The temperatures of injector, detector and oven were set at 90, 120 and 55°C, respectively. The carrier gas used was He (99.999%) at a flow rate of 10 ml min^{-1}. The supply of H_2 (99.999%) and air (clinical air) for the FID were 30 and 300 ml min^{-1}, respectively. He, H_2 and air were obtained from Hoek-Loos. The column used was a 25 m long wide-bore silica-fused column (0.53 mm inner diameter) packed with Porapak U (Varian-Chrompack). The gas chromatograph was run at its highest sensitivity, made possible by using high-purity gases. The detection limit for ethylene with the gas chromatograph was 15 to 25 ppb.

For measurements with both LPA and GC, the cyanobacteria were immobilized on a GF-F filter and placed in the thermostated gas flow-through incubator (dead volume 3 ml). The incubator has been described in detail by Staal et al. (2001). A slide projector (Leica, 250 W halogen lamp) served as light source. The slide projector contained a series of neutral-density filters (Balzers), allowing illumination of the cyanobacteria at known photon irradiances. The incubator was equipped with a gas-mixing system composed of electronic mass-flow controllers as described by Staal et al. (2001). The gases used were O_2 and N_2, each with 400 ppm CO_2 (Hoek-Loos) and analytical acetylene C_2H_2 (99.6%, Messer-Griesheim). The flow rates of the gas mixture were 1 and 2 l h^{-1} for gas chromatography detection and the LPA set-up, respectively. The gas mixtures always contained 10% C_2H_2.

Oxygen gradients. To create gradual changes in O_2 concentrations, a mixing flask (1.3 l) was placed immediately before the incubation cell. At the start of the experiment, the initial O_2 concentration in the gas flow was set to its final value. This flow was fed into the

mixing flask, so that the O_2 concentration was continuously diluted, according to:

$$C_t = C_f(1-e-tD)+C_0e-tD \quad (1)$$

where C_0, C_f and C_t are the initial and final concentrations and the concentration of O_2 at Time t, respectively, *and* D is the dilution rate, determined by $D = f/v$, where f = the flow rate (l h^{-1}) and v = the dilution volume (l). (For an example of a gradual change in O_2 concentration and the response of the acetylene reduction rate see Fig. 2.)

A Clark-type (polarographic) mini-electrode (Type 730, Diamond), connected to an O_2 monitor (Strath Kelvin) continuously monitored O_2 concentrations in the gas flow. The O_2 electrode was positioned in the gas line immediately before the photo-acoustic cell in the LPA set-up or before the sample loop in the gas chromatograph. In the latter case, a chart recorder was used for recording the O_2 signal. In the LPA set-up, O_2 concentration was recorded simultaneously with each measurement of ethylene using an analog/digital (A/D) converter card.

Dynamic changes in oxygen concentrations. Dynamic changes in O_2 concentrations were carried out in the dark and at photon irradiances of 30 and 400 µmol m^{-2} s^{-1}. Under each of these conditions, an experiment with a decreasing O_2 concentration (from 30 to 0% O_2) was first carried out, immediately followed by an increase in O_2 concentration from 0 to 30%. The whole set of measurements (increasing and decreasing O_2 concentration in the dark and at the 2 light intensities) was performed with the same sample of cyanobacteria (i.e. the same GF-F filter) and lasted approximately 4 h. To determine whether the cyanobacteria were affected by a treatment, the sequence of irradiances was changed several times. The results were the same, indicating that the effects of a treatment were not carried over to subsequent experiments.

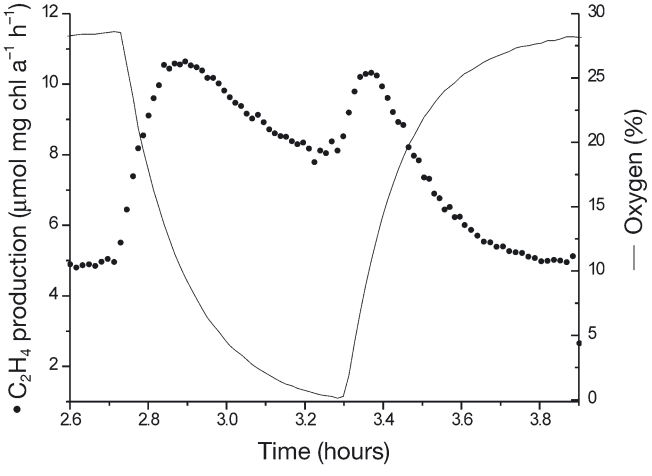

Fig. 2. Acetylene reduction and O_2 concentration plotted against time during dynamic regimes of increasing and decreasing O_2 concentration (Stn 2, 18:30 h). Acetylene reduction measured using laser-based trace-gas detection set-up (see 'Materials and methods'); O_2 measured with polarographic electrode placed in gas tube close to photo-acoustic cell

