First publ. in: Proceedings of the Royal Society of London / B, Vol. 270 (2003), pp. 1209-1214

Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*)

Eric von Elert[1*], Dominik Martin-Creuzburg[1] and Jean R. Le Coz[2]

[1]*Limnological Institute, University of Konstanz, 78434 Konstanz, Germany*
[2]*Centre IFREMER de Brest, BP 70, 29280 Plouzane, France*

A key process in freshwater plankton food webs is the regulation of the efficiency of energy and material transfer. Cyanobacterial carbon (C) in particular is transferred very inefficiently to herbivorous zooplankton, which leads to a decoupling of primary and secondary production and the accumulation of cyanobacterial biomass, which is associated with reduced recreational quality of water bodies and hazards to human health. A recent correlative field study suggested that the low transfer efficiency of cyanobacterial C is the result of the absence of long-chain polyunsaturated fatty acids (PUFA) in the diet of the zooplankton. By supplementation of single-lipid compounds in controlled growth experiments, we show here that the low C transfer efficiency of coccal and filamentous cyanobacteria to the keystone herbivore *Daphnia* is caused by the low sterol content in cyanobacteria, which constrains cholesterol synthesis and thereby growth and reproduction of the herbivore. Estimations of sterol requirement in *Daphnia* suggest that, when cyanobacteria comprise more than 80% of the grazed phytoplankton, growth of the herbivore may be limited by sterols and *Daphnia* may subsequently fail to control phytoplankton biomass. Dietary sterols therefore may play a key role in freshwater food webs and in the control of water quality in lakes dominated by cyanobacteria.

Keywords: food quality; fatty acid; polyunsaturated fatty acid; *Daphnia*; cyanobacteria; sterol

1. INTRODUCTION

The transfer of energy and carbon (C) at the autotroph–herbivore interface is a crucial parameter in the regulation of the efficiency of the transfer of energy and material in freshwater food webs. This transfer across the plant–herbivore interface is highly variable, and the mechanisms determining the efficiency of assimilation in herbivores are only partly understood (Brett & Müller-Navarra 1997). In particular, cyanobacterial C is transferred very inefficiently to zooplankton, which results in a low biomass of herbivores and an accumulation of cyanobacterial biomass (De Bernardi & Giussani 1990), leading to cyanobacterial blooms, which are associated with hazards to human health and livestock and reduced quality of recreational waters (Carmichael 1994).

Low energy assimilation by herbivorous zooplankton (e.g. *Daphnia*) can be caused by low ingestion, toxicity or the elemental and biochemical composition of the food. It is widely accepted that nutrient-limited algae (which, in freshwater systems, are mostly phosphorus (P) limited) are a low-quality food source when C : P exceeds a threshold of 300 (Sterner & Hessen 1994), which results in P limitation of *Daphnia* (Andersen & Hessen 1991); direct evidence for P limitation of *Daphnia* has been provided only recently (Urabe *et al.* 1997; Elser *et al.* 2001). However, in many lakes C : P of less than 300 is found (Brett *et al.* 2000), and food quality for *Daphnia* may be constrained by factors other than P (Sundbom & Vrede 1997). This might in particular be the case during eutrophication as a result of external P loading.

Recent correlative field studies in lakes with seston (suspended particles) with C : P of less than 300 have suggested that the transfer efficiency of C is determined by the amount of the polyunsaturated fatty acid (PUFA) 18 : 3ω3 in the seston (Wacker & Von Elert 2001); in a lake of moderate productivity (Müller-Navarra 1995) and in a highly eutrophic pond with seston dominated by cyanobacteria (Müller-Navarra *et al.* 2000) the transfer efficiency may be determined by the PUFA 20 : 5ω3. Cyanobacteria do not contain long-chain PUFAs, such as 20 : 5ω3 (Cobelas & Lechardo 1988; Ahlgren *et al.* 1992); this has led to the proposal that the well-known poor assimilation of cyanobacterial C (De Bernardi & Giussani 1990) is caused by the absence of 20 : 5ω3 (Müller-Navarra *et al.* 2000).

