

# Trophic upgrading of autotrophic picoplankton by the heterotrophic nanoflagellate *Paraphysomonas* sp.

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## Abstract

We investigated whether trophic repackaging of autotrophic picoplankton by phagotrophic protists is associated with an improvement in food quality for the metazooplankton *Daphnia magna* (i.e., whether trophic upgrading occurs in this system). The nutritional value of the autotrophic species *Microcystis aeruginosa* PCC7806, *Synechococcus* sp. strain BO8809, *Synechococcus elongatus* SAG 89.79, and *Choricystis minor* KR1988/8, and of the heterotrophic nanoflagellate *Paraphysomonas* sp. grown on these different picoplanktonic species was evaluated in standardized growth experiments with *D. magna*. In order to investigate the functional role of the flagellate in the simplified autotrophic picoplankton–*Daphnia* food chain, *Paraphysomonas* sp. was grown on the different picoplanktonic organisms and subsequently separated from the food items before being fed to *D. magna*. The presence of *Paraphysomonas* sp. as an intermediary trophic step enhanced somatic growth and reproduction of *D. magna*. Supplementation of *Synechococcus* sp. with lipids from *Paraphysomonas* sp. (grown on *Synechococcus* sp.) revealed that trophic upgrading of autotrophic picoplankton is due to the additional lipids present in the flagellate. *Paraphysomonas* sp. synthesized polyunsaturated fatty acids and sterols de novo, which most likely explains the trophic upgrading. *Paraphysomonas* sp. also improved the food quality of *M. aeruginosa* PCC7806, which is toxic for *D. magna*. The heterotrophic flagellate *Paraphysomonas* sp. is capable of trophically upgrading a poor quality food source not only by producing essential lipids, but also by detoxifying the cyanobacterial food organism.

The consumption of picoplanktonic organisms (heterotrophic bacteria and autotrophic picoplankton) by phagotrophic protists has been recognized as a major pathway of carbon flow (Azam et al. 1983). Autotrophic picoplankton (APP) accounts for the bulk of primary production in large parts of open oceans and in many oligotrophic lakes (Weisse 1993; Callieri and Stockner 2002). However, such picoplankton is largely unavailable to direct consumption by most crustacean grazers (except cladocerans), whose grazing apparatus is too coarse to retain picoplanktonic particles. In contrast, heterotrophic protists are efficiently grazed by crustacean zooplankton. By repackaging their picoplanktonic prey into particles accessible for crustacean grazers, phagotrophic protists represent a crucial link channeling picoplankton production to higher trophic levels (Sherr and Sherr 1988; Gifford 1991). The assimilation of picoplankton by protozoa leads to substantial losses in organic carbon via respiration, which has led to a debate about the quantitative significance of the transfer of picoplankton production to higher trophic levels via protists (Sherr et al. 1987). Especially in systems dominated by metazoan grazers that are able to feed directly on

picoplankton (i.e., *Daphnia*), trophic repackaging may be regarded as a sink of carbon (Stockner and Shortreed 1989). However, zooplankton production is determined not only by the quantity of the available carbon, but also its quality. A considerable amount of research has addressed phytoplankton food quality (Ahlgren et al. 1990), but surprisingly few studies have focused on the nutritional value of protozoa for metazoan grazers.

In natural systems, especially in freshwater habitats, the availability of sestonic phosphorus (Elser et al. 2001) can determine zooplankton growth. Ciliates and heterotrophic nanoflagellates (HNFs) are rich in phosphorus (Caron and Goldman 1990; Sanders et al. 1996) and, therefore, might be a high-quality food source for zooplankton. However, elemental content alone is insufficient to predict food quality; and essential lipids such as the (n-3) series of polyunsaturated fatty acids (PUFAs) (Von Elert 2002) and sterols (Von Elert et al. 2003; Martin-Creuzburg and Von Elert 2004) can limit zooplankton growth.

Little is known about the lipid composition of heterotrophic protists. The scarce data available suggest that the lipid composition of protozoa depends on the synthetic capacities particular to each species and also on the biochemical composition of their food (Desvillettes et al. 1997; Véra et al. 2001). This might explain the extreme variability in the observed food quality of protozoa for metazoan grazers (Sanders and Wickham 1993). Several studies have reported that heterotrophic protists as the sole food source are of low quality for zooplankton (Sanders et al. 1996; Bec et al. 2003a), whereas others have argued that HNFs feeding on microalgae are a high-quality food for zooplankton (Klein Breteler et al. 1999; Tang et al. 2001; Bec et al. 2003b). These latter studies have also shown that HNFs as an intermediary trophic step enhance the

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nutritional value of their microalgal diet for zooplankton. This trophic upgrading of microalgal food (Klein Breteler et al. 1999; Bec et al. 2003b) has been suggested to be due to new lipid compounds synthesized by the HNFs, but this hypothesis has not yet been tested.

In the present study, we investigated whether trophic repackaging of APP is associated with an improvement in food quality for metazooplankton. We used three strains of cyanobacteria (*Microcystis aeruginosa*, *Synechococcus* sp., and *Synechococcus elongatus*) and one picoeukaryotic alga (*Choricystis minor*) as model APP organisms. Using a simplified food chain from APP to metazooplankton, we examined whether the presence of an intermediary trophic step (the HNF *Paraphysomonas* sp.) enhanced the development and reproduction of the metazoan grazer *Daphnia magna*. Lipid analyses (fatty acids, sterols) were combined with dietary supplementation of lipids to test whether trophic upgrading by the heterotrophic flagellate was due to the synthesis of additional lipids.

## Materials and methods

*Cultures and food suspensions of autotrophic organisms*—*Synechococcus* sp. strain BO8809, *S. elongatus* SAG 89.79, and *M. aeruginosa* PCC7806 were grown in Cyano medium (Von Elert and Wolffrom 2001) at 20°C with illumination at 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . *Cryptomonas* sp. strain SAG 26.80 and *C. minor* KR1988/8 were grown in modified WC medium with vitamins (Von Elert and Wolffrom 2001) at 20°C with illumination at 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The cyanobacteria and the algae were cultured semicontinuously at a dilution rate of 0.25  $\text{d}^{-1}$  using aerated 5-liter vessels. Stock solutions of the different autotrophic organisms for the *D. magna* growth experiments were prepared by centrifugation and resuspension of the cultured cells in WC lacking vitamins. The carbon concentrations of the autotrophic food suspensions were estimated from photometric light extinction (800 nm) and from carbon-extinction regressions determined previously.