Steady-state O_2 concentrations. Light-response curves were recorded for 1 sample at different but constant levels of 20, 10, 5, 2.5 and 0% O_2, respectively. Each light-response curve consisted of measurements at 9 different photon irradiances, increasing exponentially from 0 to 400 µmol m^{-2} s^{-1}. Measurement of each light-response curve, required 45 min. After each light-response curve, the system automatically switched to the next programmed O_2 concentration. The O_2 concentration in the incubation chamber stabilized within 5 min after the mass-flow controllers had changed the gas mixture. Following the change in O_2 concentration, a dark incubation of 15 min was programmed to allow the sample to adapt to the new O_2 concentration.

Statistical tests. We used 1-way ANOVA to test for significant variation in the data. Data were tested for the assumption that the normality and homogeneity of variances were within the limits given for ANOVA tests. Fisher's least-significant difference test was used as a post-hoc test for significant differences between groups. All tests were performed with Statistica 6.0 (StatSoft).

Chlorophyll *a* determination. At the end of each experiment, the filters were stored at –20°C on board the research vessel. Upon return to the laboratory, the filters were freeze-dried and stored at –80°C until analysis. Chlorophyll *a* was extracted by 90% acetone and analyzed by HPLC as described in Staal et al. (2001).

RESULTS

Effects of dynamic changing O_2 concentrations on N_2 fixation

Fig. 2 depicts a representative oxygen-response curve (Stn 2, 18:30 h) showing the results obtained from an experiment measuring the activity of nitrogenase at a photon irradiance of 400 µmol m^{-2} s^{-1} while the cyanobacteria were exposed to dynamically changing O_2 concentrations in an experiment that lasted for 1.5 h. The results were corrected for the time lag between the change in ethylene production rate and the recording of the O_2 concentration. This delay was ~1.5 min, the time required by the system to reach 90 to 95% of the steady-state flux of ethylene (Staal et al. 2001). Maximum nitrogenase activity was achieved at an O_2 concentration

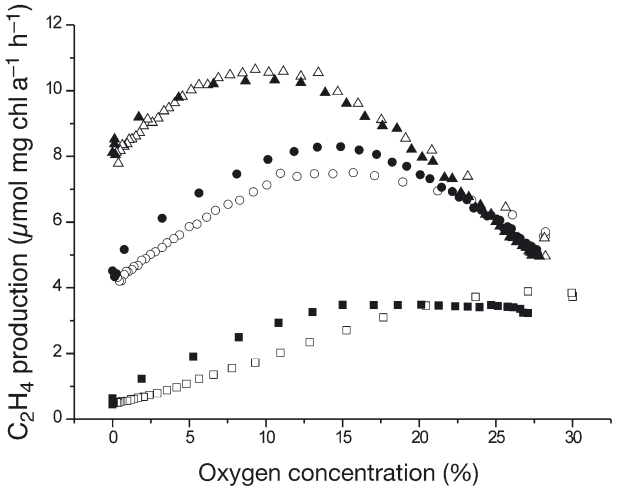

Fig. 3. Example of experiment with dynamic changes in O_2. Nitrogenase activity of natural populations of cyanobacteria (measured by acetylene reduction method) is plotted against O_2 concentration during series of dynamic O_2 regimes (Stn 1, 09:00 h). Increasing (closed symbols) and decreasing (open symbols) O_2 concentrations in the dark (squares) and at 30 (circles) and 400 (triangles) µmol photons m^{-2} s^{-1}

of ~10.5 %. This was observed both when the concentration of O_2 changed from high to low and vice versa. Also the maximum nitrogenase activity was virtually the same in both cases, amounting to ~10.5 µmol C_2H_4 mg chl a^{-1} h^{-1}. The lowest nitrogenase activity was ~4.9 µmol C_2H_4 mg chl a^{-1} h^{-1}, and was recorded at 27 % O_2 (the highest concentration applied), both at the beginning and at the end of the experiment.