The correlative evidence from field studies showing that a low availability of PUFAs constrains C transfer efficiency has been supported by the results of laboratory experiments with eukaryotic algae (Von Elert 2002), but not with cyanobacteria (Von Elert & Wolffrom 2001). Experiments in which cyanobacterial cells are supplemented with PUFAs have revealed that the added PUFAs do not improve C transfer to *Daphnia*, yet lipids from the eukaryotic alga *Scenedesmus obliquus* significantly increase assimilation of the cyanobacterial C (Von Elert & Wolffrom 2001). Therefore, a non-PUFA lipid present in eukaryotic algae, but absent in cyanobacteria, constrains assimilation of the cyanobacterial C, and the strong predictive power of the availability of 20 : 5ω3 for *Daphnia* growth on seston dominated by cyanobacteria (Müller-Navarra *et al.* 2000) does not reflect a causal dietary

*Author for correspondence (eric.vonelert@uni-konstanz.de).

deficiency for the poor assimilation of cyanobacterial C by zooplankton.

Cyanobacteria, as prokaryotes, differ from eukaryotic algae not only in the absence of long-chain PUFAs, but also in containing only traces of sterols (Urich 1990; Hai *et al.* 1996); hence, in seston dominated by cyanobacteria, low concentrations of PUFAs and of sterols will be highly correlated. Sterols are indispensable in eukaryotic lipid biostructure and serve as precursors of steroid hormones (Goad 1981). Because arthropods are not capable of *de novo* synthesis of sterols, these compounds must be obtained from their food (Goad 1981). Here, we test the hypothesis that the low content of sterols in cyanobacteria constrains the assimilation of cyanobacterial C by *Daphnia galeata*, because *Daphnia* is the most important herbivore in the zooplankton community of freshwater lakes.

2. MATERIAL AND METHODS

(a) *Growth experiments*

Synechococcus elongatus (strain SAG 89.79, Stammsammlung für Algen, Göttingen) and *Anabaena variabilis* (strain ATCC 29413) were grown in Cyano medium (Jüttner *et al.* 1983) at a dilution rate of 0.25 day^{-1} at 20 °C with illumination at 40 $\mu mol\ m^{-2}\ s^{-1}$. *Scenedesmus obliquus* (strain SAG 276-3a) was grown in batch culture (20 °C; illumination, 120 $\mu mol\ m^{-2}\ s^{-1}$) and harvested in the late exponential phase.

Growth experiments were carried out at 20 °C with third-brood juveniles of a clone of *D. galeata* originally isolated from Lake Constance (Stich & Lampert 1984). Juveniles were collected within 8 h of birth and grown to the age of 48 h in a flow-through system on *S. obliquus* (2 mg C l^{-1}) in order to make determination of dry weight more accurate; this prefeeding condition does not affect the final conclusions. The subsequent growth experiments lasted 4 days, and animals increased from *ca.* 0.7 mm to 1.6 mm in body size. Fifteen animals were transferred to 0.5 l of freshly filtered (0.45 μm pore-sized membrane filter) lake water with algal or cyanobacterial food added (2 mg C l^{-1}); the food suspensions were renewed daily so that food concentrations were never depleted to below 1 mg C l^{-1}. C concentrations of algae were estimated from photometric light extinction (800 nm) using C-extinction equations.

Somatic growth rates g (day^{-1}) were calculated according to Wacker & Von Elert (2001) as $g = (\ln W_t - \ln W_0)/t$, where W is the body weight of a subsample of the experimental animals at the beginning (W_0) and end (W_t) of the experiment. Mean individual dry weights were mean values of 10 individuals. Each treatment consisted of three replicates with 10 randomly selected animals in each, and growth rates were calculated for each replicate and subsequently averaged to give the average of the treatment. The data were analysed by one-way analyses of variance (ANOVA) and *post hoc* comparisons (Tukey).

Cyanobacteria were supplemented with PUFAs or sterols according to Von Elert (2002) with 20 mg of bovine serum albumin (BSA) dissolved in 5 ml of ultrapure water and 400 μl of an ethanolic stock solution of the fatty acid or the sterol (2.5 mg ml^{-1}). Subsequently, each solution was made up to 40 ml with the cyanobacterial suspension (4 mg of C) and culture medium, and agitated for 4 h. Excess BSA and lipid were removed by washing the cells in fresh culture medium, and the suspension was used as food in the *D. galeata* growth experiments.

