

Trophic upgrading of picocyanobacterial carbon by ciliates for nutrition of *Daphnia magna*

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ABSTRACT: Unicellular picocyanobacteria, such as species of the genus *Synechococcus*, are unsuitable for supporting growth and reproduction of *Daphnia* spp. In *Synechococcus* spp., long-chain polyunsaturated fatty acids (PUFAs) and sterols are absent, which leads to a low carbon transfer efficiency at the picocyanobacteria–*Daphnia* spp. interface. Herein, we address the question as to whether ciliates can serve as a trophic link between picocyanobacterial production and *Daphnia* spp. production, thereby upgrading the nutritional value of a picocyanobacterial food source by producing essential lipids such as PUFAs or sterols. In simplified experimental food chains consisting of 1 of 2 different *Synechococcus* strains, the ciliates *Colpidium campylum* or *Cyclidium* sp., and *D. magna*, we provided evidence that predation on ciliates by *Daphnia* spp. allows access to picocyanobacterial production. Since daphnids are primarily sterol-limited when grown on the picocyanobacteria *Synechococcus* spp., the observed trophic upgrading of *Synechococcus* food-quality by intermediary ciliates is most probably due to the addition of sterols or sterol-like compounds that (at least partly) release *Daphnia* spp. from sterol limitation. The absence of sterols in the ciliates used in the present study suggests that tetrahymanol and/or hopanoids provide functional equivalents of sterols not only in ciliates but also in *Daphnia* spp., thereby leading to enhanced growth of the cladocerans.

KEY WORDS: *Synechococcus* spp. · *Colpidium campylum* · *Cyclidium* spp. · Sterols · Tetrahymanol · Hopanoids

INTRODUCTION

The significance of autotrophic picoplankton (APP) for primary production in aquatic ecosystems has often been recognized. Unicellular picocyanobacteria are the major constituents of APP; they often dominate both the total phytoplankton biomass and the production in oligotrophic to mesotrophic lakes (Weisse 1993, Callieri & Stockner 2002). Numerous planktonic organisms are able to feed on picoplankton-sized particles. Heterotrophic nanoflagellates and nanociliates are considered to be the most important APP grazers (Sanders et al. 1989, Weisse et al. 1990, Šimek et al. 1995, Pernthaler et al. 1996), and themselves contribute significantly to zooplankton nutrition, thus transferring energy via the microbial loop to higher

trophic levels (Stoecker & Capuzzo 1990). However, the importance of protozoans for carbon-transfer efficiency in aquatic food webs is controversial. By repackaging their prey into accessible particles, protozoans are often implicated as a 'trophic link' to metazoan grazers (Gifford 1991). Copepods prey inefficiently on small particles (<5 µm) so that an intermediate protozoan level might give access to APP production that otherwise would not be available for higher trophic levels (Jack & Gilbert 1993). In contrast, both APP and most protozoans fall within the prey size spectrum of the aquatic keystone species of the genus *Daphnia* (Jürgens 1994) and, therefore, an intermediate trophic level can also be considered as a 'sink' of energy, since the efficiency with which carbon is transferred to higher trophic levels depends mainly on the

number of trophic steps through which it has to pass (Pomeroy & Wiebe 1988). However, picocyanobacteria, such as species of the genus *Synechococcus*, are unsuitable for supporting *Daphnia* spp. growth and reproduction (Lampert 1977a,b, 1981, Von Elert et al. 2003, Martin-Creuzburg & Von Elert 2004, Martin-Creuzburg et al. 2005); therefore, cladocerans might also benefit from grazing on protozoans, provided that protozoan carbon is more suitable for *Daphnia* spp.

In the genus *Synechococcus*, long-chain polyunsaturated fatty acids (PUFAs) and sterols are absent, which has led to the hypothesis that the low carbon-transfer efficiency at the cyanobacteria–*Daphnia* spp. interface is caused by the lack of either of these essential lipid classes (DeMott & Müller-Navarra 1997, Von Elert & Wolffrom 2001, Von Elert et al. 2003). Therefore, the role of protozoans as intermediary grazers might not be restricted to channelling energy; they might also upgrade the biochemical composition of food deficient in essential lipids by producing PUFAs or sterols (Klein Breteler et al. 1999, Bec et al. 2003b, Tang & Taal 2005).

Ciliates are abundant protists in freshwater ecosystems (Pace & Orcutt 1981, Beaver & Crisman 1982, Pace 1982, Porter et al. 1985, Müller 1989). Reports from laboratory (Tezuka 1974, Porter et al. 1979, Sanders et al. 1996) and field (Carrick et al. 1991, Pace & Funke 1991, Marchessault & Mazumder 1997, Jürgens et al. 1999, Zöllner et al. 2003) experiments have revealed that cladocerans are important ciliate predators. However, investigations of food-quality aspects of ciliates in *Daphnia* spp. nutrition are scarce. Available data suggest that, although rich in nitrogen and phosphorus, ciliates are less nutritious for daphnids than many algae (DeBiase et al. 1990, Sanders et al. 1996, Bec et al. 2003a). The fatty acid composition of ciliates seems to be highly diverse and points to species-specific differences in their capacity to synthesize PUFAs potentially important for zooplankton growth (Kaneshiro et al. 1979, Desvillettes et al. 1997, Sul et al. 2000, Klein Breteler et al. 2004). Although ciliates presumably lack the ability to synthesize sterols de novo, exogenously supplied sterols can be incorporated into cell membranes and metabolized into various other sterols (Conner et al. 1968, Harvey & McManus 1991, Harvey et al. 1997). In the absence of exogenous sterols, the pentacyclic triterpenoid alcohol tetrahymanol is produced; tetrahymanol is functionally equivalent to sterols as a structural component of cell membranes in ciliates (Conner et al. 1968). Ederington et al. (1995) reported the assimilation of tetrahymanol in copepod tissues when ciliates were offered as food, but the possible effects of tetrahymanol on the growth and reproduction of zooplankton with regard to sterol limitation have not yet been identified.