Fig. 3 shows nitrogenase activities in the dark and at photon irradiances of 30 and 400 µmol m^{-2} s^{-1} plotted against O_2 concentration during decreasing and increasing dynamic changes in O_2 concentration. The results show that the concentration of O_2 at which the maximum nitrogenase activity is reached is higher in the dark than at saturating irradiances. In the dark, nitrogenase reached its maximum activity at 27 % O_2 in decreasing O_2 concentrations, whereas maximum activity was already reached at 15 % during increasing O_2 concentrations. Maximum dark nitrogenase activity was ~3.9 µmol C_2H_4 mg chlorophyll a^{-1} h^{-1} in decreasing O_2 concentrations and ~3.5 µmol C_2H_4 mg chl a^{-1} h^{-1} in increasing O_2 concentrations. At a photon irradiance of 30 µmol m^{-2} s^{-1}, maximum nitrogenase activity occurred found at 14.5 % O_2, decreasing to 10 % at 400 µmol m^{-2} s^{-1}. In both cases, the O_2 concentration at which maximum nitrogenase activity was obtained was the same in increasing or decreasing O_2 concentrations. The maximum nitrogenase activity at a photon irradiance of 30 µmol m^{-2} s^{-1} was ~7.5 µmol C_2H_4 mg chl a^{-1} h^{-1} during decreasing O_2 and ~8.3 µmol C_2H_4 mg chl a^{-1} h^{-1} during increasing O_2 concentrations. At 400 µmol m^{-2} s^{-1}, these values were ~10.6 and ~10.3 µmol C_2H_4 mg chl a^{-1} h^{-1}, respectively.

A total of 7 series of measurements with dynamically changing O_2 concentrations was recorded at 3 different stations in the Baltic Sea at different times during the day (Table 1). Each set of measurements consisted of both increasing and decreasing O_2 concentrations. At saturating photon irradiances (400 µmol m^{-2} s^{-1}), maximum nitrogenase activities occurred at O_2 concentrations varying from 4 to 11.4 %. At a photon irradiance of 30 µmol m^{-2} s^{-1} a higher optimum concentration of O_2 was found, which varied from 5.5 to 15.1 %. In the dark, concentrations of 8 to 27 % O_2 were required to achieve maximum nitrogenase activity. The average O_2 optima in the dark and under photon irradiances of 30 and 400 µmol m^{-2} s^{-1} were 17.9 ± 4.9, 11.2 ± 3.6 and 8.4 ± 2.6 % (n = 14), respectively, and all differed significantly from each other (1-way ANOVA, $p < 0.05$). Only in 1 case did a photon irradiance of 30 µmol m^{-2} s^{-1} show a lower O_2 optimum than at saturating light (Stn 3, decreasing O_2 at 18:30 h). The 3 stations differed considerably in total daily irradiance. The total daily photon irradiances at Stns 1, 2 and 3 were 24.5, 59.2 and 12.5 mol m^{-2}, respectively (Table 1). At Stn 2, the highest O_2 optima were at the end

Table 1. O_2 optima (as % O_2) and corresponding nitrogenase activity rates (ratio of nitrogenase activity at saturating irradiance to activity in dark: N_{tot}/N_d) at 3 sampling stations in the Baltic Sea, measured in the dark and at 2 photon irradiances, sub-saturating (30 µmol photons m^{-2} s^{-1}) and saturating (400 µmol m^{-2} s^{-1}). Daily irradiance ratio N_{tot}/N_d was determined at 20 % O_2

