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Food quality of mixed bacteria–algae diets for *Daphnia magna*

Heike M. Freese · Dominik Martin-Creuzburg

Abstract Bacteria can comprise a large fraction of seston in aquatic ecosystems and can therefore significantly contribute to diets of filter-feeding zooplankton. To assess the effect of three heterotrophic bacteria (*Flavobacterium* sp., *Pseudomonas* sp. and *Escherichia coli*) on survival, growth and egg production of juvenile *Daphnia magna* during six-day growth experiments, five ratios of bacteria *Scenedesmus obliquus* mixtures were fed. Potential growth-limiting effects mediated by essential biochemicals were assessed upon supplementation of pure bacterial diets with a sterol (cholesterol) or a polyunsaturated fatty acid (EPA). Pure bacterial diets always had detrimental effects on *Daphnia*. However, cholesterol supplementation of *Flavobacterium* sp. enhanced growth rates of *Daphnia*. Diets containing *Pseudomonas* impaired *Daphnia* growth even at low dietary proportions (20%), indicating their toxicity. In contrast, *Daphnia* grew at relative high dietary proportions of *Flavobacterium* sp. and *E. coli* (80 50%). In fact, diets containing small proportions of these heterotrophic bacteria (*Flavobacterium* ≤50%, *E. coli* 20%) even significantly increased *Daphnia* growth rates compared to pure algal diets, indicating a nutritional upgrading by these bacteria. Our results suggest that the relative contribution of bacteria and phytoplankton to total dietary carbon as well as their phylogenetic composition strongly influence *Daphnia* fitness and potentially other filter-feeding zooplankton under field conditions.

Keywords *Flavobacterium* sp. · *Pseudomonas* sp. · *Escherichia coli* · Polyunsaturated fatty acids · Sterols · *Scenedesmus*

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Introduction

Aquatic systems are characterized by a complex food web, in which organic matter is transferred across different trophic levels. Cladocerans of the genus *Daphnia* often dominate the zooplankton in standing freshwater systems, and thus provide a crucial link between primary and secondary production (Peters & de Bernardi, 1987). As non-selective filter-feeders, *Daphnia* do not only graze on phytoplankton but also

on protozoa, bacteria and detritus (Jürgens, 1994; Cole et al., 2006). Heterotrophic bacteria often constitute a substantial part of the suspended particulate organic matter and have a key function by channelling organic carbon via incorporation into the food web (Azam et al., 1983; Biddanda et al., 2001). The aquatic bacterial communities consist of many different populations from different phyla (e.g. Rappe & Giovannoni, 2003). Freshwater ecosystems are mainly dominated by β-proteobacteria, actinobacteria and bacteroidetes, but α-proteobacteria, verrucomicrobia or planctomycetes can also be abundant (cf. Zwart et al., 2002, 2003; Newton et al., 2011). *Daphnia* can shape these microbial communities either by grazing on bacterivorous protozoans or by direct consumption of bacteria (Langenheder & Jürgens, 2001; Degans et al., 2002; Pernthaler et al., 2004).

Although bacteria can be efficiently consumed by *Daphnia* (Gophen & Geller, 1984; Brendelberger, 1991), their importance for *Daphnia* nutrition has been considered only recently. Analysis of stable isotope patterns and fatty acid biomarkers revealed that heterotrophic bacteria can significantly contribute to the diet of *Daphnia* species (Karlsson et al., 2003; Perga et al., 2006; Taipale et al., 2008, 2009). Bacteria generally have higher phosphorus to carbon (P:C) ratios than algae (Vadstein, 2000), and thus may sustain the high P demand of *Daphnia* (Andersen & Hessen, 1991; Vrede et al., 1999; Hessen et al., 2002). Besides P, *Daphnia* also require essential biochemicals, like polyunsaturated fatty acids (PUFAs) and sterols (Brett & Müller-Navarra, 1997; Martin-Creuzburg et al., 2005, 2009), which are important membrane components and serve as precursors for a number of bioactive molecules (Grieneisen, 1994; Harrison et al., 1997; Desvilettes et al., 1997; Martin-Creuzburg et al., 2007). Only few bacteria (methanotrophic bacteria, MOB) are known to produce sterols (e.g. Schouten et al., 2000; Volkman, 2003), but stable isotope analysis indicate that these bacteria may significantly contribute to *Daphnia* diet (Taipale et al., 2007, 2008). In addition, a MOB has been reported to enhance the reproduction of *Daphnia*, but the growth of *Daphnia* itself was not enhanced (Taipale et al., 2012). Only MOB parallel fed with limited quantities of phytoplankton partially support *Daphnia* growth (Deines & Fink, 2011). Since sterol supplementation of bacterial diets (including a MOB) enhanced growth of *D. magna* (Martin-Creuzburg et al., 2011), the quality of bacteria as sole food source for *Daphnia* is likely low due to the absence of sterols in bacteria. Long chain PUFAs occur also not commonly in bacteria, although some, mostly marine psychrophilic bacteria did contain PUFAs (Russell & Nichols, 1999; Okuyama et al., 2007). Consequently, *Daphnia* have been shown to be limited simultaneously by the absence of sterols and long chain PUFAs when feeding on cyanobacterial diet (Martin-Creuzburg et al., 2008, 2009). Thus, *Daphnia* are likely to be additionally restricted by the absence of these biochemicals when feeding on heterotrophic bacterial diets.