Table 1. Effect of diet on the cholesterol content of *D. galeata*. (Animals were fed on the green alga *S. obliquus* or on the cyanobacterium *Sy. elongatus* with and without cholesterol supplementation. Values are means ± s.e. ($n = 3$). Identical letters indicate cholesterol levels that are not significantly different (Tukey's *post hoc* test).)

treatment	cholesterol content (ng $individual^{-1}$)
day 0	5.63 ± 1.45 A
day 4/fed on green algae	54.52 ± 5.45 B
day 4/fed on cyanobacteria	2.70 ± 0.79 A
day 4/fed on cyanobacteria supplemented with cholesterol	90.54 ± 4.57 C

(b) *Analyses*

Sterols were analysed after extraction and saponification as free sterols using a gas chromatograph (HP 6890) equipped with an HP-5 capillary column (Agilent) and a flame ionization detector. Cholesterol was quantified by comparison with an internal standard (5-α-cholestane) and with a response factor determined for cholesterol. Sterols were identified using a gas chromatograph–mass spectrometer (Finnigan MAT GCQ) equipped with a fused silica capillary column (DB-5MS, J&W Scientific); spectra were recorded between 60 and 400 a.m.u. in the electron impact ionization mode. Cholesterol in *S. obliquus* was detected in the EI ionization mode by single-ion monitoring (m/z $[M]^+$, $[M-18]^+$ and $[M-85]^+$).

Aliquots of food suspensions were filtered onto precombusted glass-fibre filters and analysed for particulate organic C using an NCS-2500 analyser (Carlo Erba Instruments).

3. RESULTS

(a) *Sterol content of* D. galeata

In accordance with the results reported previously (Rezanka *et al.* 1986; Rzama *et al.* 1994), three major phytosterols (ergost-7-en-3-ol, stigmast-7-en-3-ol and stigmasta-7,22-dien-3-ol) but no cholesterol were found in the green alga *S. obliquus*. In *D. galeata* feeding on this green alga, cholesterol was the major sterol, with amounts per individual increasing with the time feeding on the eukaryotic algae (table 1). The cholesterol content of the animals was significantly affected by the diet (table 1; ANOVA, $F_{3,8} = 133.30$, $p < 0.001$): when animals were fed the cyanobacterium *Sy. elongatus*, no increase in cholesterol content was observed. However, when cells of *Sy. elongatus* that had been supplemented with cholesterol were fed to *D. galeata*, the amount of cholesterol in the daphnids became even higher than when they were fed the eukaryotic *S. obliquus* (table 1). This indicates that the cholesterol added to the cyanobacterial cells was assimilated by the herbivores; it was not investigated whether the added cholesterol was absorbed into or adsorbed onto the cyanobacterial cells.

(b) *Growth of* D. galeata *on* Sy. elongatus

To test whether the low sterol content of cyanobacteria prevents a more efficient assimilation of cyanobacterial C by the daphnids, cells of *Sy. elongatus* were supplemented with cholesterol. The growth of *D. galeata* was significantly affected by the food type (ANOVA, $F_{4,10} = 1056.9$,

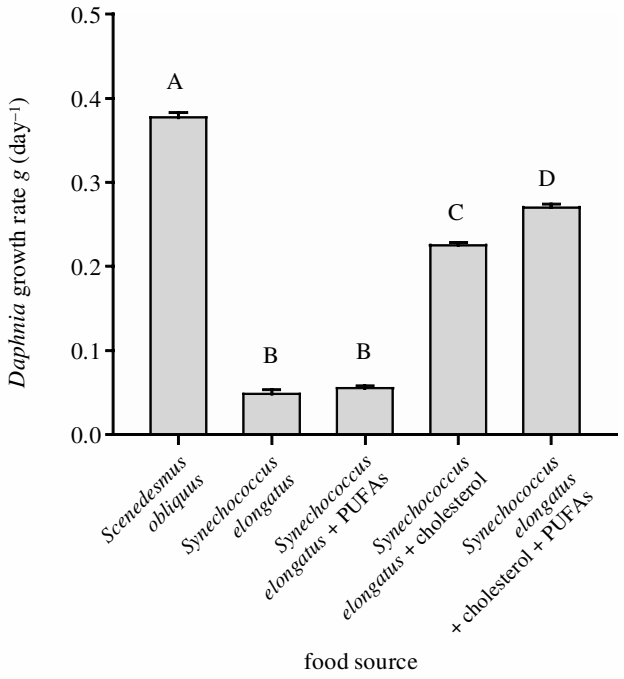

Figure 1. The effect of cholesterol and PUFAs (18 : 3ω3 and 20 : 5ω3) added to the coccal cyanobacterium *Sy. elongatus* on the growth of *D. galeata*. Values are means ($n = 3$); error bars indicate s.e. Bars labelled with the same letter are not significantly different (Tukey's *post hoc* test).