Stn	Time of day	O_2 regime	Irradiance Dark	Irradiance 30 µmol	Irradiance 400 µmol	Daily irradiance	N_{tot}/N_d
1	09:00	Increasing	18	15	11.4	24.5	2.4
1	09:00	Decreasing	19	14.7	11.0	24.5	2.8
1	18:30	Increasing	20.6	14.8	10.2	24.5	1.9
1	18:30	Decreasing	19	13.6	10.6	24.5	2.0
2	06:30	Increasing	13.3	11.5	10.8	59.2	3.5
2	06:30	Decreasing	23	10.9	8.3	59.2	2.7
2	18:30	Increasing	15	14.1	10.1	59.2	2.3
2	18:30	Decreasing	27	15.1	10.6	59.2	2.3
3	06:30	Increasing	18.6	11	7.3	12.5	3.3
3	06:30	Decreasing	22	8.2	7.5	12.5	2.7
3	12:30	Increasing	11	9.9	5.1	12.5	3.9
3	12:30	Decreasing	19	5.5	4.3	12.5	2.7
3	18:30	Increasing	8	6.3	4	12.5	3.6
3	18:30	Decreasing	17	5.5	6.8	12.5	2.2

of the day. This station also had the highest daily photon irradiance (59.2 mol m^{-2}) and there was a trend of increasing optimum O_2 concentration during the course of the day. The opposite situation was found at Stn 3, where the daily photon irradiance was only 12.5 mol m^{-2}. The O_2 optima at 30 and 400 µmol photons m^{-2} s^{-1} were significantly higher at Stns 1 and 2 than at Stn 3 (1-way ANOVA, $p < 0.05$). Furthermore, the optimal O_2 concentration in the dark was significantly lower (1-way ANOVA, $p < 0.05$) during increasing O_2 concentrations than during decreasing O_2 concentrations (Table 1). The average of all dark O_2 optima measured during decreasing O_2 concentrations was 20.9 ± 3.4%; this was only 14.9 ± 4.5% during increasing O_2 concentrations. In the light, no significant differences were observed between the optima during incrreasing or decreasing O_2 concentrations. Not only did the O_2 concentration at which nitrogenase reached its optimal activity differ as a function of irradiance, but activity sometimes differed also at this optimum, depending on whether it was measured during increasing or decreasing O_2 concentration. Nitrogenase activities during incubation at 30 µmol m^{-2} s^{-1} were somewhat higher (5 to 17%) during increasing O_2 concentration than during decreasing O_2 concentration. A higher nitrogenase activity in O_2 gradients with increasing O_2 concentrations was also found for all dark incubations at Stn 1 (up to 20% more activity). The other 2 stations did not show any difference in nitrogenase activity between the 2 types of dynamic O_2 changes. At 400 µmol m^{-2} s^{-1}, neither maximum nitrogenase nor the optimum O_2 concentration varied.

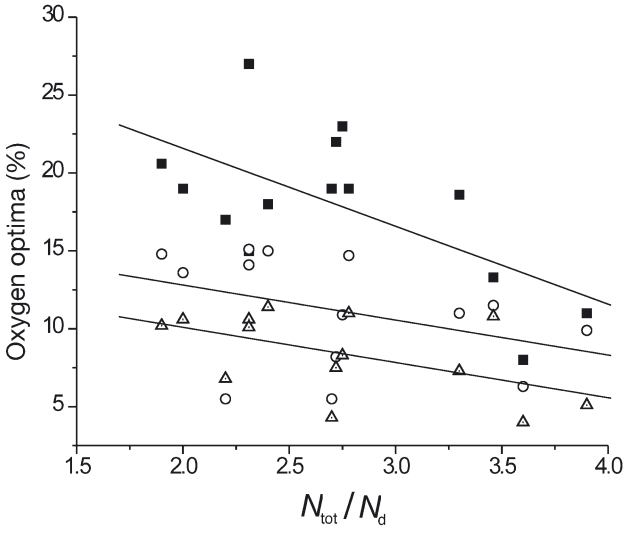

Fig. 4. Relationship between O_2 optima and N_{tot}/N_d (ratio of nitroganese activity at saturating irradiance to activity in dark) in the dark (■), at 30 (○) and 400 (△) µmol photons m^{-2} s^{-1}. Linear regressions ($p < 0.05$) significant for dark (upper line) and 400 µmol photons m^{-2} s^{-1} (lower line); at 30 µmol photons m^{-2} s^{-1} (centre) the relationship was not significant