*Cultures and food suspensions of Paraphysomonas sp.*—The chrysoomonad *Paraphysomonas* sp. was obtained from the culture collection of the Limnological Institute (University of Konstanz, Germany). *Paraphysomonas* sp. was cultivated on different food organisms (*M. aeruginosa*, *Synechococcus* sp., *S. elongatus*, *C. minor*). These *Paraphysomonas* sp. cultures were grown semicontinuously in 300 mL of WC medium with vitamins in 500-mL flasks at 20°C. Each of the four different food organisms was fed to the flagellate by replacing 10% of the culture volume with WC medium with vitamins and the food organism every 12 h. Every other day, the *Paraphysomonas* sp. cultures were transferred into clean flasks. Two days before the *Paraphysomonas* sp. cultures were harvested for the *D. magna* growth experiments, the supply of medium and food was stopped to allow *Paraphysomonas* sp. to reduce the concentration of autotrophic picoplankton in the flagellate cultures. However, at the end of this 2-d period, picoplanktonic cells were still observed in the culture medium and in the *Paraphysomonas* sp. food vacuoles. For the

growth experiments *Paraphysomonas* sp. cells were separated from their food source by repeated centrifugation (4 min at 2,000  $\times$  g) and resuspension in fresh medium, taking advantage of their negative geotaxis. Subsequently, the flagellate suspensions were slowly filtered through a 5- $\mu\text{m}$  membrane filter without vacuum, and cells retained on the filter were immediately resuspended in WC without vitamins to obtain protist stock suspensions for the *D. magna* growth experiments. Cell concentrations of *Paraphysomonas* sp. were determined with an electronic particle counter, and *Paraphysomonas* sp. carbon concentrations offered to daphnids were estimated using the carbon conversion factor proposed by Menden-Deuer and Lessard (2000). Contamination of the flagellate suspensions with heterotrophic bacteria and autotrophic cells was determined using the method described by Porter and Feig (1980). The carbon contributions of heterotrophic bacteria and cyanobacteria were calculated using the conversion factors proposed by Bratbak (1985) and Verity et al. (1992). The carbon concentration of *Paraphysomonas* sp. ranged from 0.7 to 1.5  $\text{mg C L}^{-1}$  and comprised 92–98% of the total carbon concentration of the food suspensions. The contamination with autotrophic particles never exceeded 4% of the total carbon of either of the *Synechococcus* strains and 6.8% of *M. aeruginosa* and *C. minor*; the contamination with heterotrophic bacteria was negligible in all treatments (<2% of the total carbon).

*Daphnia growth experiments*—Growth experiments were conducted with third-clutch juveniles (born within 10 h) of a clone of *D. magna*. The experiments were carried out at 20°C and an illumination of 60  $\mu\text{mol m}^{-2} \text{s}^{-1}$  in glass beakers filled with 200 mL of filtered lake water (0.45  $\mu\text{m}$  pore-sized membrane filter). Each treatment consisted of three replicates with seven animals each. Somatic growth rates (g) were determined as the increase in dry weight (W) during the experiments using the equation:

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

Subsamples of the experimental animals consisting of seven individuals were taken at the beginning ( $W_0$ ) and at the end ( $W_t$ ) of an experiment. After 24 h in a drying chamber, subsamples were weighed on an electronic balance (Mettler UMT 2;  $\pm 0.1 \mu\text{g}$ ). Growth rates were calculated as means for each treatment ( $n = 3$ ).

In the first set of growth experiments, we investigated the nutritional value for *D. magna* of the autotrophic species *M. aeruginosa*, *Synechococcus* sp., *S. elongatus*, and *C. minor* and of *Paraphysomonas* sp. grown on each of these autotrophic species. Starved daphnids and daphnids fed high-quality food (*Cryptomonas* sp.) served as references. The food suspensions, containing 1.5  $\text{mg C L}^{-1}$  of the autotrophic species and 0.7–1.5  $\text{mg C L}^{-1}$  of *Paraphysomonas* sp., were renewed daily during the 6-d experiments.

A second set of growth experiments was designed to test whether lipids synthesized by *Paraphysomonas* sp. were involved in the trophic upgrading of picocyanobacteria. *D. magna* was reared on *Synechococcus* sp., *Synechococcus* sp.

supplemented with *Paraphysomonas* sp. lipids, and *Synechococcus* sp. supplemented with its own lipids. Because bovine serum albumin (BSA) was used for the lipid amendment, *Synechococcus* sp. was also supplemented with BSA without additional lipids to exclude possible effects of BSA on the growth of *D. magna*.

**Extraction and supplementation of lipids**—Twenty micrograms of particulate organic carbon (POC) of *Paraphysomonas* sp. (grown on *Synechococcus* sp.) or of *Synechococcus* sp. were loaded onto precombusted GF/F filters (Whatman) and extracted three times with a mixture of dichloromethane/methanol (2 : 1, v/v). The pooled cell-free extracts were evaporated with nitrogen to dryness, weighed to the nearest 0.1  $\mu\text{g}$ , and resuspended in ethanol (2.5 mg mL<sup>-1</sup>). To enrich *Synechococcus* sp. with lipids of *Paraphysomonas* sp. or with its own lipids, 15 mg of BSA was dissolved in 1 mL of ultrapure water, and 300  $\mu\text{L}$  of the lipid stock solution was added. After addition of 1 mg POC of *Synechococcus* sp., the volume was brought to 10 mL with Cyano medium. The resulting suspension was incubated on a rotary shaker (100 revolutions per minute min<sup>-1</sup>) for 4 h. Surplus lipids and BSA were removed by washing the cells three times in 10 mL of fresh medium using to the method described by Von Elert (2002). The resulting *Synechococcus* sp. suspensions were used as food in the *D. magna* growth experiments.

**Analysis of fatty acids and sterols**—To analyze fatty acids and sterols, the food suspensions (sampled on Day 6) were filtered on precombusted GF/F filters (Whatman, 25 mm diameter). POC was measured using an NCS-2500 analyzer (Carlo Erba Instruments). The total lipids were extracted with dichloromethane/methanol as described above and evaporated to dryness with nitrogen. The lipid extracts were transesterified with 3 mol L<sup>-1</sup> methanolic HCl (60°C, 15 min) for the analysis of fatty acids or saponified with 0.2 mol L<sup>-1</sup> methanolic KOH (70°C, 1 h) for the analysis of sterols. Subsequently, fatty acid methyl esters (FAMES) were extracted three times with 2 mL of isohexane; the neutral lipids (sterols) were extracted with isohexane : diethyl ether (9 : 1, v/v). The isohexane lipid fractions were evaporated to dryness under nitrogen and resuspended in 10–20  $\mu\text{L}$  of isohexane. Lipids were analyzed by gas chromatography on an HP 6890 GC equipped with a flame ionization detector; a DB-225 (J&W Scientific) capillary column was used to analyze fatty acids (Von Elert 2002) and an HP-5 (Agilent) capillary column was used to analyze sterols (Martin-Creuzburg and Von Elert 2004). Lipids were quantified by comparison to internal standards (C17 : 0 and C23 : 0 methyl esters; 5 $\alpha$ -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 for FAMES; DB-5MS, Agilent for sterols). Sterols were analyzed as free sterols and as their acetate derivatives. Spectra were recorded between 50 and 600 amu (atomic mass units) in the EI ionization mode. Mass spectra were identified by comparison with mass spectra of reference

substances (purchased from Sigma, Supelco, or Steraloids) or spectra found in the literature. The C24 epimers poriferasterol/stigmasterol and the *cis/trans* isomers isofucosterol/fucosterol could not be identified with certainty. The absolute amount of each lipid was related to the POC, which was determined from another aliquot of the food suspensions used for lipid analysis.

