Diurnal Nitrogenase Modification in the Cyanobacterium 
Anabaena variabilis*

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Received: October 2, 1989; Accepted: January 26, 1990

Abstract

The nitrogen-fixing cyanobacterium Anabaena variabilis (ATCC 29413) was cultivated as continuous culture under a 12 h:12 h light-dark cycle. In the light, photosynthetic activity resulted in a continuous increase in cellular glycogen content, followed by an almost complete dissimilation of the polysaccharide during the dark period. Nitrogenase activity, assayed by the acetylene reduction technique, was low at the end of the dark period and increased quickly upon illumination to reach a maximum after 4 to 6 h of light. The activity rapidly declined after darkening the culture. Increase and decrease of activity were accompanied by a change in the electrophoretic mobility of the Fe-protein of nitrogenase (dinitrogenase reductase) indicative of enzyme modification being involved in the diurnal control of nitrogenase activity. Modification and demodification of the Fe-protein were not coupled to the cell cycle since they followed darkening and illumination when the light or dark periods were changed. Addition of fructose increased nitrogenase activity even in darkness and caused demodification of the Fe-protein. Ammonium chloride supplied at the onset of illumination slowed down the increase of nitrogenase activity. A delayed inhibition of the enzyme was accompanied by partial Fe-protein modification only. The reaction was completed after transfer to darkness. The function of enzyme modification in maintaining a constant C: N ratio is discussed and a dominating role of carbohydrate supply in this regulation is indicated by the reported findings.

Key words

Cyanobacteria (blue-green algae), heterocysts, nitrogenase, Fe-protein modification, diurnal growth, C: N-ratio.

Abbreviations and Symbols

Chl: chlorophyll a
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

The enzyme complex nitrogenase (E.C. 1.18.6.1) consists of a dimeric Fe-protein (the dinitrogenase reductase) functioning as an electron carrier to the tetrameric MoFe-protein (the dinitrogenase) which reduces molecular nitrogen to ammonia. Both enzymes are highly oxygen-sensitive. The intrinsically anaerobic character of the nitrogenase complex requires special adaptation in cyanobacteria which produce oxygen in a plant-type photosynthesis. Heterocysts of filamentous cyanobacteria provide such an anaerobic environment by creating a diffusion barrier for gases, enhanced respiratory activity and the lack of the oxygenic photosystem II (reviewed by Scherer et al., 1988). Reductant supply of nitrogenase via ferredoxin is provided by photosynthates transported from vegetative cells to the heterocysts and ATP is generated by photosystem-I activity or oxidative phosphorylation in the heterocysts (for review see Stewart and Rowell, 1986).

Under a light-dark regime most heterocystous strains described so far preferentially fix nitrogen in the light (e.g. Mullineaux et al., 1981; Stal and Krumbein, 1985; Khamees et al., 1987). Also, natural blooms dominated by heterocystous cyanobacteria exhibit higher nitrogenase activity in the light than in the dark (Ganf and Horne, 1975; Vanderheef et al., 1975; Peterson et al., 1977; Horne, 1979). The low activity of nitrogenase in darkness was assigned to the inability of metabolism to sufficiently generate reductants under these conditions (Ernst and Bühme, 1984). However, during dark treatment an oxygen-dependent loss of nitrogenase activity was shown which persisted after reillumination under assay conditions (Weare and Bemmann, 1974; Ernst et al., 1984; Ramos et al., 1985). Treatment with 100 % oxygen (Smith et al., 1986) caused the ADP-ribosylation of Fe-protein of nitrogenase observed in Rhodospirillaceae after transfer from light to darkness or after addition of ammonia (Kanemoto and Böger, 1989) were recently shown not only to diminish enzyme activity but also to alter the electrophoretic mobility of the Fe-protein from two Anabaena strains. In many respects this modification resembles the ADP-ribosylation of Fe-protein of nitrogenase observed in Rhodospirillaceae after transfer from light to darkness or after addition of ammonia (Kanemoto and Ludden, 1984 and references therein). In these phototrophic bacteria an ADP-ribose group derived from NAD is en-
zymatically attached to or removed from an $^{131}$-arg residue which is located in a highly conserved region of the Fe-protein of nitrogenase (Saari et al., 1984; Pope et al., 1985; Lowery and Ludden, 1988).