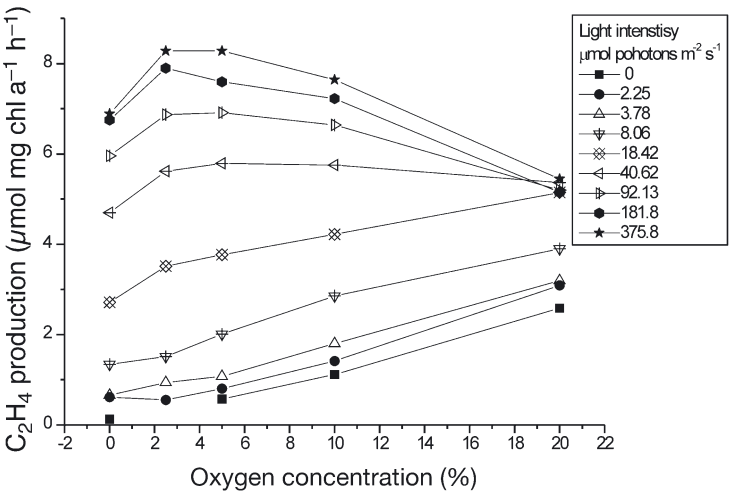

Fig. 5. Example of a steady-state experiment in which nitrogenase activity rates of cyanobacteria versus O_2 concentrations were measured by light-response curves at decreasing steady-state O_2 concentrations

Light-response curves made at the same time showed that irradiances above 200 µmol m^{-2} s^{-1} almost saturated nitrogenase activity; hence, a photon irradiance of 400 µmol m^{-2} s^{-1} was considered sufficient to achieve maximum activity at a specific O_2 concentration. Nitrogenase activity at saturating irradiance is called $N_{tot,}$ while N_d denotes dark activity (Staal et al. 2002). The ratio N_{tot}/N_d at 20% O_2 in each series of measurements was calculated and plotted against the O_2 optima (Fig. 4). Linear regression revealed a significant negative relationship between the ratio N_{tot}/N_d and the O_2 optima in the dark and at an irradiance of 400 µmol m^{-2} s^{-1} ($p < 0.05$). Irradiance of 30 µmol m^{-2} s^{-1} included a similar trend, but this trend was not significant ($p = 0.17$).

Light-response curves at different steady-state levels of oxygen

Light-response curves were measured at all 3 stations, applying different steady-state concentrations of O_2, ranging from 20 to 0% (Fig. 5). In the dark and at irradiances up to 40 µmol m^{-2} s^{-1}, highest nitrogenase activities were observed at 20% O_2, the highest concentration tested in this experiment. At 40 µmol m^{-2} s^{-1}, nitrogenase activity was virtually the same at all O_2 concentrations, except under anaerobic conditions, where the activity was somewhat lower. Above an irradiance of 100 µmol m^{-2} s^{-1}, 2.5 to 5% O_2 yielded the highest nitrogenase activities. This was somewhat

Table 2. *Nodularia spumigena*. Effect of prolonged darkness on O_2 optima of nitrogenase activity in cultured heterocystous cyanobacterium *N. spumigena*. At t = 24 h, light was turned on. Time shown is time after light was turned off. Light-response curves showed that 18 µmol photons m^{-2} s^{-1} was close to saturation. Only at t = 36 h did the nitrogenase activity increase by 20% at higher irradiances

Time (h)	N_{tot}/N_d	Irradiance Dark	4 µmol	9 µmol	18 µmol
Dark					
0	–	20	10	10	0
12	1.8	20	5	2.5	0
18	3	10	2.5	0	0
24	3.7	5	2.5	0	0
Light					
30	2.3	20	5	0	0
36	1.8	20	10	2.5	2.5

lower then that observed during the measurements with a dynamic O_2 gradient. In the dark, under anaerobic conditions, nitrogenase activity was zero. It increased with increasing O_2 concentration, indicating that, in the dark, aerobic respiration is indispensable for N_2 fixation.

At 20% O_2, light-saturation of nitrogenase activity was already achieved at a photon irradiance of 20 to 40 µmol m^{-2} s^{-1}, while at 10% O_2 and lower, nitrogenase activity required much higher irradiances (200 to 300 µmol m^{-2} s^{-1}) in order to become light-saturated.