In the present study, we investigated the potential of ciliates to serve as a trophic link between *Synechococcus* spp. and zooplankton production. In experimental tritrophic food chains consisting of 1 of 2 different *Synechococcus* strains, the ciliates *Colpidium campylum* or *Cyclidium* sp., and *Daphnia magna*, we tested the ability of ciliates as intermediary grazers to upgrade the food quality of cyanobacterial APP for *D. magna*, by producing essential lipids such as PUFAs or sterols.

MATERIALS AND METHODS

Cultivation of cyanobacteria and algae. The green alga *Scenedesmus obliquus* (SAG 276-3a; Sammlung von Algenkulturen Göttingen, Germany) was used as food for stock cultures of *Daphnia magna*; it was grown in batch cultures in Cyano medium (Jüttner et al. 1983) at 20°C with illumination at 120 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, and harvested in the late-exponential phase. The coccoid cyanobacteria *Synechococcus elongatus* (SAG 89.79) and *Synechococcus* sp. Strain BO8809 (isolated from Lake Constance by A. Ernst in 1988; for detailed information see Ernst et al. 1991) were each grown in Cyano medium at 20°C with illumination at 60 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The flagellate *Cryptomonas* sp. (SAG 26.80) was grown in modified Woods Hole (WC) medium containing vitamins (Guillard 1975) at 20°C with illumination at 120 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. The cyanobacteria and *Cryptomonas* sp. were cultured semi-continuously at a dilution rate of 0.25 d^{-1} in aerated 5 l vessels. Stock solutions of these organisms for the growth experiments were prepared by concentrating the cells by centrifugation and resuspension in WC medium lacking vitamins. Carbon concentrations of the food suspensions were estimated from photometric light extinction (800 nm) and from carbon-extinction equations determined previously.

Cultivation of protozoans. *Colpidium campylum* was obtained from the Laboratoire de Biologie des Protistes (Université Blaise Pascal, France) and *Cyclidium* sp. was obtained from the culture collection of the Limnological Institute (University of Konstanz, Germany). The protozoans were cultivated semi-continuously at 20°C in mineral water (Volvic®). Ciliates were fed with 1 of the 2 *Synechococcus* strains at approximately 2 mg C l^{-1} and, in order to maintain exponential growth, 20 to 40% of the medium was renewed every other day.

For the growth experiments, ciliate cells were separated from their food source by repeated centrifugation (1600 to 1900 $\times g$, 3 to 5 min, for *Colpidium campylum*; 2000 to 2300 $\times g$, 3 to 5 min, for *Cyclidium* sp.) and resuspension in fresh medium, taking advantage of their negative geotaxis. Subsequently, the ciliate sus-

pensions were slowly filtered through a 12 μm (*Colpidium campylum*) or 5 μm (*Cyclidium* sp.) membrane filter without vacuum, and cells retained on the filter were immediately resuspended in mineral water to obtain protist stock suspensions. Subsamples of the stock suspensions were taken to estimate the number of cells in the food suspensions using a Sedgewick-Rafter chamber. Cell sizes were determined by measuring the length and width of at least 50 unpreserved cells using an image-analysis system (see Table 1); the cell volume was computed using the geometric formula of a prolate spheroid (Hillebrand et al. 1999). To estimate the contamination of the *Daphnia magna* food suspension with *Synechococcus* spp., subsamples were DAPI-stained and enumerated by epifluorescence microscopy. In the ciliate suspensions used to feed *D. magna*, *Synechococcus* spp. comprised <30% of the total carbon. Ciliate suspensions used for analysis were prepared separately and more thoroughly, leading to a contamination of <15% of the total carbon. Contamination with bacteria was negligible in all treatments (<0.6% of total carbon; bacterial carbon was estimated according to Bratbak 1985).