In this study regulation of cyanobacterial nitrogenase under diurnal growth conditions (cyclostat culture) was examined with respect to substrate limitation in the dark and/or enzyme modification. For substrate limitation nitrogenase activity was assayed under air or molecular hydrogen in light and in darkness. Hydrogen can substitute for endogenous reductants in whole filaments (Bothe et al., 1977) as well as in isolated heterocysts (Schrautemeier et al., 1984). Consumption of hydrogen in the light also provides ATP for nitrogenase activity due to the coupling of ATP synthesis and photosynthetic electron transport in heterocysts (Ernst and Böhme, 1984). Dark assays were performed under air to determine nitrogenase activity supplied by dark carbon metabolism and oxidative phosphorylation. Fe-protein modification was examined by immunospecific Western blotting of cell extracts using a polyclonal antibody raised against the Fe-protein of *Anabaena* nitrogenase (Reich and Böger, 1989).

### Materials and Methods

#### Organism and cultivation

*Anabaena variabilis* (ATCC 29413) was continuously cultivated as a cyclostat at 30°C in a whole-glass fermenter (Kummer, Freiburg, FRG) in a mineral medium without combined nitrogen (Arnon et al., 1974). White light was supplied for 12 h in a diurnal cycle by two circular fluorescent tubes (Osram L32/W25) with an incident photon fluence rate of 135 μE m$^{-2}$s$^{-1}$ measured at the inside surface of the vessel with a quantum sensor LI 185B (LiCOR, Inc., Lincoln, Neb., USA). The culture volume (1.7 l) was continuously diluted (dilution rate, D = 0.028 h$^{-1}$) with mineral medium. CO$_2$ was supplied by aeration through a gas disperser mounted at the bottom of the culture vessel at a flow rate 2041 h$^{-1}$ which allowed the filaments to remain in suspension without additional stirring (for further details see Reich and Mur, 1978). In some experiments the medium was supplemented with ammonium chloride (10 mM, ammonium-supplied culture) or fructose (100 mM) using a minipump at a flow rate of 10 ml h$^{-1}$ and 1 ml h$^{-1}$, respectively. Continuous addition of ammonia increased the dilution rate to D = 0.034 h$^{-1}$; the dilution rate marginally changed with addition of fructose. The pH was continuously monitored with a pH controller (model pH40, New Brunswick, Edison, N.J., USA). The pH increased to 8.5 during the light period and decreased to 7.5 in the dark indicative of CO$_2$-limitation of the culture; during addition of ammonia, the pH remained at 7.2.

#### Analytical procedures

Nitrogenase activity was assayed by the acetylene reduction technique. Within 5 min after removal from the fermenter the filaments (1 ml) were placed into glass vials (7.3 ml) which were previously filled with air or hydrogen and closed with rubber stoppers (Suba Seal, Freeman, Barneysley, U.K.). The reaction was started by injection of acetylene (1 ml). After a 15-min incubation at 30°C aliquots of the gas phase were analyzed by gas chromatography (Carlo-Erba, Hofheim, FRG, model 118, equipped with a flame ionization detector and a Porapak N column).

For NH$_4$ determination the medium was cleared by centrifugation (10 min, 11,000 × g) and the ammonia was measured in the supernatant according to Chaney and Marbach (1962).

Total nitrogen was estimated by the Kjeldahl method from two 20-ml aliquots concentrated by centrifugation (10 min, 5,000 × g), with the ammonia obtained from cell constituents determined as above. C:N ratio was measured with a CHN-Analyzer (Carlo Erba, Hofheim, model 1106). For glycogen measurement 1 ml of cell suspension was centrifuged at 10,000 × g for 10 to 15 min immediately after removal from the culture. 950 μl of the supernatant were removed and the remaining pellet was frozen in liquid nitrogen. Carbohydrates were extracted from duplicate samples by KOH treatment, then the polymers were precipitated and washed with ethanol. Glycogen was quantified after enzymic hydrolysis with a coupled enzymic assay of glucose. The method is described in detail in Ernst et al. (1984). Protein content of whole filaments was determined according to Lowry et al. (1951) and Benzadown and Weinstein (1976). To ensure complete protein extraction the filaments were incubated with tolue (final concentration 1%, v/v) for 5 min at room temperature prior to the precipitation of proteins by trichloroacetic acid. Dry weight was determined on glass fiber filters (Schleicher and Schill, Dassel, FRG) and chlorophyll was extracted with methanol (Ernst et al., 1984).