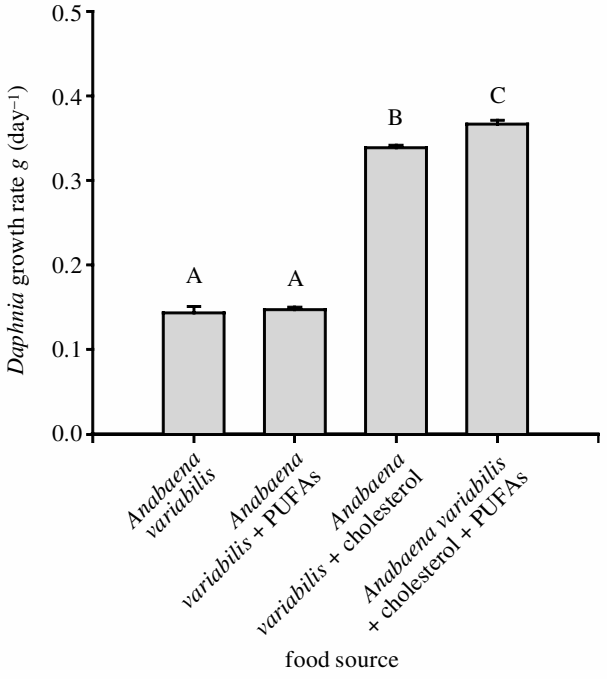

Figure 2. The effect of cholesterol and PUFAs (18 : 3ω3 and 20 : 5ω3) added to the filamentous cyanobacterium *A. variabilis* on the growth of *D. galeata*. Values are means ($n = 3$); error bars indicate s.e. Bars labelled with the same letter are not significantly different (Tukey's *post hoc* test).

$p < 0.001$). The almost maximal possible growth rate of the herbivore of 0.5 day^{-1} (Wacker & Von Elert 2001) was observed when fed on the green alga *S. obliquus*; growth when fed on the cyanobacterium was significantly lower ($p < 0.001$; figure 1). Growth on cholesterol-supplemented *Sy. elongatus* increased significantly compared with growth on the unsupplemented cyanobacterium ($p < 0.001$; figure 1), which indicates that the absence of sterols constrained the assimilation of cyanobacterial C by the daphnid. The low content of sterols constrained both growth and reproduction, as evidenced by the increase in the number of eggs per 6-day-old individual from 0 on unsupplemented *Sy. elongatus* to 1.3 on *Sy. elongatus* supplemented with cholesterol. Supplementation with PUFAs (18 : 3ω3 and 20 : 5ω3) alone did not improve the nutritional quality of the cyanobacterium, but when it was supplemented with cholesterol and PUFAs together, an additional significant increase in the growth of *D. galeata*, above that seen for cholesterol supplementation alone, was observed ($p < 0.001$; figure 1).

(c) *Growth of* D. galeata *on* A. variabilis

Somatic growth of *D. galeata* on a suspension of the filamentous cyanobacterium *A. variabilis* (mean length of filaments of 536 μm) was low, but was significantly affected by the kind of supplementation (ANOVA, $F_{3,8} = 634.6$, $p < 0.001$), which indicated that mechanical interference of the filaments by the grazer was not the cause of the poor assimilation of the cyanobacterial C. Somatic growth rates of *D. galeata* were significantly enhanced by supplementation of *A. variabilis* with cholesterol ($p < 0.001$; figure 2). Similarly, egg numbers in 6-day-old animals increased from 0 with no supplementation to 4.5 per individual on *A. variabilis* supplemented with cholesterol. Supplementation of *A. variabilis* with PUFAs (18 : 3ω3 and 20 : 5ω3) enhanced the growth of *D. galeata* only when the shortage of sterols in the cyanobacterial cells had been overcome by supplementation with cholesterol (figure 2).