***Daphnia magna* growth experiments.** Growth experiments were conducted with third-clutch juveniles (born within 10 h) of a clone of *D. magna* originally isolated from Großer Binnensee, Germany (Lampert 1991). The experiments were carried out at 20°C in glass beakers filled with 200 ml of filtered lake water (0.45 μm pore-sized membrane filter). Each treatment consisted of 3 replicates with 7 daphnids each. The food suspensions, containing 1.5 mg C l⁻¹ of *Synechococcus* spp. or *Cryptomonas* sp. (1.4×10^4 cells ml⁻¹) and 1 mg C l⁻¹ (9×10^2 *Colpidium campylum* ml⁻¹; 1×10^4 *Cyclidium* sp. ml⁻¹) of ciliate biomass, were renewed daily during the 6 d experiments. Somatic growth rates (g) were determined as the increase in dry weight (W) during the experiments using:

$$g = (\ln W_t - \ln W_0) / t$$

Subsamples of the experimental daphnids consisting of 7 individuals were taken at the beginning (W_0) and at the end (W_t) of an experiment, where t is the duration of the experiments (6 d). After 24 h in a drying chamber, subsamples were weighed on an electronic balance (Mettler UMT 2; ± 0.1 μg). Growth rates were calculated as means for each treatment.

Chemical analysis. For the analysis of fatty acids and neutral lipids (sterols and sterol-like compounds, e.g. tetrahymanol), 0.5 mg particulate organic carbon (POC) of the food suspensions was filtered on precombusted GF/F filters (Whatman, 25 mm diameter). Total lipids were extracted 3 times from filters with dichloromethane/methanol (2:1, v/v), and the pooled cell-free extracts were evaporated to dryness with

nitrogen. The lipid extracts were transesterified with 3 mol methanolic HCl l⁻¹ (60°C, 15 min) for the analysis of fatty acids or saponified with 0.2 mol methanolic KOH l⁻¹ (70°C, 1 h) for the analysis of sterols. Subsequently, fatty acid methyl esters (FAMES) were extracted 3 times with 2 ml *iso*-hexane; neutral lipids were partitioned into *iso*-hexane:diethyl ether (9:1, v/v). The lipid-containing fraction was evaporated to dryness under nitrogen and resuspended in 10 to 20 μl *iso*-hexane.

Lipids were analyzed by gas chromatography on an HP 6890 GC equipped with a flame ionization detector and either a DB-225 (J&W Scientific) capillary column to analyze FAMES or an HP-5 (Agilent) capillary column to analyze sterols. Details of GC configurations are given elsewhere (Von Elert 2002 for fatty acids, Martin-Creuzburg & Von Elert 2004 for sterols). Lipids were quantified by comparison to internal standards (C17:0 and C23:0 methyl esters; 5 α -cholestan) and identified by their retention times and their mass spectra, which were recorded with a gas chromatograph/mass spectrometer (Finnigan MAT GCQ) equipped with a fused-silica capillary column (DB-225MS, J&W Scientific for FAMES; DB-5MS, Agilent for sterols). Sterols and sterol-like compounds were analyzed in their free form and as their trimethylsilyl derivatives. Spectra were recorded between 50 and 600 atomic mass units in the EI ionization mode. Mass spectra were identified by comparison with mass spectra of reference substances (e.g. tetrahymanol and diplopterol, provided by M. Rohmer) or spectra found in a self-generated spectra library or in the literature (e.g. Ten Haven et al. 1989, Venkatesan 1989, Harvey & McManus 1991). The detection limit was 20 ng of fatty acid or sterol. It was not possible to distinguish between petroselinic acid (C18:1n-12) and oleic acid (C18:1n-9). The absolute amount of each lipid was related to the POC. Therefore, aliquots of the food suspensions were filtered onto precombusted glass-fiber filters (Whatman GF/F, 25 mm diameter) and analyzed for POC and nitrogen using an NCS-2500 analyzer (ThermoQuest). For determination of particulate phosphorus, aliquots were collected on acid-rinsed polysulfon filters (HT-200; Pall) and digested with a solution of 10% potassium peroxodisulfate and 1.5% sodium hydroxide for 60 min at 121°C, and soluble reactive phosphorus was determined using the molybdate-ascorbic acid method (Greenberg et al. 1985).

Data analysis. The somatic growth rates of *Daphnia magna* were analyzed using 1-way analysis of variance (ANOVA). Raw data met the assumption of homogeneity of variance (Levene's test); single-treatment effects were tested by Tukey's HSD post-hoc test ($p = 0.05$).

RESULTS

Microscopy revealed high grazing activities of both ciliate species on the 2 *Synechococcus* strains. The red cells of *Synechococcus* sp. Strain BO8809 and the green cells of *S. elongatus* could easily be observed in the food vacuoles of the ciliates.

The 2 ciliate species differed significantly in size (Table 1), but cell sizes were not affected by the food source of the ciliates. The cell sizes of *Cryptomonas* sp. and *Cyclidium* sp. were similar.