#### Immunological methods

An antibody was prepared against purified Fe-protein of nitrogenase from *Anabaena variabilis* (Reich and Böger, 1989) and used for immunospecific Western blotting. 15 ml of cells were harvested by two centrifugations (4 min, 5,000 × g and 1 min, 10,000 × g). The pellet was immediately frozen in liquid nitrogen. Extraction of proteins was performed by addition of 300 μl sampling buffer (Laemmli, 1970), by vigorous shaking on a Vortex shaker and by boiling (4 min). An aliquot of the extract containing 1 μg Chl (in case of diatrophic cultures) or 2 μg Chl (ammonia-supplied cultures) was applied on an 18% SDS-polyacrylamide gel containing 3M urea using the gel electrophoresis apparatus GE 2/4 LS (Pharmacia, Uppsala, Sweden). With this instrument increased conductivity at the edges of the gels caused bending of protein fronts, which neither deteriorated separation nor its reliability. After electrophoretic separation the proteins were transferred to a PVDF-filter (Millipore) and incubated with the Fe-protein antibody followed by incubation with an peroxidase-coupled anti-rabbit antibody (Dianova, Hamburg, FRG); for further details see Reich and Böger (1989).

The results show data of single 24-h time courses. Standard deviations are given for three replicates of an individual determination in the nitrogenase assays. Other determinations were performed in duplicate to minimize the material required during one diurnal sampling course. The standard deviation was ±13% for dry weight and chlorophyll determinations, glycogen standards and protein determination varied by ±10% and ±7%, respectively.

#### Chemicals

Enzymes and glycogen (from molluscs, used as standard) were purchased from Boehringer, Mannheim (FRG), other chemicals used were of the highest analytical grade available.

#### Results

##### Biomass composition in the diurnal cycle

When a light-dark cycle was applied to a continuously diluted culture (cyclostat) the biomass of *Anabaena variabilis*, determined as dry weight, increased during illumination due to C- and N-assimilation and decreased in the dark due to continuous culture dilution and dissimilation of stored carbohydrates (Fig. 1A). Accumulation of glycogen accounted for most of the biomass increase during the light period in the diazotrophic culture. Addition
of ammonia prior to the onset of illumination decreased glycogen accumulation and protein accounted for most of the increase of biomass during the light period (Fig. 1B). Both nitrogen-fixing (Table 1) and ammonia-supported cultures (data not shown) exhibited a constant N-content of biomass during the diurnal cycle, indicating that the diazotrophic culture was not N-limited during glycogen accumulation. Therefore, a smaller protein synthesis and enhanced glycogen accumulation in the diazotrophic culture indicates that only part of the newly fixed nitrogen and carbonates are incorporated into proteins during the light period. About 20% of the proteins were formed in darkness (Table 2) using stored carbohydrates and nitrogen. Obviously, the latter was stored transiently (see constant C : N ratio, Table 1), and could not be detected by the protein assay used. Table 2 demonstrates the change of selected parameters in the cyclostat culture described in Fig. 1A by correcting the actual amounts present in the fermenter for continuous culture dilution (comp. Rhee, 1982). It should be mentioned that chlorophyll, the parameter used as reference for nitrogenase activity, does not exactly follow protein synthesis.

### Nitrogenase activity in the diurnal cycle

In the cyclostat nitrogenase activity exhibited strong diurnal changes (Fig. 2A). At the end of the dark period, enzyme activity was low when measured in a 15-min assay in the light. The activity rapidly increased after illumination of the culture reaching its maximum 4 h to 6 h later. Following this increase the activity per ml culture volume remained constant or dropped slightly due to culture dilution (data not shown); the activity referred to Chl a (presented in Fig. 2A) dropped due to continuous increase of chlorophyll and biomass during the light period (Fig. 1A). After transfer to darkness nitrogenase activity (which was assayed in the light) decreased more rapidly than expected from culture dilution. It was noteworthy that the diurnal characteristic of nitrogenase was not eliminated by the presence of hydrogen, which is a potent reductant for nitrogenase (Fig. 2A, upper graph). Dark activity exhibited diurnal variation corresponding to that in the light. The rates attained 21 ± 2% and 28 ± 4% of the activity measured in the light under hydrogen and air, respectively, throughout the 24 h period. Apparently, the periodic change in nitrogenase activity, as observed under all assay conditions, cannot be attributed to reductant or light limitation.