**Data analysis**—Growth rates and clutch sizes were analyzed by one-way analyses of variance (ANOVA) and post-hoc comparisons (Tukey's HSD,  $\alpha = 0.05$ ). Raw data met the assumption of homogeneity of variance (Levene's test).

## Results

**Growth of *D. magna***—Juvenile somatic growth rates (g) of *D. magna* were significantly affected by the food supplied in the growth experiments (ANOVA,  $F_{8,18} = 649$ ,  $p < 0.001$ ; Fig. 1). Growth rates of *D. magna* ranged from  $-0.06 \text{ d}^{-1}$  (starving) to  $0.51 \text{ d}^{-1}$  (*Cryptomonas* sp.). Somatic growth of *D. magna* on both *Synechococcus* strains was poor. However, when *Paraphysomonas* sp. was introduced as an intermediary trophic level between the picocyanobacterial strains and the metazoan grazer, growth of *D. magna* was increased sevenfold to eightfold, which indicated a significant improvement in cyanobacterial food quality by the HNF (Tukey's honestly significant difference [HSD],  $p < 0.05$ ). Daphnids fed on the toxic *M. aeruginosa* did not survive the 6-d experiment. In contrast, daphnids fed on *Paraphysomonas* sp. and grown on *M. aeruginosa* survived and grew relatively well. Growth of *D. magna* on *C. minor* was substantially higher than it was on picocyanobacteria (Tukey's HSD,  $p < 0.05$ ; Fig. 1), but did not increase when the HNF was interposed as intermediary grazer (Tukey's HSD,  $p = 0.10$ ).

**Clutch sizes of *D. magna***—*D. magna* did not produce eggs when grown on *Synechococcus* sp., *S. elongatus*, or *Paraphysomonas* sp. fed with *M. aeruginosa* (Fig. 2). In contrast, when the HNF was interposed between either of the *Synechococcus* strains and *D. magna*, the daphnids produced 3.4–4.6 eggs per individual. Daphnids fed on *C. minor* produced comparatively small clutches (Fig. 2). When *Paraphysomonas* sp. was introduced as an intermediary grazer, clutch sizes increased threefold to fourfold (Fig. 2; Tukey's HSD,  $p < 0.05$  following ANOVA,  $F_{4,10} = 87.9$ ,  $p < 0.001$ ). The largest clutch sizes were produced when *Cryptomonas* sp. was offered as food.

**Effects of lipid supplementation on growth of *D. magna***—The supplementation of *Synechococcus* sp. with BSA did not affect *D. magna* growth (Fig. 3; Tukey's HSD following ANOVA,  $F_{4,10} = 361$ ,  $p < 0.001$ ). Similarly, supplementation of *Synechococcus* sp. with its own lipids did not improve growth of the grazer, which indicates that the food quality of *Synechococcus* sp. was not due to a general shortage of lipids. When *Synechococcus* sp. was supplemented with lipids from *Paraphysomonas* sp. growth

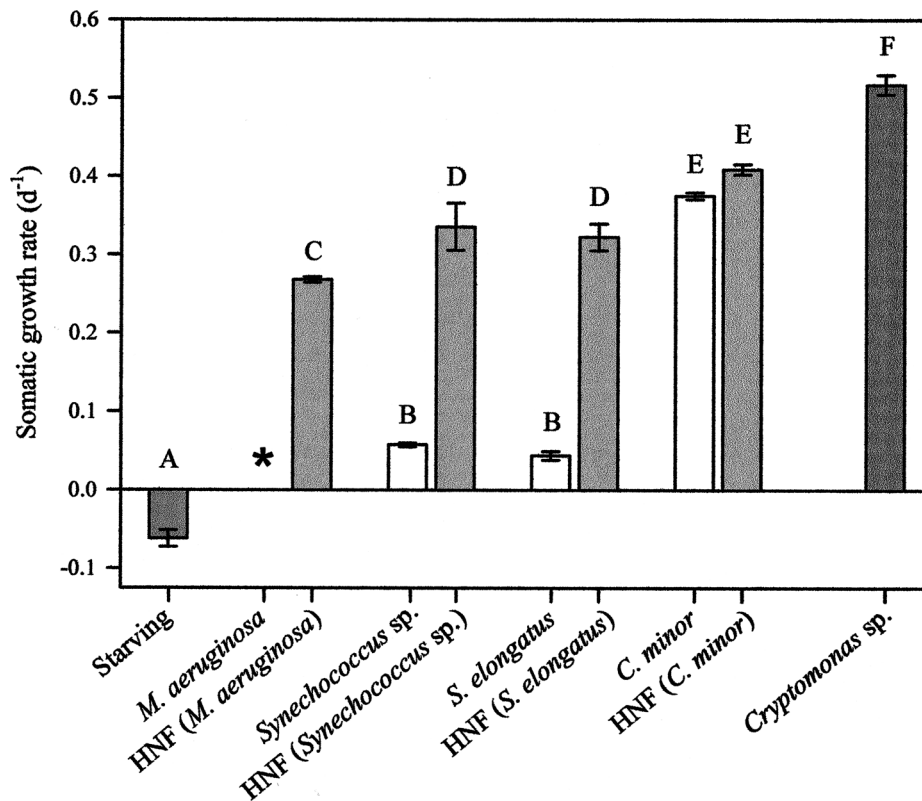


Fig. 1. Juvenile somatic growth rates of *D. magna* grown on *M. aeruginosa*, *Synechococcus* sp., *S. elongatus*, *C. minor*, and on the HNF *Paraphysomonas* sp. fed with one of these autotrophic species. Growth without food supply (starving) and growth on *Cryptomonas* sp. are shown for comparison. Daphnids grown on the toxic *M. aeruginosa* did not survive the 6-d experiment (indicated by an asterisk). Data are means of  $n = 3$ ; error bars indicate significant difference. Bars labeled with the same letters are not significantly different (Tukey's HSD,  $p < 0.05$  following ANOVA).

of *D. magna* increased (Fig. 3; Tukey's HSD,  $p < 0.05$ ), which indicates that the low food quality of *Synechococcus* sp. was due to the absence of a lipid that is present in the flagellate.