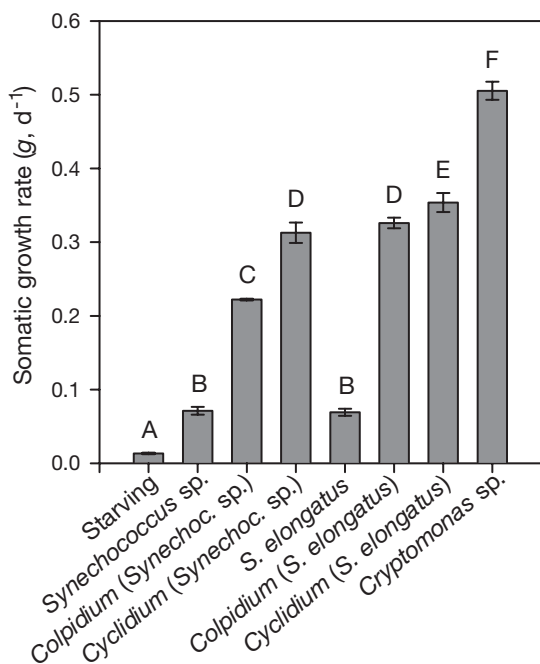


Fig. 1. *Daphnia magna*. Mean \pm SD ($n = 3$) juvenile somatic growth rates of daphnids grown on *Synechococcus* sp. Strain BO8809, *S. elongatus*, or ciliates previously fed 1 of the 2 *Synechococcus* strains (given in parentheses). Growth without food supply ('Starving') and growth on flagellate *Cryptomonas* sp. are shown for comparison. Bars labelled with same letter are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA)

Growth experiments

Juvenile somatic growth rates (g) of *Daphnia magna* were significantly affected by the food supplied in the growth experiments (ANOVA, $F_{7,16} = 1146$; $p < 0.001$; Fig. 1). Growth rates of *D. magna* ranged from 0.01 d^{-1} without food supply to 0.51 d^{-1} when fed on *Cryptomonas* sp. The cyanobacteria *Synechococcus* sp. Strain BO8809 and *S. elongatus* did not differ in their food quality for *D. magna* (Tukey's HSD, $p = 1$); growth on both strains was in general poor ($g = 0.07 \text{ d}^{-1}$). In comparison to growth on pure *Synechococcus* spp., *D. magna* exhibited significantly higher growth rates on ciliates fed either of the 2 *Synechococcus* strains. Growth of *D. magna* was significantly higher on either of the ciliates fed on *S. elongatus* than on either of the ciliates fed on *Synechococcus* sp. Strain BO8809 (Tukey's HSD, $p < 0.05$). Regardless of the food source of the ciliates, *Cyclidium* sp. improved the growth of *D. magna* more than *Colpidium campylum* (Tukey's HSD, $p < 0.05$).

Elemental nutrient ratios

The carbon:nitrogen (C:N) and carbon:phosphorus (C:P) ratios of the various food suspensions are given in Table 2. The 2 *Synechococcus* strains were characterized by high nitrogen and phosphorus content; however, the C:N and C:P ratios were slightly lower in *Synechococcus* sp. Strain BO8809 than in *S. elongatus*. The C:N ratios of ciliates ranged from 4.40 to 4.92. The C:P ratios were in general lower in *Colpidium campylum* than in *Cyclidium* sp. The nutrient ratios of the ciliates seemed to be affected by the nutrient ratios of their food source: the C:N and C:P ratios tended to be lower when the nitrogen- and phosphorus-rich *Synechococcus* sp. Strain BO8809 was offered as food. The nitrogen content of the flagellate *Cryptomonas* sp. was lower (higher C:N ratio) than that of the ciliates, whereas the C:P ratio was comparable to that of *Cyclidium* sp.

Table 1. *Colpidium campylum* and *Cyclidium* sp. Cell sizes of ciliates grown on 1 of 2 different *Synechococcus* strains (*Synechococcus* sp. = Strain BO8809) and cell size of flagellate *Cryptomonas* sp. ($n = 50$ for all species)

| Species | Length (μm) | | | Width (μm) | | | Vol. (μm^3) Mean |
|--------------------------------|--------------------------|------|------|-------------------------|------|------|----------------------------------|
| | Mean \pm SD | Max. | Min. | Mean \pm SD | Max. | Min. | |
| <i>Colpidium campylum</i> fed: | | | | | | | |
| <i>Synechococcus</i> sp. | 43.9 \pm 3.9 | 52.3 | 36.2 | 20.0 \pm 2.4 | 24.3 | 14.9 | 9164.7 |
| <i>S. elongatus</i> | 44.4 \pm 4.2 | 52.5 | 27.2 | 20.0 \pm 2.7 | 25.8 | 12.1 | 9301.2 |
| <i>Cyclidium</i> sp. fed: | | | | | | | |
| <i>Synechococcus</i> sp. | 18.0 \pm 2.1 | 22.3 | 12.4 | 8.9 \pm 1.5 | 15.7 | 6.6 | 739.9 |
| <i>S. elongatus</i> | 17.8 \pm 2.2 | 22.2 | 10.4 | 8.1 \pm 1.1 | 11.3 | 5.6 | 611.8 |
| <i>Cryptomonas</i> sp. | 19.1 \pm 1.7 | 23.1 | 15.0 | 8.8 \pm 1.1 | 11.4 | 7.0 | 778.3 |

Sterols and sterol-like compounds

Sterols were not detected in the 2 *Synechococcus* strains or in the 2 ciliate species. Neutral lipids of *Colpidium campylum* were characterized by the triterpenoid alcohol tetrahymanol (gammaceran-3 β -ol) and its hopanoid isomer diplopterol. In *Cyclidium* sp., tetrahymanol was accompanied by hopan-3 β -ol (Table 3). Stigmasterol (24-ethylcholesta-5,22-dien-3 β -ol) and epibrassicasterol (24-methylcholesta-5,22-dien-3 β -ol) were the principal sterols found in *Cryptomonas* sp. Epibrassicasterol, the 24 α -epimer of brassicasterol, occurs in cryptophycean algae, such as species of *Cryptomonas* and *Rhodomonas* (Goad et al. 1983, Gladu et al. 1990). Although we did not determine the stereochemistry of the side chain at C-24, a 24 α -configuration was also assumed.