In natural environments, bacteria are unlikely the sole food source for *Daphnia* and instead are ingested along with phytoplankton species. The bacteria/phytoplankton proportions vary strongly among lakes and within lakes depending on nutrient state and season (0.02 16 bacterial-C/phytotplakton C; cf. Simon et al., 1992; Hessen et al., 2003). Here, to investigate the response of survival, growth and egg production of *D. magna* to differences in food quality of mixed bacteria-phytoplankton diets, we conducted standardized growth experiments with juvenile animals feeding on different bacteria provided either as sole food source or in combination with the green alga *Scenedesmus obliquus*. By experimentally increasing the proportion of different bacteria in the diet, we determined the bacteria to phytoplankton ratio at which *Daphnia* were negatively affected indicating the effect of declined food quality. We hypothesized that higher proportions of heterotrophic bacteria in the food suspension will restrict growth and egg production of *Daphnia* because of a limitation by sterols and PUFAs and that low proportions will either not affect or even benefit the animals. In addition, to assess the significance of a dietary sterol or PUFA deficiency caused by increasing the proportion of bacterial carbon, bacteria were supplemented either with cholesterol, the predominant animal sterol, or eicosapentaenoic acid (EPA), a long chain PUFA known to be of particular importance for *Daphnia* growth and reproduction.

Materials and methods

Cultivation of food organisms and preparation of food suspensions

Growth experiments were conducted with three strains of heterotrophic bacteria, i.e. *Pseudomonas* sp. DD1

Table 1 Experimental setup and composition of food suspension

Treatment	Supplementation of bacteria with	Bacterial carbon (mg C l^{-1})	Abundance of *Flavobacterium* sp. DD5b (ml^{-1})	Abundance of *Pseudomonas* sp. DD1 (ml^{-1})	Abundance of *E. coli* (ml^{-1})
Starving	Nothing	0	0	0	0
Scenedesmus	*S. obliquus*	0	0	0	0
20/80	*S. obliquus*	0.4	4×10^6	4×10^6	4.4×10^6
50/50	*S. obliquus*	1	10×10^6	10×10^6	11.1×10^6
80/20	*S. obliquus*	1.6	16×10^6	16×10^6	17.8×10^6
Bacteria	Nothing	2	20×10^6	20×10^6	22.2×10^6
+Liposoms	Control liposomes	2	20×10^6	20×10^6	22.2×10^6
+Cholesterol	Cholesterol containing liposomes	2	20×10^6	20×10^6	22.2×10^6
+EPA	EPA containing liposomes	2	20×10^6	20×10^6	22.2×10^6

(NCBI: HQ113379) and *Flavobacterium* sp. DD5b (NCBI: HQ113381), both representing typical pelagic bacteria (Glöckner et al., 2000; Van der Gucht et al., 2005; Pearce et al., 2005), and *E. coli* (wild-type strain), which is regularly found in aquatic ecosystems (LaLiberte & Grimes, 1982 and cited references; Hamelin et al., 2007). The bacterial strains were grown in mineral medium as described in Martin-Creuzburg et al. (2011), but with 20 mM glucose and trace element solution SL12a (2 ml l^{-1}) which was slightly modified with 1.1 g l^{-1} $FeCl_2 \cdot 4H_2O$, 0.07 g l^{-1} $ZnCl_2$ and 0.1 g l^{-1} $MnCl_2$ from SL 12 (Overmann et al., 1992). Bacteria were grown at 20°C and harvested daily in the late exponential and early stationary growth phase. Cells were centrifuged (10 min, 5,000*g*, 15°C), and resuspended in sterile-filtered and autoclaved Lake Constance water. Aggregates were dissolved by vortexing and short sonication. In order to add defined numbers (carbon concentrations) of bacteria to the growth experiments (Table 1), cell numbers were determined in a Helber counting chamber with a Zeiss Axiophot microscope. Carbon concentrations of defined bacterial cell numbers (i.e. bacterial carbon content) were estimated before the start of the experiment to adjust the carbon concentrations and were repeated during the experiment.