**Sterol compositions**—No sterols were detected in any of the cyanobacterial strains. Stigmasterol (24 $\alpha$ -ethylcholesta-5,22-dien-3 $\beta$ -ol) and epibrassicasterol (24 $\alpha$ -methylcholesta-5,22-dien-3GB $\beta$ -ol) were the principal sterols in *Cryptomonas* sp. (Table 1). Epibrassicasterol, the 24 $\alpha$ -epimer of brassicasterol, occurs in cryptophycean algae such as *Cryptomonas* and *Rhodomonas* (Goad et al. 1983). Although we did not determine the side-chain stereochemistry at C-24, the presence of epibrassicasterol rather than brassicasterol in the *Cryptomonas* sp. strain used here was assumed. Three major sterols were detected in the green alga *C. minor*: fungisterol (24 $\beta$ -methylcholesta-7-en-3 $\beta$ -ol); ergosterol (24 $\beta$ -methylcholesta-5,7,22-trien-3 $\beta$ -ol); and chondrillasterol (24 $\beta$ -ethylcholesta-7,22-dien-3 $\beta$ -ol), all of which are common in chlorophycean species (Cranwell et al. 1990).

The major sterol of the chrysomonad *Paraphysomonas* sp. was 24-ethylcholesta-5,22-dien-3 $\beta$ -ol. The two C-24 epimers of 24-ethylcholesta-5,22-dien-3 $\beta$ -ol—poriferasterol

(24 $\beta$ ) and stigmasterol (24 $\alpha$ )—could not be fully separated on the gas chromatograph–flame ionization detector and gas chromatograph–mass spectrometer systems used; therefore, the stereochemistry of the ethyl group at C-24 was not defined. The presence of poriferasterol in the related chrysomonad *Ochromonas* (Gershengorn et al. 1968) suggests that the 24 $\beta$  configuration of 24-ethylcholesta-5,22-dien-3 $\beta$ -ol is also present in *Paraphysomonas* sp. Another sterol found in *Paraphysomonas* sp. was 24-ethylidenecholesta-5,24(28)-dien-3 $\beta$ -ol. To assess the configuration of the C-24 ethylidene group, which can either be *cis* (24Z, isofucosterol) or *trans* (24E, fucosterol), sterols were analyzed as their steryl acetates. The mass spectra of the acetates showed a dominant  $m/z$  296 ion and a much less abundant  $m/z$  394 ion, which indicates the presence of isofucosterol rather than fucosterol (Knights and Brooks 1969).

The sterol composition of the HNF did not change with its food source (i.e., *Paraphysomonas* sp. grown on a sterol-free cyanobacterial diet exhibited the same sterol pattern as *Paraphysomonas* sp. grown on the sterol-containing green alga *C. minor* [Table 1]). Only trace amounts of the sterols found in *C. minor* were detected in *Paraphysomonas* sp. grown on this eukaryotic alga. These traces could be

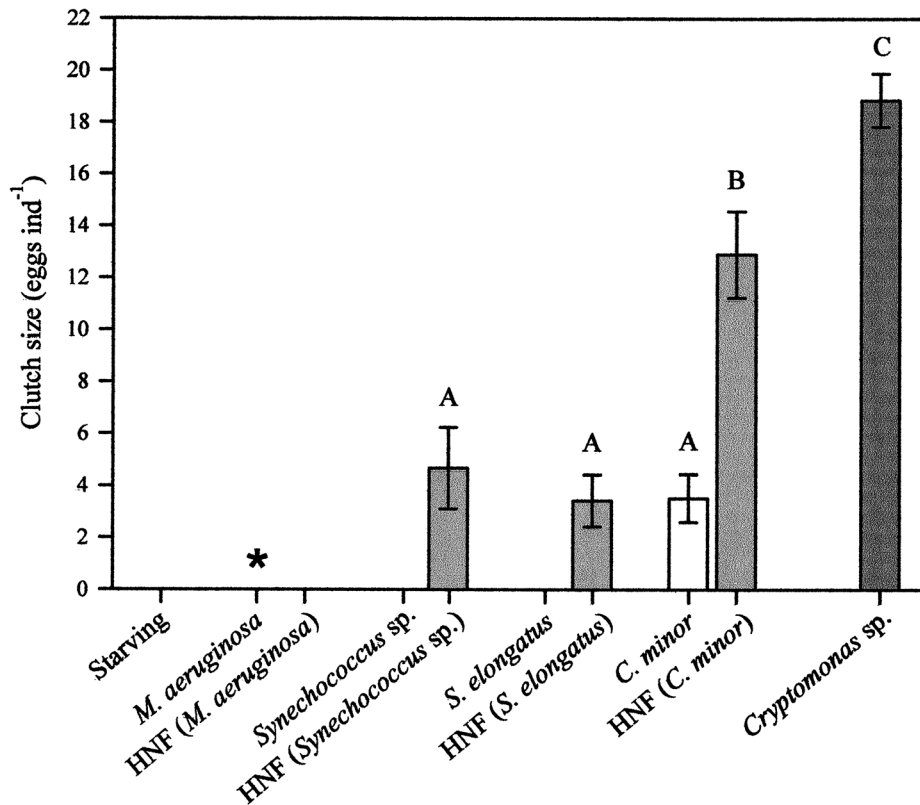


Fig. 2. Clutch sizes of *D. magna* grown on *M. aeruginosa*, *Synechococcus* sp., *S. elongatus*, *C. minor*, and on the HNF *Paraphysomonas* sp. fed with one of these autotrophic species. The clutch size of *D. magna* grown on *Cryptomonas* sp. is shown for comparison. Daphnids grown on the toxic *M. aeruginosa* did not survive the 6-d experiment (indicated by an asterisk). Data are means of  $n = 3$ ; error bars indicate significant difference. Bars labeled with the same letters are not significantly different (Tukey's HSD,  $p < 0.05$  following ANOVA).

attributed to the slight contamination of the flagellate suspensions with *C. minor* cells, or to undigested material in the HNF food vacuoles, or both. The total sterol content of *Paraphysomonas* sp. was generally higher than the total sterol content of *C. minor* and *Cryptomonas* sp. (Table 1).