### Table 1  
Nitrogen content and C : N ratio of A. variabilis cultivated diazotrophically in continuous culture with a 12 h : 12 h light-dark cycle (cyclostat).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Light period</th>
<th>Dark period</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-content (Kjeldahl)</td>
<td>7.12 ± 0.33</td>
<td>6.98 ± 0.25</td>
</tr>
<tr>
<td>N-content (CHN analyzer)</td>
<td>7.75 ± 0.03 (n = 17)</td>
<td>7.71 ± 0.20 (n = 17)</td>
</tr>
<tr>
<td>C : N ratio</td>
<td>5.23 ± 0.37</td>
<td>5.21 ± 0.52</td>
</tr>
</tbody>
</table>

The nitrogen content was determined by either the Kjeldahl method (samples removed in 4 h intervals) or with a CHN-analyzer (samples removed in 2 h intervals). Data are given as % of dry weight (biomass).

#### Table 2  
Specific rates for the change of parameters determined from A. variabilis grown diazotrophically in a 12 h : 12 h light-dark cyclostat.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>( \mu_{\text{light}} )</th>
<th>( \mu_{\text{dark}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a</td>
<td>0.055 ± 0.002</td>
<td></td>
</tr>
<tr>
<td>Dry weight (biomass)</td>
<td>0.066 ± 0.012</td>
<td>0.076 ± 0.012</td>
</tr>
<tr>
<td>Glycogen</td>
<td>0.253 ± 0.195</td>
<td></td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>0.063 ± 0.015</td>
<td>0.076 ± 0.012</td>
</tr>
<tr>
<td>Protein</td>
<td>0.042 ± 0.008</td>
<td>0.076 ± 0.012</td>
</tr>
</tbody>
</table>

Positive values denote net assimilation of constituents, negative values indicate net dissimilation of material.

Specific rates of parameter changes (μ) in the light and dark were calculated from data of Fig. 1A and further data obtained in 2 h or 4 h intervals according to \( \mu = \frac{(\text{ln} x_n) - \text{ln} x_0}{n \cdot (t_n - t_0)} \). The cyclostat (12 h: 12 h light-dark cycle) is defined by the equation \( D = 1/2 \left( \mu_{\text{light}} + \mu_{\text{dark}} \right) \) with a dilution rate \( D = 0.028 \text{ h}^{-1} \) (Rhee, 1982); deviations of D result from measuring errors of μ.
Influence of ammonia on diurnal nitrogenase activity

Ammonia was added prior to the onset of light, and a 0.4–1 mM concentration was maintained by continuous supply throughout the 24-h cycle. Upon illumination nitrogenase activity increased slower than in the control culture without ammonia, resulting in a 45% inhibition of nitrogenase activity within the first 4 h (Fig. 2B). In the following hours nitrogenase activity disappeared more rapidly than expected from culture dilution. Activity was completely lost after 14 h with continuous addition of ammonia (Fig. 2B).

Modification of the Fe-protein of nitrogenase during the diurnal cycle

Aliquots of the culture were removed every hour and examined for Fe-protein modification by immunospecific Western blotting. After a 12-h dark period one band is seen on the Western blot of cell extract representing the modified Fe-protein polypeptide with an apparent molecular weight of 33 kDa (Fig. 3A, first lane, comp. Reich and Böger, 1989). A faster moving Fe-protein species (lower band; apparent molecular weight 32 kDa) emerged after illumination, while the upper band of the Fe-protein disappeared (Fig. 3A, following lanes). This indicates complete demodification of the Fe-protein polypeptides after illumination. Upon darkening the 32-kDa Fe-protein polypeptides were again modified to a 33-kDa species.