The green alga *S. obliquus* (SAG 276-3a), which contains sterols, but is deficient in C-20 PUFAs and thus of intermediate food quality, was used as food for stock cultures of *D. magna* and as a reference food in the growth experiments. It was grown in semi-continuous batch cultures as described in Martin-Creuzburg et al. (2005) and harvested in the late exponential growth phase. Carbon contents of the autotrophic food suspensions were estimated from photometric light extinctions (800 nm) and from previously determined carbon-extinction equations.

Daphnia growth experiments

Stock cultures of *D. magna* (originally isolated by Lampert, 1991) were raised in filtered lake water (0.2-µm pore-sized membrane filter) containing saturating concentrations of *S. obliquus*. Growth experiments were carried out with third-clutch neonates (born ± 6 h) at 20°C in glass beakers filled with 200 ml of filtered lake water (<0.2 µm). Each treatment consisted of three replicates with six *D. magna* per beaker. Animals were transferred daily into new beakers with freshly prepared food suspensions over a period of 6 days, after which eggs were produced and the effect of different food qualities were pronounced.

Daphnia magna were raised on *Pseudomonas* sp. DD1, *Flavobacterium* sp. DD5b, or *E. coli* either as sole food source (100:0%, i.e. 2 mg C l^{-1}) or in different combination with *S. obliquus* (bacteria:phytoplankton carbon proportions: 80:20%, 50:50%, 20:80%, 0:100%; Table 1). The total carbon concentration in all treatments was 2 mg C l^{-1}. In addition, pure bacterial food suspensions were supplemented with either 50 µl of control liposomes (no sterols, no PUFAs), 50 µl cholesterol-containing liposomes, or 50 µl EPA-containing liposomes per beaker. Liposome stock suspensions were prepared as described in

Martin-Creuzburg et al. (2008). The experiment was completed by a concomitant starvation treatment.

To estimate somatic growth rates of *D. magna*, subsamples of the experimental animals were taken at the beginning and at the end of the experiment, dried for 24 h, and weighed on an electronic balance (Mettler Toledo XP2U; ±0.1 µg). Somatic growth rates (g) were determined as the increase in dry mass from day 0 (M_0) to day 6 (M_6) of the experimental period ($t = 6$ days) using the equation: $g = (\ln M_6 - \ln M_0)\ t^{-1}$. Egg production was estimated by counting the eggs in the brood chambers of the animals at the end of the experiment.

Biochemical analyzes of food suspensions

For analysis of fatty acids and sterols, at least 5×10^9 cells were harvested by centrifugation, washed, freeze-dried, and stored at −80°C at two time points. Total lipids were extracted thrice from freeze-dried samples with dichloromethane/methanol (2:1, v/v) and the pooled cell-free extracts were evaporated to dryness with nitrogen. Lipid extracts were transesterified with 3 mol l^{-1} methanolic HCl (60°C, 15 min) for analysis of fatty acids, or saponified with 0.2 mol l^{-1} methanolic KOH (70°C, 1 h) for analysis of sterols. Subsequently, fatty acid methyl esters (FAMEs) were extracted three times with 2 ml *iso*-hexane; the 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 a volume of 10 20 µl *iso*-hexane. Lipids were analyzed by gas chromatography on a HP 6890 GC equipped with a flame ionization detector (FID) and a DB-225 (J&W Scientific) capillary column to analyze FAMEs or with a HP-5 (Agilent Technologies) capillary column to analyze sterols. Details of GC configurations are given elsewhere (for fatty acids, Martin-Creuzburg et al. (2010); for sterols, Martin-Creuzburg et al. (2009)). Lipids were quantified (FID) by comparison to internal standards (C23:0 ME, 5α-cholestan) of known concentrations using multipoint standard calibration curves previously established for each compound (Sigma-Aldrich). The few non-purchasable lipid compounds were quantified using calibration curves of structurally related lipids with similar retention times. Lipids were identified by their retention times and their mass spectra, which were recorded with a gas chromatograph-mass spectrometer (Agilent Technologies, 7890A GC, 5975C inert MSD) equipped with a fused-silica capillary column (DB-225MS, J&W for FAMEs; DB-5MS, Agilent for sterols). Sterol samples were analyzed in their free form and as their trimethylsilyl derivatives. Spectra were recorded between 50 and 600 amu in the EI ionization mode. The limit of quantitation was ~20 ng for fatty acids or sterols. The absolute amount of each lipid was related to the particulate organic carbon (POC). At three times during the experiment, carbon, nitrogen (N) and sulphur (S) were determined in duplicates from bacterial and algal suspensions concentrated in tin capsules for liquid samples using an elemental analyser (EuroEA3000, HEKAtech GmbH, Germany). For determination of particulate phosphorus, two aliquots per food suspension were collected on acid-rinsed polysulfone filters (HT-200; Pall) and digested with a solution of 10% potassium peroxodisulfate and 1.5% sodium hydroxide for 60 min at 121°C; soluble reactive phosphorus was determined using the molybdate-ascorbic acid method (Greenberg et al. 1985).