**Fatty acid compositions**—Both *Synechococcus* strains had high amounts of 16:0 and 16:1(n-7) fatty acids and lacked PUFAs (Table 2). In contrast, *M. aeruginosa* was characterized by significant amounts of (n-6) PUFAs, primarily represented by 18:2(n-6) and 18:3(n-6). Like other Chlorophyceae (Ahlgren et al. 1992), *C. minor* had significant amounts of 16:3(n-3), 18:2(n-6), and 18:3(n-3) fatty acids. *Cryptomonas* sp. was characterized by a clear dominance of (n-3) series PUFAs, primarily 18:3(n-3), 18:4(n-3), and 20:5(n-3).

*Paraphysomonas* sp. grown on the different autotrophic species had substantial amounts of 20:4(n-6), 20:5(n-3), and 22:6(n-3) fatty acids. Both *Synechococcus* strains lack (n-3) PUFAs, which demonstrated that *Paraphysomonas* sp. is able to synthesize these long-chain PUFAs de novo. The fatty acid composition of the HNF was furthermore influenced by its diet. Like *C. minor*, *Paraphysomonas* sp. grown on this picoeukaryotic alga is characterized by significant amounts of 16:3(n-3), 18:2(n-6), and 18:3(n-3)

fatty acids. This result is in good agreement with a previous study that reported that the HNF *Aulacomonas submarina* accumulates C<sub>16</sub> and C<sub>18</sub> PUFAs when fed the chlorophycea *Chlorogonium elongatum* (Bec et al. 2003b). *C. minor* showed a higher content of 18:2(n-6) than of 18:3(n-3) (Table 2). However, this ratio is reversed in *Paraphysomonas* sp. when feeding on *C. minor*, which indicates a preferential assimilation of 18:3(n-3) by the HNF.

## Discussion

APP is the major primary producer in many oligotrophic to mesotrophic marine and freshwater systems. In marine systems, where copepods are the major metazoan grazers, this production becomes available only after assimilation by protozoa as an intermediate trophic level. This trophic situation differs from that of freshwater systems, where in many cases the major metazoan grazers are representatives of the genus *Daphnia* rather than copepods. Daphnids can either feed directly on APP or gain indirect access to APP production when they consume protists feeding on picoplankton. Here we experimentally investigated these two contrasting situations for *D. magna* in simplified bitrophic and tritrophic food chains. When *Paraphysomonas* sp. fed on the different autotrophic picoplanktonic

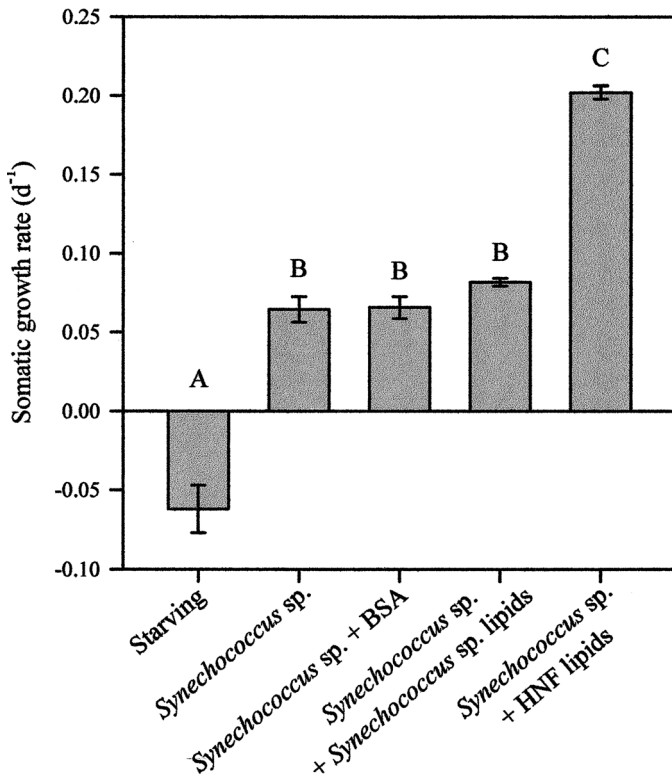


Fig. 3. Juvenile somatic growth rates of *D. magna* grown on *Synechococcus* sp. and on *Synechococcus* sp. supplemented with BSA, a total lipid extract gained from *Synechococcus* sp., and a total lipid extract gained from the HNF *Paraphysomonas* sp. that previously fed on *Synechococcus* sp. Growth without food supply (starving) is shown for comparison. Data are means of  $n = 3$ ; error bars indicate significant difference. Bars labeled with the same letters are not significantly different (Tukey's HSD,  $p < 0.05$  following ANOVA).

organisms, the dietary carbon provided to daphnids consisted primarily of the HNF. Contamination of picoplanktonic cells never exceeded 10% carbon and ranged between 2% and 8%, which allowed us to clearly identify the effects on growth of *D. magna* of the various *Paraphysomonas* sp. food suspensions. Even though daphnids were offered a lower quantity of food when fed the different preparations of the *Paraphysomonas* sp. than when they were directly fed the different autotrophic species, the food quantity was well above the incipient limiting level that is reported to be approximately 0.5 mg of carbon per liter (Lampert 1978). Therefore, we could interpret differences in growth and reproduction of *D. magna* as an effect of food quality and not of food quantity.

Growth rates and clutch sizes of *D. magna* fed on prokaryotic APP were low, which is in accordance with the well-known low food quality of cyanobacteria for daphnids (Von Elert and Wolffrom 2001; Von Elert et al. 2003). The introduction of *Paraphysomonas* sp. as an intermediary trophic level led to a sevenfold to eightfold increase in somatic growth of *D. magna* and to increased clutch sizes. Hence, the HNF *Paraphysomonas* sp. clearly upgraded the food quality of autotrophic picocyanobacteria.

Table 1. Sterol content of the HNF *Paraphysomonas* sp. grown on different food sources (*M. aeruginosa*, *Synechococcus* sp., *S. elongatus*, or *C. minor*) and of *C. minor* and *Cryptomonas* sp. No sterols were detected in the two *Synechococcus* strains and in *M. aeruginosa*. The values are means of  $n = 3$ ;  $\pm$ SD.