![Figure 2](image)

**Fig. 2.** Diurnal variation of nitrogenase activity from Anabaena variabilis in a 12 h:12 h light-dark cycle. 
A. Cyclostat culture grown without combined nitrogen as described in Fig. 1A. 
B. Diazotrophic cyclostat culture continuously supplied with ammonia (from t = 0) as described in Fig. 1B. The nitrogenase assays were performed in the light in presence of either air/C2H2 (△--△) or H2/C2H2 (O---O) or darkness under air/C2H2 (△--△). White and black bars on top of the figure indicate light and dark phase, respectively.

The amount of Fe-protein detected by the antibody was significantly lowered after addition of ammonia, but still demodification (appearance of the lower band) was observed after illumination (Fig. 3B). However, after 6 h the modified form of the Fe-protein (upper band) developed even when the light was still on. A double band indicating that approximately half of the Fe-protein polypeptides present were modified remained until the light was switched off. The remaining Fe-protein polypeptides then became modified.

Activation and inactivation of nitrogenase under a changed diurnal regime

A cyclostat culture grown in a regular 12 h:12 h light-dark cycle was illuminated 6 h in advance or 6 h after the normal illumination time to see whether the induction of nitrogenase activity may follow endogenous signals. Fig. 4 shows that under each regime nitrogenase activity immediately rises upon illumination. The increase of nitrogenase activity was always accompanied by demodification of the Fe-protein polypeptides. In diazotrophic cultures the immunological analysis revealed no modification occurring in the light until darkening. When the light period was prolonged, modification was delayed (Fig. 3C) and nitrogenase activity per ml culture volume remained stable or showed a slight decrease due to continuous dilution (data not shown).

Heterotrophic activation of nitrogenase

After a standard 12 h dark period fructose was added to a culture and subsequently kept in darkness. The exogenously supplied carbohydrate induced glycogen storage in the dark. Concurrently, nitrogenase activity (assayed in the light) increased to reach a maximum after 6 h, which, however, was significantly lower than the activity observed under autotrophic conditions in the light (Fig. 5). Demodification of the Fe-protein after fructose addition in the dark is demonstrated in Fig. 3D.

Discussion

Nitrogenase measurements of cultures growing under diurnal conditions are usually performed under the light or dark conditions of the respective growth phase (see e.g. Mullineaux et al., 1981; Khamees et al., 1987). Such assay conditions reflect the energetic situation of the culture, but not the state of nitrogenase activity. Here, we show that nitrogenase activity exhibited strong diurnal changes under all assay conditions applied (Fig. 2A). A diurnal behaviour under optimum ATP and reductant supply (in the light under hydrogen) strongly suggests regulation of nitrogenase activity at the enzyme level. Analysis of cell extracts by immunospecific Western blotting showed that nitrogenase from *Anabaena* was composed of a 32-kDa dimer of the Fe-protein in the light and a 33-kDa dimer in the dark and both forms together after the light was switched on or off (Fig. 3). We conclude that nitrogenase activity is regulated at the enzyme level by modification of the Fe-protein polypeptides under diurnal conditions. A similar light-dark regulation was first observed with *Rhodospirillum*. ADP-ribose is attached to one of the two identical subunits of the dimeric Fe-protein resulting in the formation of a double...
Diurnal Nitrogenase Modification in the Cyanobacterium

**Fig. 3** Demonstration of a reversible modification of the Fe-protein from nitrogenase during light-dark cycles. Filaments were removed from A. variabilis cultures, cell extracts were subjected to SDS-PAGE (see Methods); the dinitrogen reductase was visualized by Western blotting with an antiserum against the Fe-protein form A. variabilis.

A) Diazotrophic culture grown as cyclostat in a 12 h:12 h light-dark cycle.

B) Light-dark cycle as in (A) but NH$_4$Cl was added continuously to a culture previously grown as diazotrophic cyclostat starting at t = 0 (comp. Fig. 1B).

C) Diazotrophic cyclostat with a prolonged light period.

D) Darkened diazotrophic culture supplied with fructose (1 mM) starting at t = 0 (comp. Fig. 4). Hours of removal from culture are indicated, black bars mark the dark period. The arrows indicate the modified (upper band) and the unmodified (lower band) Fe-protein polypeptide of nitrogenase.