Fatty acids

PUFAs were not detected in either *Synechococcus* strain, except for low amounts of 16:2n-6 in both strains and traces of 16:2n-4 in *Synechococcus* sp. Strain BO8809 (Fig. 2). Instead, both strains were characterized by high amounts of short-chain saturated fatty acids and the monounsaturated fatty acid 16:1n-7.

Table 2. Mean \pm SD (n = 3) carbon:nitrogen (C:N) and carbon:phosphorus (C:P) molar ratios of different food suspensions offered to *Daphnia magna*. *Synechococcus* sp. = Strain BO8809

| Food | C:N | C:P |
|--------------------------------|-----------------|--------------------|
| <i>Synechococcus</i> sp. | 2.89 \pm 0.62 | 79.87 \pm 34.39 |
| <i>S. elongatus</i> | 4.46 \pm 0.03 | 156.29 \pm 12.13 |
| <i>Colpidium campylum</i> fed: | | |
| <i>Synechococcus</i> sp. | 4.40 \pm 0.16 | 58.78 \pm 2.78 |
| <i>S. elongatus</i> | 4.73 \pm 0.03 | 71.79 \pm 3.61 |
| <i>Cyclidium</i> sp. fed: | | |
| <i>Synechococcus</i> sp. | 4.80 \pm 0.08 | 113.19 \pm 3.92 |
| <i>S. elongatus</i> | 4.92 \pm 0.35 | 161.96 \pm 3.73 |
| <i>Cryptomonas</i> sp. | 5.49 \pm 0.07 | 123.95 \pm 6.14 |

Compared to the fatty acid composition of the cyanobacteria, *Colpidium campylum* contained high amounts of n-6 PUFAs such as 16:2n-6, 18:2n-6, and 18:3n-6, and, in addition, moderate amounts of 16:2n-4, 16:3n-4, 18:1n-7, and 18:2n-7. In contrast, *Cyclidium* sp. was characterized by high amounts of n-3 PUFAs (18:3n-3, 18:4n-3) rather than n-6 PUFAs (Fig. 2). Dietary effects on the fatty acid content of the ciliates were negligible. The fatty acid composition of *Cryptomonas* sp. was dominated by high amounts of n-3 PUFAs, such as 18:3n-3, 18:4n-3, and 20:5n-3. The total fatty acid content was highest in *Cryptomonas* sp. (211.0 \pm 12.6 $\mu\text{g mg}^{-1}$ C) and lowest in the *Synechococcus* strains (79.3 \pm 4.9 $\mu\text{g mg}^{-1}$ C in *Synechococcus* sp. Strain BO8809; 98.8 \pm 4.6 $\mu\text{g mg}^{-1}$ C in *S. elongatus*). The ciliates showed intermediate contents of total fatty acids: 121.7 \pm 2.3 $\mu\text{g mg}^{-1}$ C in *Colpidium campylum* fed on *Synechococcus* sp. Strain BO8809; 112.0 \pm 4.7 $\mu\text{g mg}^{-1}$ C in *C. campylum* fed on *S. elongatus*; 131.7 \pm 13.0 $\mu\text{g mg}^{-1}$ C in *Cyclidium* sp. fed on *Synechococcus* sp. strain BO8809; and 161.4 \pm 18.4 $\mu\text{g mg}^{-1}$ C in *Cyclidium* sp. fed on *S. elongatus*.

DISCUSSION

In oligotrophic to mesotrophic lakes, picocyanobacteria, such as *Synechococcus* species, are abundant autotrophic prokaryotes that often contribute significantly to primary production, thereby forming the base of a complex pelagic food web (Weisse 1993, Callieri & Stockner 2002). Grazing by ciliates can be considered as an important loss process controlling biomass and production of APP in freshwater ecosystems (Fahnenstiel et al. 1991, Sherr et al. 1991, Šimek et al. 1995, Hadas & Berman 1998).

The 2 ciliate species *Colpidium campylum* and *Cyclidium* sp. used in the present study showed high grazing activities on both *Synechococcus* strains. Although bacterial contamination was marginal, the consumption of bacteria could not be excluded since none of the ciliate cultures was axenic. However, the importance of picocyanobacteria as a potential carbon source for ciliates (e.g. *Cyclidium* sp.) has already been

Table 3. *Colpidium campylum* and *Cyclidium* sp. Mean \pm SD (n = 3) neutral lipid content of ciliates ($\mu\text{g mg}^{-1}$ C). nd: not detected (*Synechococcus* sp. = Strain BO8809)

| Lipid | <i>Colpidium campylum</i> fed: | | <i>Cyclidium</i> sp. fed: | |
|---------------------|--------------------------------|---------------------|---------------------------|---------------------|
| | <i>Synechococcus</i> sp. | <i>S. elongatus</i> | <i>Synechococcus</i> sp. | <i>S. elongatus</i> |
| Tetrahymanol | 5.87 \pm 1.64 | 8.41 \pm 1.51 | 1.95 \pm 0.31 | 2.73 \pm 0.32 |
| Diplopterol | 10.48 \pm 2.45 | 12.99 \pm 2.39 | nd | nd |
| Hopan-3 β -ol | nd | nd | 5.01 \pm 0.17 | 5.28 \pm 0.23 |
| Total | 16.35 \pm 4.09 | 21.40 \pm 3.90 | 6.96 \pm 0.15 | 8.01 \pm 0.51 |