Sterol	<i>Paraphysomonas</i> ( <i>M. aeruginosa</i> )		<i>Paraphysomonas</i> ( <i>S. elongatus</i> )		<i>Paraphysomonas</i> ( <i>C. minor</i> )		<i>C. minor</i>		<i>Cryptomonas</i> sp.	
	( $\mu\text{g mg C}^{-1}$ )	(%)	( $\mu\text{g mg C}^{-1}$ )	(%)	( $\mu\text{g mg C}^{-1}$ )	(%)	( $\mu\text{g mg C}^{-1}$ )	(%)	( $\mu\text{g mg C}^{-1}$ )	(%)
Poriferasterol	10.9 $\pm$ 0.1	85.0	8.4 $\pm$ 1.0	66.7	6.0 $\pm$ 0.8	70.2	—	—	—	—
Isofucosterol	1.9 $\pm$ 0.0	15.0	4.2 $\pm$ 0.1	33.3	2.6 $\pm$ 0.1	29.8	—	—	—	—
Fungsterol	—	—	—	—	Trace	Trace	2.3 $\pm$ 0.1	45.9	—	—
Ergosterol	—	—	—	—	Trace	Trace	1.7 $\pm$ 0.5	33.9	—	—
Chondrillasterol	—	—	—	—	—	—	1.0 $\pm$ 0.1	20.2	—	—
Stigmasterol	—	—	—	—	—	—	—	—	4.0 $\pm$ 0.2	57.7
Epibrassicasterol	—	—	—	—	—	—	—	—	2.9 $\pm$ 0.1	42.3
Total	12.8 $\pm$ 0.1	100	12.6 $\pm$ 0.9	100	8.6 $\pm$ 0.8	100	5.0 $\pm$ 0.7	100	6.9 $\pm$ 0.3	100

Table 2. Fatty acid composition of the HNF *Paraphysomonas* sp. grown on different food sources (*M. aeruginosa*, *Synechococcus* sp., *S. elongatus*, or *C. minor*) and of *M. aeruginosa*, *Synechococcus* sp., *S. elongatus*, *C. minor*, and *Cryptomonas* sp.\*

Fatty acid	<i>M. aeruginosa</i>	<i>Paraphysomonas (M. aeruginosa)</i>	<i>Synechococcus sp.</i>	<i>Paraphysomonas (Synechococcus sp.)</i>	<i>S. elongatus</i>	<i>Paraphysomonas (S. elongatus)</i>	<i>C. minor</i>	<i>Paraphysomonas (C. minor)</i>	<i>Cryptomonas sp.</i>
16:0	36.7	15.8	16.4	14.2	26.2	16.9	15.2	15.2	9.0
16:1n-7	4.7	15.2	46.0	25.3	51.3	21.6	1.5	4.8	2.2
16:3n-3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.8	3.8	n.d.
17:1n-7	0.6	3.4	0.8	1.8	0.5	1.5	n.d.	1.8	0.5
18:0	9.9	4.8	2.3	3.3	1.6	3.7	1.0	5.6	1.5
18:1n-9	12.9	5.8	1.0	2.9	0.7	4.8	7.8	7.0	2.1
18:1n-7	4.5	18.8	2.1	18.8	1.9	17.5	0.6	8.1	3.1
18:2n-6	15.5	4.0	n.d.	3.9	n.d.	4.1	37.2	14.7	7.2
18:3n-6	11.1	n.d.	n.d.	2.1	n.d.	1.7	n.d.	0.4	0.5
18:3n-3	0.4	1.8	n.d.	1.9	n.d.	1.6	26.4	18.0	32.6
18:4n-3	n.d.	3.0	n.d.	n.d.	n.d.	n.d.	n.d.	1.6	21.0
20:1n-9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.7
20:2n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5	n.d.
20:4n-6	n.d.	10.2	n.d.	5.2	n.d.	6.9	n.d.	2.1	0.4
20:3n-3	n.d.	n.d.	n.d.	n.d.	n.d.	0.8	n.d.	1.8	0.4
20:5n-3	n.d.	4.4	n.d.	3.9	n.d.	6.0	n.d.	3.4	15.0
22:6n-3	n.d.	5.9	n.d.	7.2	n.d.	7.2	n.d.	3.4	1.4
Total	100	100	100	100	100	100	100	100	100
Total (n-3)	0.4	15.0	n.d.	12.7	n.d.	15.2	28.5	30.1	68.4

\* Values are given in percentages as means of  $n = 3$ . n.d. = not detected.

The effects of an additional intermediary trophic level on growth and reproduction of *D. magna* differed when the picoalga *C. minor* was supplied as food: *Paraphysomonas* sp. raised on *C. minor* did not affect the somatic growth of *D. magna*, but led to an increase in reproduction. It is generally assumed that effects of food quantity on somatic growth and on reproduction are highly correlated in juvenile *Daphnia* (Lampert and Trubetskova 1996). However, it has been shown that mineral and biochemical food quality affect somatic growth and reproduction differently (Becker and Boersma 2003; Martin-Creuzburg and Von Elert 2004).

Growth and reproduction of *D. magna* differed when it was fed the different *Paraphysomonas* sp. preparations, which indicates that the nutritional value of the HNF varies with its picoplanktonic prey. Hence, the high quality of dietary *Paraphysomonas* sp. for *D. magna* is primarily due to the ability of *Paraphysomonas* sp. to upgrade its diet of APP, and to a smaller extent, the food quality of this HNF is determined by the respective APP species on which it fed.

Trophic upgrading of microalgae has been hypothesized to be due to the lipid composition of the considered HNFs (Klein Breteler et al. 1999; Bec et al. 2003b), but this had not yet been tested. In the present study, *Synechococcus* sp. was supplemented with its own lipids and with lipids of *Paraphysomonas* sp. (grown on *Synechococcus* sp.), which clearly demonstrated that trophic upgrading is due to additional lipids present in the HNF.

Sterols are involved in a wide range of physiological processes in arthropods. However, arthropods are incapable of synthesizing sterols de novo and, therefore, must obtain these essential compounds from their diet. In contrast to *Paraphysomonas* sp., the three cyanobacterial strains used here do not contain sterols. It has recently been shown that the absence of sterols in cyanobacteria is a major food quality constraint for daphnids (Von Elert et al. 2003; Martin-Creuzburg and Von Elert 2004). In line with these findings, we now argue that the presence of sterols in *Paraphysomonas* sp. lipids is a primary explanation for the trophic upgrading of picocyanobacterial food. This hypothesis is supported by the high quantities of sterols in the HNF ranging from 8.6  $\mu\text{g mg C}^{-1}$  (*Paraphysomonas* sp. grown on *C. minor*) to 12.6  $\mu\text{g mg C}^{-1}$  (*Paraphysomonas* sp. grown on *S. elongatus*), which exceed even those of eukaryotic algae (*Cryptomonas* sp., *C. minor*). Moreover, *Paraphysomonas* sp. sterols are predominantly  $\Delta^5$  sterols, which when supplemented to *S. elongatus*, support somatic growth and reproduction of daphnids (Martin-Creuzburg and Von Elert 2004).