**Fig. 4** Nitrogenase activity under a changed light-dark regime. Diazotrophic cultures grown perivously as 12 h:12 h light-dark cyclostats were subjected to shifts in the regime of illumination and darkness. Illumination is indicated by an arrow.

1) Standard 12 h:12 h light-dark cycle (indicated by the bars at the top of the figure).
2) Light was switched on 6 h earlier than in (1).
3) Dark period was 5 h longer than in (1).

All assays were performed in the light under air/C$_2$H$_2$.

**Fig. 5** Nitrogenase activity after addition of fructose in continuous darkness. Fructose was added to an autotrophic cyclostat culture after a 12 h dark period. The consumption of fructose (■—■), formation of glycogen (○—○) and nitrogenase activity (△—△) were followed over a 24 h dark period. Then the culture was illuminated.

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band in the Western blot (Kanemoto and Ludden, 1984), and, as shown transiently in our experiments, after light was switched on or off (Fig. 3). Modification of both Fe-protein polypeptides was reported from cyanobacteria transferred to 100% oxygen (Smith et al., 1986; Reich and Boger, 1989). In our cultures modification of both polypeptides occurs at air level of oxygen, although in the dark only. In contrast to the light condition, carbon supply is limited in heterocysts in the dark leading to a decrease of respiratory oxygen consumption. Presumably this causes a similar effect as the increased oxygen concentration in the experiments mentioned above. Inactivation and reactivation of nitrogenase was shifted according to changes in the illumination regime (Fig. 4), thus excluding the cell cycle in triggering the modification process. The activation of nitrogenase in the dark by fructose and by illumination strongly suggests that carbohydrate supply of the heterocysts is a prerequisite for activation (comp. also Ernst et al., 1990).
The pH-dependence of the ammonia switch-off has been attributed to enzyme modification at alkaline pH (7.2). Addition of ammonia (mMolar) causes rapid inhibition of nitrogenase by (partial) enzyme modification at alkaline pH only (Reich et al., 1986). The pH-dependence of the ammonia switch-off has been attributed to the differential permeability of cell membranes for ammonia (present at pH 10) and the ammonium ion (dominant at pH 7) (comp. Kleiner, 1985). In our experiment, the Fe-protein exhibits modification after 6 h of continuous addition of ammonia at neutral pH. Conceivably, a prolonged presence of ammonium ions may change metabolism to resemble that of a rapid ammonia inundation at pH 10, then resulting in partial modification of the Fe-protein polyamides.

This study shows for the first time that Fe-protein modification is correlated with the diurnal regulation of cyanobacterial nitrogen fixation. This regulation may be involved in the maintenance of a constant C:N ratio under diurnal conditions (Table 1). A close coupling of photosynthesis and nitrogen assimilation is also observed with ammonia and nitrate as nitrogen sources (Romero et al., 1985). This regulation was assigned to an ammonia-induced feedback inhibition (see Guerrero and Lara, 1989, for a review of current knowledge of nitrogen assimilation). Accordingly, Ramos et al. (1985) tried to assign regulation of nitrogenase activity to an ammonia-mediated control system. In our experiments, a constant C:N ratio (Table 1) was maintained in the diazotrophic culture during high glycogen accumulation and low protein synthesis (Fig. 1A) indicating that not all fixed nitrogen was detected by the protein assay. (Cyanophycin, a nitrogen-enriched polymer consisting of arginine and aspartic acid, is not detected by the protein assay. Evidence exists of an increased turnover of this polymer in heterocysts indicating its dynamic role in nitrogen fixation (Gupta and Carr, 1981). This finding will be discussed in detail in a forthcoming paper). The fixation of nitrogen did not lead to enzyme modification even after prolonged illumination (Fig. 3C). On the other hand, cessation of photosynthesis induced nitrogenase modification. Thus, the signal causing modification of the Fe-protein of nitrogenase is more likely to be related to the cessation of photosynthetic supply than with a product of nitrogen fixation. This conclusion agrees with recent studies on nitrogenase regulation in batch cultures (Ernst et al., 1990).

Acknowledgements

This study was supported by the Deutsche Forschungsgemeinschaft through the Sonderforschungsbereich 248 “Stoffhaushalt des Bodensees”. The authors thank Regina Grimm for excellent technical assistance.

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