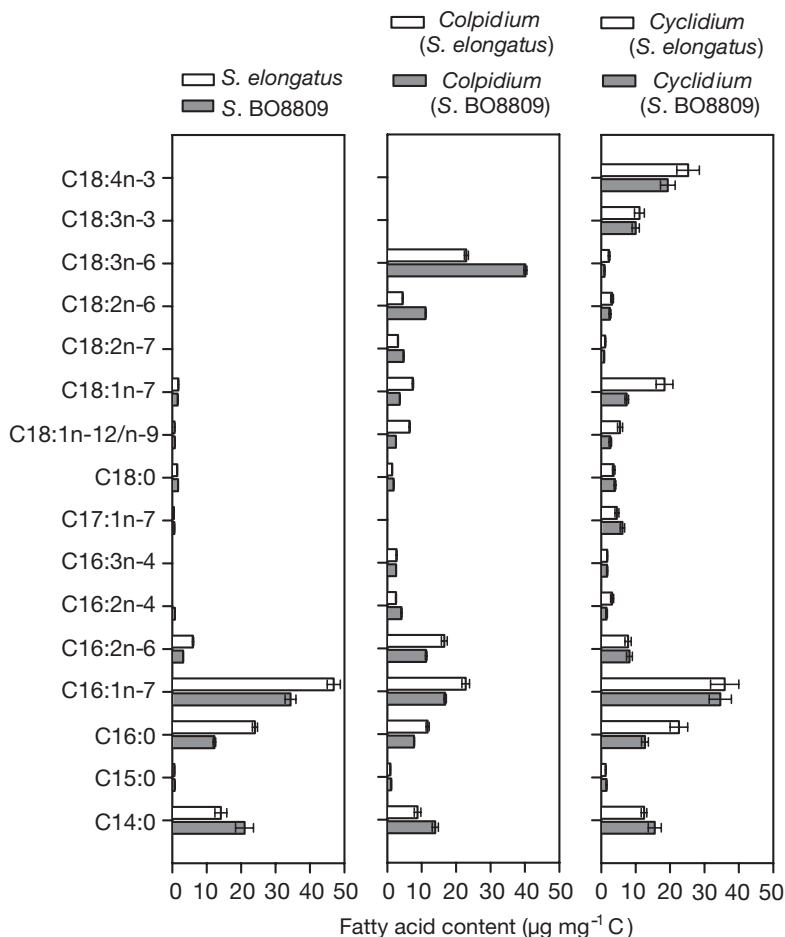


Fig. 2. *Synechococcus* sp. Strain BO8809 and *S. elongatus*, and *Colpidium campylum* and *Cyclidium* sp. previously fed either *Synechococcus* sp. Strain BO8809 or *S. elongatus* (given in parentheses). Mean \pm SD; (n = 3) fatty acid content

demonstrated (Šimek et al. 1996), and, moreover, Šimek et al. (1996) reported a strong size selection towards larger picoplankton prey; therefore, the consumption of bacteria in the present study appears to have been negligible.

Invertebrate grazers, in particular cladocerans of the genus *Daphnia*, feed on a wide size-range of particles that includes picocyanobacteria and small ciliates (Jürgens 1994). It is well established that picocyanobacteria such as *Synechococcus* spp. are unsuitable for supporting growth of *Daphnia* spp. (Lampert 1977a,b, DeMott & Müller-Navarra 1997, Von Elert et al. 2003, Martin-Creuzburg & von Elert 2004, Martin-Creuzburg et al. 2005), whereas effects of ciliates on *Daphnia* spp. nutrition are controversial. In the field, ciliates are suppressed during population peaks of *Daphnia* spp., and several experimental studies have shown that daphnids effectively prey on ciliates up to a

certain size (Tezuka 1974, Porter et al. 1979, Jack & Gilbert 1993, Sanders et al. 1996). However, ciliates are less nutritious for *Daphnia* spp. than many algae (DeBiase et al. 1990, Wickham et al. 1993, Sanders et al. 1996). In the present study, we addressed whether ciliates can serve as a trophic link between *Synechococcus* spp. production and *Daphnia* spp. production, thereby upgrading the nutritional value of a picocyanobacterial food source. Both *Synechococcus* strains used here were unsuitable as a sole food source for *D. magna*, whereas when either of the 2 ciliate species that fed on the *Synechococcus* strains was used as a food source, it significantly enhanced the somatic growth of the daphnid. The results of this simplified experimental food chain (*Synechococcus* spp. \rightarrow ciliates \rightarrow *D. magna*) suggest that predation on ciliates by *Daphnia* species allows access to picocyanobacterial production and provides a linkage of carbon flow to higher trophic levels. Moreover, ciliates obviously improve the quality of the supplied food by the addition of essential components that are absent in *Synechococcus* spp.