The effect of a decrease in energy availability on O_2 optima was tested on a culture of *Nodularia spumigena*. This culture was incubated for 24 h in the dark to lower the internal energy sources in the heterocyst. During this dark period, irradiance versus nitrogenase activity curves were recorded at different O_2 levels after 0, 12, 18 and 24 h, as described for the steady-state incubations of the field samples. The optima for O_2 in the dark and at non-saturating irradiances (<18 µmol photons m^{-2} s^{-1}) decreased with increasing duration of the dark period (Table 2). In the cultured samples, optima were found at 0% O_2 at photon irradiances >10 to 20 µmol m^{-2} s^{-1}. The optima in the dark decreased from 20% O_2 at t = 0 to 5% O_2 after 24 h of darkness. The culture was subsequently incubated in the light again (60 µmol m^{-2} s^{-1}), and light-response curves at the different O_2 concentrations were made at 6 and 12 h after the light was turned on. This resulted in an increase in the optima for O_2 at the lower irradiances with time (Table 2). The ratios N_{tot}/N_d were also determined at 20% O_2. An increase in N_{tot}/N_d was found after prolonged dark incubation, but after the light was turned on, N_{tot}/N_d decreased again. The highest O_2 optima coincided with the lowest N_{tot}/N_d.

DISCUSSION

This investigation studied the effects of O_2 on nitrogenase activity in a natural sample of heterocystous bloom-forming cyanobacteria. Our first approach was to apply dynamically increasing and decreasing changes of O_2 concentrations while simultaneously monitoring nitrogenase activity using an on-line and near real-time technique of acetylene reduction. Incubation of the organisms on a filter speeds up gas exchange compared to incubations in water, and therefore steady-state ethylene fluxes were achieved within 1.5 min (Staal et al. 2001). As a result, the measured ethylene concentrations were directly proportional to the enzyme activity, while preventing changes in O_2 concentration due to photosynthesis or respiration. As shown by Staal et al. (2001), this treatment does not affect the performance of the incubated organisms, and can be applied to benthic as well as pelagic cyanobacteria. The high frequency of sampling by the laser photo-acoustic trace-gas detection system allowed a high accuracy in determining optimal O_2 concentrations for N_2 fixation in heterocystous cyanobacteria that cannot be achieved by gas chromatographic determination of ethylene.

Our second approach was to study the effect of O_2 applying steady-state O_2 concentrations. The set-up for the acetylene reduction assay was the same as described above, except that a gas chromatograph was used to measure of ethylene, since a high sampling frequency was not required in this case. The optimum O_2 concentrations at specific irradiances differed, depending on the approach that was applied — dynamic or steady-state O_2 concentration. The different results may be ascribed to the differences in O_2 regimes. It can be argued that when heterocysts are incubated for a prolonged time under low O_2, fewer electrons will be consumed by respiration and, hence, more electrons will be available for N_2 fixation. Using a dynamic O_2 regime, it was shown that exposure to low O_2 concentrations for a short time could increase maximum nitrogenase activity or change the optimal O_2 concentration for N_2 fixation. During steady-state incubations at decreased O_2 levels for prolonged periods (1 h per O_2 level), nitrogenase activity increased with time and may thus have been overestimated, resulting in lower O_2 optima. Therefore, we conclude that the dynamic O_2 regime gives the best estimate of the optimum O_2 concentration of nitrogenase activity because it largely excludes the effects of prolonged exposure to low O_2 levels. It is possible that the rate of O_2 change in the dynamic measuring system might also affect the optimal O_2 concentration. The dilution rate used was imposed by the limitations of the set-up. A higher dilution rate would have caused 2 problems: there would

have been a lower number of measurement points (decreasing the accuracy of the determination) and the relative delay due to the ethylene exchange between the sample and overlying gas would have increased. A lower dilution rate would decrease the number of O_2 response curves that can be measured at a station. The rates at which O_2 concentrations change in aggregates in nature are unknown, and depend on mixing rates in the eupothic zone and on rotation rates of the aggregates themselves.