(d) *Sterol requirement of* D. galeata

Because growth on the cyanobacterium *Sy. elongatus* was constrained by the availability of sterols, dose-dependent effects of cholesterol on the growth of *D. galeata* were investigated in order to estimate the sterol requirements of the daphnids. Somatic growth was not constrained by the availability of cholesterol for cholesterol contents above 2 μg mg^{-1} of cyanobacterial C (figure 3). Assuming that the three major phytosterols in the green alga *S. obliquus* (ergost-7-en-3-ol, stigmast-7-en-3-ol and stigmasta-7,22-dien-3-ol) are all converted to cholesterol by *D. galeata* in a 1 : 1 ratio, the phytosterols in the green alga were quantified as cholesterol equivalents. *Scenedesmus obliquus* contained 10.60 ± 0.6 μg (mean ± s.e., $n = 3$) cholesterol equivalents per mg of algal C, which suggests that *ca.* 20% of the eukaryotic algal C in the daphnids' diet is required to compensate for the low sterol content of the cyanobacterial food.

4. DISCUSSION

Cholesterol was the major sterol in *D. galeata*, which is in accordance with reports for marine crustaceans (Goad 1981). The increased cholesterol content of *Daphnia* fed on the green alga *S. obliquus*, which contained phytosterols, but no cholesterol, indicated that *D. galeata* uses the phytosterols of eukaryotic algae as precursors for the synthesis of cholesterol. No cholesterol was synthesized by

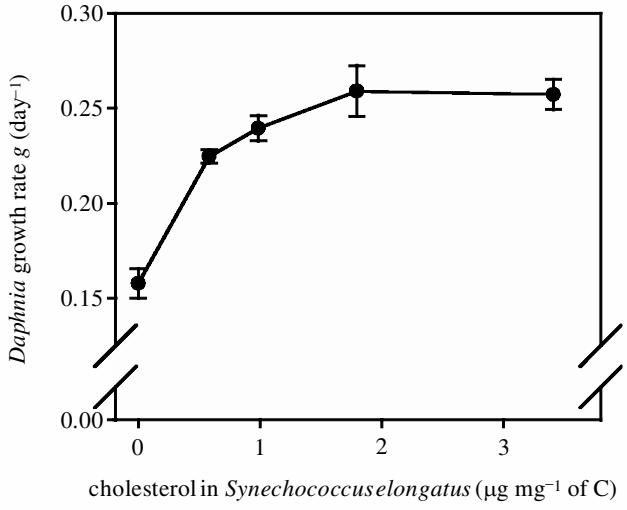

Figure 3. Somatic growth of *D. galeata* as a function of the cholesterol content of the coccal cyanobacterium *Sy. elongatus,* which was supplemented with different concentrations of cholesterol before feeding to *D. galeata*. Values are means ($n = 3$); error bars indicate s.e.

D. galeata fed on unsupplemented cyanobacteria, but the daphnids feeding on cholesterol-supplemented cyanobacteria contained cholesterol, thereby confirming that somatic growth and, hence, the transfer efficiency of cyanobacterial C, were constrained by the availability of sterols. These results also confirm earlier findings that the food quality of *Sy. elongatus* is constrained by the absence of a non-PUFA lipid present in the eukaryotic alga *S. obliquus* (Von Elert & Wolffrom 2001). In accordance with somatic growth of juvenile *Daphnia* being highly correlated with estimates of fitness (Lampert & Trubetskova 1996), both juvenile growth and reproduction were constrained by the availability of sterols when the daphnids were fed on unsupplemented cyanobacteria. Because clutch size was determined in 6-day-old animals regardless of whether they had reached the stage of first reproduction, it remains unclear whether the cyanobacterially mediated reduction in reproduction was caused by a reduction in egg numbers or a delay in the time of first reproduction.

It is well known that cyanobacteria are a poor-quality food for herbivorous zooplankton for a variety of reasons, including the toxicity of some strains, poor digestibility, feeding interference and biochemical deficiencies (Ahlgren *et al.* 1990). Although potential causes have been identified in laboratory experiments (Lampert 1987; De Bernardi & Giussani 1990), the relative importance of these causes has been poorly understood (reviewed by Haney 1987).