Synechococcus elongatus is a nontoxic picocyanobacterium that is assimilated well by *Daphnia* spp. (Lampert 1977a,b, 1981). The elemental nutrient ratios determined show that both *Synechococcus* strains and, possibly as a consequence, both ciliate species, were rich in phosphorus and nitrogen, which is in accordance with previous data (DeBiase et al. 1990, Sanders et al. 1996, DeMott 1998). In all treatments, C:P ratios

were far below those determined for P-limited growth of *Daphnia* spp. (C:P > 300; Sterner & Schulz 1998), and C:N ratios were considerably lower than those usually found in phytoplankton species (e.g. *Cryptomonas* sp. in the present study). Thus, the growth-enhancing trophic upgrading effect is unlikely to be due to the elemental nutrient supply.

Stoecker & Capuzzo (1990) proposed that ciliates might be an important source of essential lipids, thus representing a supplementary diet to enhance growth of *Daphnia* spp. The lack of sterols in *Synechococcus* spp. has recently been identified as the major food quality constraint in *Daphnia* species (Von Elert et al. 2003, Martin-Creuzburg et al. 2005), which implies that the observed trophic upgrading of picocyanobacterial food quality by intermediary ciliates is due to the addition of sterols. However, available data suggest that ciliates lack the ability to synthesize sterols de novo (Conner et al. 1968, Harvey & McManus 1991,

Harvey et al. 1997, Klein Breteler et al. 2004). Instead, the pentacyclic triterpenoid alcohol tetrahymanol is produced, which is functionally equivalent to sterols as a structural component of cell membranes. Numerous studies have shown that exogenously supplied sterols can be incorporated into cell membranes of ciliates and, as a consequence, inhibit tetrahymanol synthesis (first demonstrated by Conner et al. 1968). Hence, the occurrence of sterols in ciliates depends on their diet: ciliates feeding on bacteria or picocyanobacteria cannot rely on a dietary source of sterols and, therefore, are expected to produce tetrahymanol. Sterols were not detected in either of the 2 ciliate species used in the present study; this lack of sterols can be attributed to the absence of sterols in their food source, *Synechococcus* spp. Instead, relatively high amounts of tetrahymanol and its isomer diplopterol were detected in *Colpidium campylum*; *Cyclidium* sp. contained considerably lower amounts of tetrahymanol and no diplopterol. Tetrahymanol was first identified in *Tetrahymena pyriformis* (Mallory et al. 1963), a ciliate related to *C. campylum*, but has recently also been observed in several marine scuticociliates, including *Cyclidium* sp. (Harvey & McManus 1991). Neutral lipids of *Cyclidium* sp. were dominated by hopan-3 β -ol, a hopanoid that seems to be common among scuticociliates (Harvey & McManus 1991, Harvey et al. 1997).

Like all arthropods, crustaceans are incapable of synthesizing sterols de novo and therefore must acquire these essential nutrients from their diet (Goat 1981). Daphnids feeding on bacterivorous or picocyanobacterivorous ciliates cannot rely on a dietary source of sterols and, therefore, are expected to be sterol limited. However, tetrahymanol and related compounds that functionally replace sterols as membrane reinforcers in ciliates (Raederstorff & Rohmer 1988) might also be suitable as sterol surrogates in crustacean tissues. Thus, the observed trophic upgrading of *Synechococcus* spp. food quality by intermediary ciliates might be due to the addition of tetrahymanol and hopanoids, which at least partly release *Daphnia* spp. from sterol limitation. Ederington et al. (1995) reported the assimilation of tetrahymanol in tissues of ciliate-fed copepods (mainly in eggs) and suggested that tetrahymanol can provide functional equivalence to cholesterol, thereby maintaining minimal egg production. Beside their role as structural components of cell membranes, sterols serve as precursors for many bioactive molecules, such as ecdysteroids, which are involved in the process of molting (Goat 1981). Whether tetrahymanol and related compounds affect growth and/or reproduction of crustaceans remains to be tested, possibly by supplementation of a sterol-free diet with these compounds. Recently, for copepods, Klein Breteler et al. (2004) found

no evidence for trophic upgrading of a sterol-deficient diet by the marine ciliate *Strombidium sulcatum*. However, neither sterols, tetrahymanol nor other sterol surrogates were detected in *S. sulcatum*, which possibly makes this bacterivorous ciliate unsuitable as a single food source for copepods, and corroborates the finding of this study that trophic upgrading of a sterol-free diet can be attributed to tetrahymanol-related compounds in ciliates.

Von Elert et al. (2003) have shown that when the shortage of sterols in *Synechococcus* spp. is overcome by supplementation with cholesterol, growth of *Daphnia galeata* is limited by the availability of long-chain PUFAs. Hence, the addition of PUFAs by the intermediary ciliates might have further improved the trophic upgrading effect, provided that the daphnids were released from sterol limitation.