Despite the differences found with the different methods, both approaches clearly demonstrated that O_2 is required to achieve highest nitrogenase activity in the field. In the dark, 19 to 30% O_2 is required to obtain maximum nitrogenase activities (Fig. 3). Theoretically, in the dark, the concentration of O_2 decreases due to the absence of photosynthetic O_2 production, less respiration takes place, and thus less ATP is produced and, hence, less nitrogenase activity can be supported. This is what was observed in this study. At increasing irradiances, photosynthesis supplies increasing amounts of energy for N_2 fixation. Hence, a decrease in the optimal O_2 concentrations would be expected with increasing irradiance. Although lower O_2 concentrations are required in the light than in the dark for optimal nitrogenase activity, it was unexpected that even at saturating irradiances a certain amount of O_2 was needed to obtain the highest nitrogenase activity in the field samples. This can only be explained by the fact that respiration contributes to the energy demand of N_2 fixation in natural samples of cyanobacteria in the light. This contradicts the hypothesis proposed by Bottomley & Stewart (1977), who proposed that Photosystem I-mediated energy generation could supply all ATP required for maximum nitrogenase activity. Indeed, many cultured heterocystous cyanobacteria have their O_2 optimum between 0 and 5% at saturating irradiances (Murry et al. 1984, Fay 1992, Prosperi 1994, authors' pers. obs.). On the other hand, cultures of *Anabaena cylindrica* grown at elevated O_2 levels (60% O_2 in the gas phase) increased their O_2 optimum for acetylene reduction to 10% in the light (Murry et al. 1984).

In many environments, cyanobacteria may become exposed to elevated levels of O_2. This has been reported for cyanobacterial scums (Ibelings & Mur 1992), aggregates (Paerl & Bebout 1988) and microbial mats (Epping & Jørgensen 1996). Therefore, it is likely that O_2 super-saturation also occurred in aggregates of heterocystous cyanobacteria in the Baltic Sea (E. H. G. Epping pers. comm.). This could explain the high O_2 optimum at saturating irradiances.

The ratio N_{tot}/N_d represents the proportion of ATP produced by respiration relative to the total ATP demand of nitrogenase. A high N_{tot}/N_d reflects a high light-dependency of nitrogen fixation, while a low value shows that respiration is necessary to fulfill the energy demand of nitrogenase. The extreme situation will be that $N_{tot}/N_d = 1$, indicating that all energy used by nitrogenase originates from respiration. In such extreme case, N_d is no longer ATP-limited, but is electron-limited. As shown when incubating *Nodularia spumigena* for 24 h in the dark, the N_{tot}/N_d increased with time during a prolonged period of darkness, and decreased again after the light was turned on. Therefore, we conceive that a high N_{tot}/N_d reflects a low energy status in the heterocyst and that N_2 fixation depends strongly on light. The ratio N_{tot}/N_d was negatively correlated with the O_2 optima. Hence, a low-energy status could result in the observed drop in O_2 concentrations that allowed maximum nitrogenase activity at Stn 3 (Table 1), the station with the lowest daily irradiance. This is in agreement with the results of Murry et al. (1984), who found that the heterocystous cyanobacterium *Anabaena cylindrica* had lower O_2 optima for N_2 fixation when grown under light-limited conditions. This would imply that the high O_2 optima at Stns 1 and 2 might be caused by the combined effect of elevated O_2 levels and an increased energy status of the heterocysts.

For a physiological interpretation of the results, it is necessary to understand the theoretical basis of the physiological and physical processes involved. The interior of the heterocyst is virtually anaerobic due to the low permeability of the cell wall in combination with high respiration rates (Haury & Wolk 1978, Walsby 1985). This implies that the respiration rate is limited by the diffusion of O_2, resulting in a linear relationship between the external O_2 concentration and the respiration rate. Hence, the ATP production should increase proportionally to the external O_2 concentration. Because at 20% O_2 nitrogenase activity is stimulated by light, nitrogenase activity in the dark is limited by ATP (Ernst et al. 1984). The ratio of N_{tot}/N_d at 20% O_2 was 2.3 in the dynamic O_2 regime experiment (Fig. 3). A ratio of 2.3 indicates that 43% of the ATP that is needed to achieve the maximum nitrogenase activity originates from respiration. Regarding the N_d value in a Michaelis–Menten enzyme kinetic model of nitrogenase, its activity in the dark is below the half-saturation (K_m) constant for ATP (Staal et al. 2002). The fact that nitrogenase was not stimulated by the additional influx of O_2, although the enzyme was not even half-saturated by ATP, implies that a process other than respiration, i.e. a process that consumes O_2 but does not produce ATP, must play a role in the O_2 dependency of N_2 fixation. Some heterocystous cyanobacteria possess O_2 uptake mechanisms that are not inhibited by cyanide and, hence, not associated with cytochrome oxidase-dependent respiration. It has