Mechanical interference with the filtering process of the zooplankton is regarded as the main reason for the inferior assimilation of filamentous cyanobacteria (Porter & McDonough 1984) even though experimental evidence is contradictory. We tested the relative importance of mechanical interference versus biochemical dietary deficiency by supplementing the filamentous cyanobacterium *A. variabilis* with cholesterol at a cyanobacterial density of 2 mg C l^{-1}, which is well below the density of the typical biomass in eutrophic waterbodies (e.g. Müller-Navarra *et al.* 2000). The positive effect of supplementary cholesterol clearly ruled out mechanical interference as the cause of poor assimilation of this filamentous cyanobacterium and indicated a nutritional deficiency. The absence of mechanical interference in our experiments is in accordance with the results of Arnold (1971) and contradictory to the results of a laboratory study in which mechanical interference by a filamentous cyanobacterium was observed and nutritional deficiencies were largely ruled out (Gliwicz & Lampert 1990). However, these experiments were performed with the cyanobacterium *Cylindrospermopsis raciborskii,* which has rigid filaments that are substantially more resistant to ingestion by a rotifer than the relatively soft filaments of *A. flos-aquae* (Rothhaupt 1991), and hence the results should not be generalized for all filamentous cyanobacteria.

In natural seston, evidence for interference (DeMott *et al.* 2001) and for no interference (Knisley & Geller 1986; Müller-Navarra *et al.* 2000) of cyanobacterial filaments with assimilation by *Daphnia* has been demonstrated. Epp (1996) concluded that interfering effects in natural phytoplankton assemblages are highly dependent on the species of filamentous cyanobacteria. Therefore, nutritional deficiencies might be much more relevant to the well-known poor growth of herbivorous zooplankton feeding on natural assemblages of cyanobacteria than hitherto considered.

For *Daphnia* feeding on the virtually ubiquitous coccal cyanobacterium *Synechococcus* (Thierry *et al.* 2002) and the filamentous cyanobacterium *A. variabilis*, sterol limitation preempts PUFA limitation, which suggests a general significance of sterols as a cause for the low food quality of cyanobacterial C for grazers. Natural seston contains eukaryotic components that can also be ingested by filter-feeding zooplankton and thus might ameliorate the lack of sterols in cyanobacteria. Assuming that the sterol content of *S. obliquus* is representative of eukaryotic phytoplankton species and that the eukaryotic phytosterols are fully available for the synthesis of cholesterol in *Daphnia,* it can be roughly estimated that sterols constrain the quality of the ingested natural seston if more than 80% of the biomass is prokaryotic, as is often the case with bloom-forming cyanobacteria (Oliver & Ganf 2000). However, sterol content can differ considerably, even within green algae (Wright *et al.* 1980), and for a selectively feeding terrestrial herbivorous insect it has been shown that the chemical nature of the phytosterols greatly determines their degree of transformation to cholesterol by the herbivore (Behmer *et al.* 1999). Hence, the assumption that phytosterols from eukaryotic phytoplankton are fully converted to cholesterol by daphnids probably overestimates the compensating effects of eukaryotic phytoplankton; therefore, even less than a cyanobacterial share of 80% ingested biomass might lead to sterol limitation in the non-selective grazer *Daphnia*.

Eutrophication often results in proliferations of cyanobacteria, many of which replace other species by forming heavy blooms. Where zooplankton populations are unable to grow because of unsuitable food conditions, grazing ceases to be a factor in the control of phytoplankton. This uncoupling of primary and secondary production at the phytoplankton–herbivore interface is a frequently encountered problem during lake restoration, when the food chain is manipulated by altering higher trophic levels in

order to increase the grazing pressure of herbivorous zooplankton on phytoplankton and thus to improve water quality. In many cases, the seston becomes dominated by cyanobacteria, which support only a low biomass of herbivorous zooplankton, which, in turn, fails to control phytoplankton biomass (Moss *et al.* 1991; Hansson *et al.* 1998). The observation that the low sterol content of nontoxic cyanobacteria constrains the transfer efficiency of C from autotrophs to heterotrophs can be viewed as a biochemical bottom-up process that affects trophic transfer and species succession. The identification of sterols as a resource that may constrain growth and reproduction of *Daphnia* could be useful for the biological restoration of lakes, as added sterols might substantially increase secondary production in herbivorous zooplankton in lakes dominated by cyanobacteria and thus increase the ability of the zooplankton community to control phytoplankton biomass and water clarity. It remains to be seen whether in oligotrophic lakes, where most of the autotroph biomass may be picocyanobacteria, sterols similarly may determine food quality for *Daphnia*.

This article benefited from critical reading of an earlier version by K. O. Rothhaupt, W. Lampert and A. Wacker. We thank P. Merkel for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (DFG 179/4-2).

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