However, cyanobacteria not only lack sterols, but most of them are also a poor source of PUFAs. Like sterols, (n-3) PUFAs are of particular physiological importance for metazoans and are considered essential for zooplankton (Arts 1998). Several studies have underlined the importance of (n-3) PUFAs and especially of long-chain PUFAs (e.g., 20:5(n-3)), for growth and reproduction of cladocerans (Ahlgren et al. 1990; Brett and Müller-Navarra 1997; Von Elert 2002). In this regard, Von Elert et al. (2003) have previously shown that daphnids feeding on cyanobacteria are limited primarily by the absence of sterols, and when

this dietary deficiency is fully compensated by supplementary sterols, growth and reproduction of daphnids become limited by the availability of PUFAs. Because *Paraphysomonas* sp. differed from its picocyanobacterial prey by having both sterols and PUFAs, it is tempting to speculate that the high sterol content of *Paraphysomonas* sp. fully compensated this cyanobacterial deficiency and that the PUFAs in the lipids of *Paraphysomonas* sp. further contributed to the trophic upgrading of picocyanobacteria.

Trophic upgrading by *Paraphysomonas* sp. was also observed for the picoalga *C. minor*, although this effect was detected only for reproduction and not for somatic growth of *D. magna*. *C. minor* and *Paraphysomonas* sp. had similar amounts, but a different composition of sterols. Such qualitative differences may result in different degrees of utilization of sterols by the daphnids (Martin-Creuzburg and Von Elert 2004). However, reproduction is less sensitive than somatic growth to sterol limitation (Martin-Creuzburg et al. 2005), and hence the trophic upgrading is probably due to the presence of long-chain PUFAs in *Paraphysomonas* sp. that are absent in *C. minor*. In *C. minor* no PUFAs longer than C18 were found, whereas *Paraphysomonas* sp. feeding on *C. minor* contained six different C20 and C22 long-chain PUFAs. Hence, trophic upgrading of picocyanobacteria may be assigned to the synthesis of long-chain PUFAs only, whereas the amelioration of picocyanobacterial carbon by *Paraphysomonas* sp. involves the synthesis of both sterols and (n-3) PUFAs. More generally, trophic upgrading of APP by this HNF can be attributed to its lipid metabolism, which implies not only de novo synthesis, but also accumulation and bioconversion of dietary compounds. Indeed, the PUFA content of *Paraphysomonas* sp. had a marked dietary influence, which could largely explain the variability of the HNF nutritive value.

The effects of feeding *M. aeruginosa* to the intermediary *Paraphysomonas* sp. compared with direct feeding to daphnids point to factors other than lipid metabolism that should be considered. *M. aeruginosa* PCC7806 was toxic for *Daphnia* when *D. magna* fed directly on *M. aeruginosa*; however, when daphnids fed on *Paraphysomonas* sp. that had been raised on *M. aeruginosa*, no mortality was observed, and the daphnids exhibited higher growth rates than those fed nontoxic cyanobacteria. Growth of the HNF was not affected by *M. aeruginosa* toxicity. Several phagotrophic protists graze and grow efficiently on toxic phytoplankton species (Stoecker et al. 2002), and it has been suggested that some reduce the toxicity of their prey for copepods (Jeong et al. 2003). In accordance with these findings, we showed here that *Paraphysomonas* sp. as an intermediary trophic step reduces *M. aeruginosa* toxicity for *D. magna*. However, the growth and clutch size of daphnids fed on *Paraphysomonas* sp. that had been raised on *M. aeruginosa* were significantly lower than when fed *Paraphysomonas* sp. that had been raised on nontoxic picocyanobacteria. This might be attributed to residual toxicity caused by incomplete removal of *M. aeruginosa* cells in the daphnids' diet or to undigested material contained in the food vacuoles of the HNF.

In conclusion, we showed that HNFs can trophically upgrade prokaryotic and eukaryotic picoplankton for metazoan grazers by producing sterols and fatty acids. We furthermore provided evidence that the HNF *Paraphysomonas* sp. is capable of detoxifying cyanobacterial food for *D. magna*. Hence, phagotrophic protists should not only be considered as a link that repackages their prey into accessible particles for metazoans, but also as a biosynthetic step that can potentially improve the nutritional value of picoplankton. Evidently, having an intermediary trophic level of phagotrophic protists still leads to a carbon loss. However, little is known about the assimilation efficiency of picoplankton by phagotrophic protists (Pomeroy 2000). Note that another consequence of protistan grazing is the release of dissolved organic matter and inorganic nutrients (Caron and Goldman 1990), which might enhance picoplankton productivity in oligotrophic systems. Thus, the phagotrophic protist sink as well as the protozoan nutritional value for metazoans should be more precisely estimated. In this regard, further investigations are needed to explore the lipid metabolism of phagotrophic protists and the trophic transfers of essential compounds in microbial food webs.

## References

- AHLGREN, G., I. GUSTAFSSON, AND M. BOBERG. 1992. Fatty acid and chemical composition of freshwater microalgae. *J. Phycol.* **28**: 37–50.
- , L. LUNDSTEDT, M. BRETT, AND C. FORSBERG. 1990. Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. *J. Plankton Res.* **12**: 809–818.
- ARTS, M. T. 1998. Lipids in freshwater zooplankton: Selected ecological and physiological aspects, p. 71–90. *In* M. T. Arts and B. C. Wainman [eds.], *Lipids in freshwater ecosystems*. Springer-Verlag.
- AZAM, F., T. FENCHEL, J. G. FIELD, J. S. GRAY, L. A. MEYER-REIL, AND F. THINGSTAD. 1983. The ecological role of water-column microbes in the sea. *Mar. Ecol. Prog. Ser.* **10**: 257–263.
- BEC, A., C. DESVILLETES, A. VÉRA, D. FONTVIELLE, AND G. BOURDIER. 2003a. Nutritional value of different food sources for the benthic daphnidae *Simocephalus vetulus*: Role of fatty acids. *Arch. Hydrobiol.* **156**: 145–163.
- , ———, ———, C. LEMARCHAND, D. FONTVIELLE, AND G. BOURDIER. 2003b. Nutritional quality of a freshwater heterotrophic flagellate: Trophic upgrading of its microalgal diet for *Daphnia hyalina*. *Aquat. Microb. Ecol.* **32**: 203–207.
- BECKER, C., AND M. BOERSMA. 2003. Resource quality effects on life history of *Daphnia*. *Limnol. Oceanogr.* **48**: 700–706.
- BRATBAK, G. 1985. Bacterial biovolume and biomass estimations. *Appl. Environ. Microbiol.* **49**: 1488–1493.
- BRETT, M. T., AND D. C. MÜLLER-NAVARRA. 1997. The role of highly unsaturated fatty acids in food web processes. *Freshw. Biol.* **38**: 483–499.
- CALLIERI, C., AND J. G. STOCKNER. 2002. Freshwater autotrophic picoplankton: A review. *J. Limnol.* **61**: 1–14.
- CARON, D. A., AND J. C. GOLDMAN. 1990. Protozoan nutrient regeneration, p. 283–306. *In* G. M. Capriulo [ed.], *Ecology of marine protozoa*. Oxford Univ. Press.
- CRANWELL, P. A., G. H. M. JAWORSKI, AND M. BICKLEY. 1990. Hydrocarbons, sterols, esters and fatty acids in six freshwater chlorophytes. *Phytochemistry* **29**: 145–151.