been shown that enzymes other than cytochrome oxidase are capable of transferring electrons to O_2 in heterocysts (Houchins & Hind 1982, Scherer et al. 1988b). Electron donors present in cyanobacteria that have been proposed to be involved in the reduction of O_2 include ferredoxin (Houchins & Hind 1982) and cyanoglobin coupled to a terminal cytochrome oxidase (Thorsteinsson et al. 1999). Another mechanism that can reduce O_2 is via the autoprotective pathway by nitrogenase itself (Thorneley & Ashby 1989, Bergman et al. 1997). All these alternative pathways do not yield ATP. Moreover, autoprotection of nitrogenase is at the expense of ATP, which could explain the decrease of N_2 fixation activity at O_2 concentrations above the optimum. This decrease in nitrogenase activity caused by O_2 at saturating irradiances as well as in the dark indicates that a competition for O_2 and electrons exists between the different pathways, rather than that the enzyme becoming inactivated. This was also suggested by the fact that the decrease of nitrogenase activity in the dynamic O_2 gradients was reversible.

Competition between different O_2-uptake mechanisms could also explain the hysteresis effect found between the dynamic increasing and decreasing O_2 regimes in the dark. In order to be able to compete for O_2, cytochrome oxidase must be in a reduced state. The relative amount of enzymes in their reduced state will depend on the reaction rate with O_2 and the time required for the enzyme to become reduced again. This amount decreases when the supply of electrons is slower than the reaction rate with O_2, i.e. at a high O_2 flux in the heterocyst. A lowered amount of reduced cytochrome oxidases will result in a lower ATP yield per O_2 respired, since we assume that all O_2 within the heterocyst is respired in order to maintain an anaerobic environment. During decreasing O_2 concentration, a lower amount of cytochrome oxidase will be in a reduced state than during increasing O_2 concentration. Therefore, more reduced cytochrome oxidases will be present during a regime of increasing O_2 concentration in order to compete with non-ATP yielding pathways. These other pathways do not show an increase in the amount of reduced enzymes because they are oxidized by processes such as N_2 fixation, which is active under anaerobic conditions. This will result in a higher ATP yield at the same O_2 flux during a regime of increasing O_2 concentration. In the light, only part of the ATP is generated by respiration, which explains the absence of hysteresis under these conditions.

The formation of heterocysts is thought to be an optimal adaptation for diazotrophic growth in oxygenic phototrophic microorganisms (Gallon 2001). However, it seems that the formation of aggregates or colonies in natural conditions counteracts this optimization. Because of its higher biomass concentration and the creation of a less turbulent environment, heterocysts within an aggregate or colony will experience stronger O_2 gradients relative to single trichomes. This will cause lower levels of O_2 in the dark, when the O_2 optima are highest, and O_2 over-saturation when the O_2 optima are lowest. Thus, in both situations, aggregation will result in less optimal conditions for N_2 fixation compared to single trichomes. Therefore, we conclude that aggregation will decrease the daily N_2 fixation rate. The effect of O_2 on N_2 fixation should be taken into account when models are used to calculate the daily depth-integrated N_2 fixation rate. Combining photosynthesis models with N_2 fixation can achieve this when O_2 diffusion and aggregate size are included in the model.

CONCLUSIONS

This research has demonstrated that under aerobic conditions N_2 fixation in heterocystous cyanobacteria is supported for a considerable part by respiration, even when light is available. The results suggest that respiration coupled to a terminal electron transport chain is not the only O_2-consuming process in the heterocyst. Other, not yet identified, O_2-scavenging mechanisms must be present that do not yield ATP or even require energy. Moreover, it has been demonstrated that O_2 levels lower than in air can stimulate nitrogenase activity, although in field samples in the light anoxic conditions were sub-optimal. Laboratory cultures showed optimal oxygen concentration for nitrogenase to be mostly 0 to 2.5% O_2 under saturating irradiance. Because O_2 partial pressure may fluctuate considerably in natural communities of N_2-fixing cyanobacteria, O_2 dynamics and their effect on N_2 fixation should be taken into account when constructing models to estimate N_2 fixation in natural communities.

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