Long-chain PUFAs were not detected in either of the *Synechococcus* strains. However, the fatty acid composition of *Colpidium campylum* was characterized by relatively high amounts of n-6 PUFAs (18:2n-6, 18:3n-6), which suggested a de novo synthesis of these fatty acids by *C. campylum*. This is corroborated by the finding that species of *Tetrahymena* synthesize n-6 PUFAs when grown on fatty-acid-free media (Sul & Erwin 1997). In contrast, *Cyclidium* sp. synthesized high amounts of n-3 PUFAs (18:3n-3, 18:4n-3), which have previously been detected in marine scuticociliates: 18:3n-3 in *Pleuronema* sp. (Ederington et al. 1995), and 18:3n-3 and 18:4n-3 in *Parauronema acutum* (Sul & Erwin 1997, Sul et al. 2000).

In most animals, the PUFAs 18:2n-6 and 18:3n-3 are essential dietary compounds that play important roles in animal physiology (Cook 1996). In *Daphnia* species, the n-6 PUFAs found in *Colpidium campylum* (18:2n-6, 18:3n-6) might be further converted into 20:4n-6, an intermediate in prostaglandin synthesis (Weers et al. 1997). However, laboratory experiments and correlative field studies suggest either 18:3n-3 or 20:5n-3 as a potentially limiting resource that constrains proper growth of *Daphnia* spp. (Müller-Navarra 1995, Müller-Navarra et al. 2000, Wacker & Von Elert 2001, Von Elert 2002, Becker & Boersma 2003, Ravet et al. 2003). In *Cyclidium* sp., comparatively high amounts of n-3 PUFA (18:3n-3, 18:4n-3) were detected, which, in *Daphnia* spp., can be converted into 20:5n-3 through a process of elongation and desaturation. Although the conversion of 18:3n-3 into 20:5n-3 is low (Weers et al. 1997, Von Elert 2002), the availability of these PUFAs might be adequate to meet metabolic demands. Even though *Cyclidium* sp. contains less tetrahymanol and related compounds than *Colpidium campylum*, it resulted in a higher trophic upgrading of picocyanobacterial carbon, which leads to the conclusion that the amount of tetrahymanol-related compounds present in

Cyclidium sp. was sufficient to release daphnids from the sterol limitation observed on pure *Synechococcus* spp. (Von Elert et al. 2003, Martin-Creuzburg et al. 2005). Hence, the superior quality of *Cyclidium* sp. must be attributed to compounds present in *Cyclidium* sp. but not in *C. campylum*. In accordance with the finding that when sterol requirements are met, growth of daphnids on *Synechococcus* spp. becomes limited by n-3 PUFAs (Von Elert et al. 2003), the synthesis of n-3 PUFAs in *Cyclidium* sp. but not in *Colpidium campylum* provides a reasonable explanation for the superior food quality of the former.

Differences in the food quality of *Colpidium campylum* and *Cyclidium* sp. for *Daphnia magna* might also be due to their different cell sizes. Jack & Gilbert (1993) reported that large ciliates are less susceptible to *Daphnia* spp. predation, and showed that clearance rates decrease with increasing ciliate size. *C. campylum* might be close to the upper size range of food particles that can be ingested as a whole by juvenile *D. magna*. Large particles up to a certain size have to be manipulated, which results in a reduced ingestion efficiency (Porter et al. 1979). However, ciliates much larger than *C. campylum* have been shown to be ingestible by *D. pulex*, and among various ciliates tested, a ciliate similar in size to *C. campylum* (*Tetrahymena pyriformis*) was most vulnerable to *D. pulex* predation (Jack & Gilbert 1993). Therefore, it seems unlikely that the observed differences in food quality of the 2 ciliates derive exclusively from differences in cell sizes.

Although the ciliates used herein improved *Synechococcus* spp. food quality for *Daphnia magna*, growth rates achieved on a ciliate diet rank far below those achieved with the flagellate *Cryptomonas* sp., which contained comparatively high amounts of the Δ^5 sterols stigmasterol and epibrassicasterol. Δ^5 sterols such as stigmasterol have been shown to support somatic growth and reproduction of daphnids when used as supplements to the sterol- and PUFA-deficient *S. elongatus* (Martin-Creuzburg & Von Elert 2004). In addition, the fatty acid composition of *Cryptomonas* sp. was dominated by high levels of n-3 PUFAs, especially 18:3n-3, 18:4n-3, and 20:5n-3, which is in accordance with previous data (e.g. Von Elert & Stampfl 2000). This implies that the high food quality of *Cryptomonas* sp. is a combined effect of its sterol and PUFA composition.

In summary, the presented data clearly show that predation on ciliates by *Daphnia magna* (and presumably other species of this genus) can provide a linkage between picocyanobacterial production and zooplankton production. Especially in oligotrophic to mesotrophic lakes, where APP species often dominate phytoplankton assemblages, this might be an impor-

tant pathway channelling carbon and essential nutrients to higher trophic levels. Daphnids have been shown to be primarily sterol-limited when grown on the picocyanobacteria *Synechococcus* spp., which implies that the observed trophic upgrading of APP food quality by intermediary ciliates is due to the addition of sterols or sterol-like compounds that (at least partly) release *Daphnia* spp. from sterol limitation. The absence of sterols in ciliates suggests that tetrahymanol and/or hopanoids are functional equivalents to sterols not only in ciliates but also in *Daphnia* spp., thereby leading to enhanced growth.

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