- DESVILLETES, C., G. BOURDIER, C. AMBLARD, AND B. BARTH. 1997. Use of fatty acids for the assessment of zooplankton grazing on bacteria, protozoan and microalgae. *Freshw. Biol.* **38**: 629–637.
- ELSER, J. J., K. HAYAKAWA, AND J. URABE. 2001. Nutrient limitation reduces food quality for zooplankton: *Daphnia* response to seston phosphorus enrichment. *Ecology* **82**: 898–903.
- GERSHENGORN, M. C., A. R. H. SMITH, G. GOULSTON, L. J. GOAD, T. W. GOODWIN, AND T. H. HAINES. 1968. The sterols of *Ochromonas danica* and *Ochromonas malhamensis*. *Biochemistry* **7**: 1698–1706.
- GIFFORD, D. J. 1991. The protozoan-metazoan trophic link in pelagic ecosystems. *J. Protozool.* **38**: 81–86.
- GOAD, L. J., G. G. HOLZ JR., AND D. H. BEACH. 1983. Identification of (24S)-24-methylcholesta-5,22-dien-3 $\beta$ -ol as the major sterol of a marine cryptophyte and a marine prymnesiophyte. *Phytochemistry* **22**: 475–476.
- JEONG, H. J., K. H. PARK, J. S. KIM, H. KANG, C. H. KIM, H. J. CHOI, Y. S. KIM, J. Y. PARK, AND M. G. PARK. 2003. Reduction in the toxicity of the dinoflagellate *Gymnodinium catenatum* when fed on by the heterotrophic dinoflagellate *Polykrikos kofoidii*. *Aquat. Microb. Ecol.* **31**: 307–312.
- KLEIN BRETELER, W. C. M., N. SCHOOT, M. BAAS, S. SCHOUTEN, AND G. W. KRAAY. 1999. Trophic upgrading of food quality by protozoans enhancing copepod growth: Role of essential lipids. *Mar. Biol.* **135**: 191–198.
- KNIGHTS, B. A., AND C. J. W. BROOKS. 1969. Isomers of 24-ethylidenecholesterol: Gas chromatographic and mass spectrometric characterization. *Phytochemistry* **8**: 463–467.
- LAMPERT, W. 1978. A field study on the dependence of the fecundity of *Daphnia* sp. on food concentration. *Oecologia* **36**: 363–369.
- , AND I. TRUBETSKOVA. 1996. Juvenile growth rate as a measure of fitness in *Daphnia*. *Funct. Ecol.* **10**: 631–635.
- MARTIN-CREUZBURG, D., AND E. VON ELERT. 2004. Impact of 10 dietary sterols on growth and reproduction of *Daphnia galeata*. *J. Chem. Ecol.* **30**: 483–500.
- , A. WACKER, AND E. VON ELERT. 2005. Life history consequences of sterol availability in the aquatic keystone species *Daphnia*. *Oecologia* **144**: 362–372.
- MENDEN-DEUER, S., AND E. J. LESSARD. 2000. Carbon to volume relationships for dinoflagellates, diatoms, and other protist plankton. *Limnol. Oceanogr.* **45**: 569–579.
- POMEROY, L. R. 2000. Food web connections: Links and sinks, p. 81–87. In C. R. Bell, M. Brylinsky and P. Johnson-Green [eds.], *Microbial biosystems: New frontiers*. Atlantic Canada Society for Microbial Ecology.
- PORTER, K. G., AND Y. S. FEIG. 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25**: 943–948.
- SANDERS, R. W., AND S. A. WICKHAM. 1993. Planktonic protozoa and metazoa: Predation, food quality and population control. *Mar. Microb. Food Webs.* **7**: 197–223.
- , C. E. WILLIAMSON, P. L. STUTZMAN, R. E. MOELLER, C. E. GOULDEN, AND R. AOKI-GOLDSMITH. 1996. Reproductive success of “herbivorous” zooplankton fed algal and non algal food resources. *Limnol. Oceanogr.* **41**: 1295–1305.
- SHERR, B. F., E. B. SHERR, AND L. J. ALBRIGHT. 1987. Bacteria: Link or sink? *Science* **235**: 88–89.
- SHERR, E. B., AND B. F. SHERR. 1988. Role of microbes in pelagic food webs: A revised concept. *Limnol. Oceanogr.* **33**: 225–227.
- STOCKNER, J. G., AND K. S. SHORTREED. 1989. Algal picoplankton production and contribution to food webs in oligotrophic British Columbia lakes. *Hydrobiologia* **173**: 151–166.
- STOECKER, D. K., M. W. PARROW, J. M. BURKHOLDER, AND H. D. GLASGOW. 2002. Grazing by microzooplankton on *Pfiesteria piscida* cultures with different histories of toxicity. *Aquat. Microb. Ecol.* **28**: 79–85.
- TANG, K. W., H. H. JAKOBSEN, AND A. W. VISSER. 2001. *Phaeocystis globosa* (prymnesiophyceae) and the planktonic food web: Feeding, growth, and trophic interactions among grazers. *Limnol. Oceanogr.* **46**: 1860–1870.
- VÉRA, A., C. DESVILLETES, A. BEC, AND G. BOURDIER. 2001. Fatty acid composition of freshwater heterotrophic flagellates: An experimental study. *Aquat. Microb. Ecol.* **25**: 271–279.
- VERITY, P. G., C. Y. ROBERSON, C. R. TRONZO, M. G. ANDREWS, J. R. NELSON, AND M. E. SIERAKI. 1992. Relationships between cell volume and the carbon and nitrogen content of marine photosynthetic nanoplankton. *Limnol. Oceanogr.* **37**: 1434–1446.
- VON ELERT, E. 2002. Determination of limiting fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnol. Oceanogr.* **47**: 1764–1773.
- , AND T. WOLFFROM. 2001. Supplementation of cyanobacterial food with polyunsaturated fatty acids does not improve growth of *Daphnia*. *Limnol. Oceanogr.* **46**: 1552–1558.
- , D. MARTIN-CREUZBURG, AND J. R. LE COZ. 2003. Absence of sterols constrains carbon transfer between cyanobacteria and a freshwater herbivore (*Daphnia galeata*). *Proc. R. Soc. Lond. B* **270**: 1209–1214.
- WEISSE, T. 1993. Dynamics of autotrophic picoplankton in marine and freshwater ecosystems. *Adv. Microb. Ecol.* **13**: 327–369.