Statistical analysis

Somatic growth rates and egg production of *Daphnia magna* were analyzed using one-way analyses of variance (ANOVA) and post hoc tests (Tukey's HSD or Dunnett's T3 if variances were not equal (*E. coli*)). Treatments in which only one or none animal per beaker survived were excluded from the ANOVAs. Raw data met the assumptions for ANOVA. Statistical analyses were carried out using the General Linear Model module of SPSS 11 (SPSS Inc.).

Results

Characteristics of food sources

The three bacterial strains used for diet mixing experiments hardly differed in their C, N, and S-content per cell (Table 2). The phosphorus content per cell was lowest in *E. coli* and highest in *Flavobacterium* sp. Compared to *S. obliquus*, the bacteria were characterized by lower C:N (33 45%) and C:P ratios (22 46%), indicating a higher N and P content of the bacteria (Table 2).

Table 2 Elemental composition and proportion of bacterial strains in comparison to the green algae *Scenedesmus obliquus*

	Flavobacterium sp. DD5b	*Pseudomonas* sp. DD1	*E. coli*	*S. obliquus*
fg C/cell	90.6 ± 18.4	81.9 ± 24.1	77.7 ± 9.9	
fg N/cell	21.2 ± 4.3	21.9 ± 5.2	22.9 ± 2.6	
fg P/cell	4.4 ± 0.1	3.0 ± 0.02	2.3 ± 0.04	
fg S/cell	3.7 ± 1.0	3.3 ± 1.0	3.4 ± 0.9	
C:N (mol:mol)	4.9	4.2	4.0	7.3
C:P (mol:mol)	64	82	92	117

Data represent means of three replicates (for phosphate one) over time from which each two subsamples were measured ± standard deviation

The fatty acid (FA) composition of the bacterial strains was dominated by saturated and monounsaturated fatty acids (Table 3). The fatty acid profiles of the γ-proteobacteria *E. coli* and *Pseudomonas* sp. were similar except for the saturated fatty acid 19:0 which only occurred in *E. coli*, a higher proportion of palmitoleic (16:1n-7) acid in *Pseudomonas* sp., and a higher proportion of cyclopropanoic acid (17:0Δ) in *E. coli*. The FA profile of *Flavobacterium* sp. was more diverse than the profiles of the other two bacteria and contained also high amounts of branched-chain and hydroxy-fatty acids. PUFAs and sterols could not be detected in any of the bacterial strains. In contrast, *S. obliquus* contained high amounts of 18:2n-6 and 18:3n-3, but no PUFAs with more than 18 carbon atoms. Chondrillasterol (IUPAC name: (22E)-5α-poriferasta-7,22-dien-3β-ol; mean ± SD: 58.3 ± 3.8%), fungisterol (5α-ergost-7-en-3β-ol; 19.4 ± 1.6%), 22-dihydrochondrillasterol (5α-poriferast-7-en-3β-ol; 9.7 ± 0.9%), and schottenol (5α-stigmast-7-en-3β-ol; 12.5 ± 1.1%) were the principal sterols found in the green alga.

The supplemented liposomes did not differ in their palmitic acid (16:0) and oleic acid (18:1n-9) content, which both are components of the phospholipids used to prepare the liposomes (Martin-Creuzburg et al., 2008). Liposomes prepared either in the presence of EPA or in the presence of cholesterol contained 14.5 ± 1.9 μg EPA or 11.2 ± 1.1 μg cholesterol per 50 μl of liposome stock suspension, respectively.

Survival of *D. magna*

On a pure *S. obliquus* diet, all *Daphnia* survived the experimental period. Without food, about 40% of the animals survived (Fig. 1). On pure bacterial diets, survival differed depending on the bacterial strain used. In general, survival of *D. magna* was highest on *Flavobacterium* sp. (∼60%), intermediate on *E. coli* (∼15%), and lowest on *Pseudomonas* sp. (0%). When provided in combination with *S. obliquus*, survival of *D. magna* decreased with increasing proportions of bacteria in the food suspension (Fig. 1). This decrease in survival was most pronounced with *Pseudomonas* sp.; none of the animals survived the experimental period when ≥50% of the available carbon was provided as *Pseudomonas* sp. In contrast, survival on diets containing increasing proportions of *Flavobacterium* sp. or *E. coli* was not reduced until 80% of the available carbon was bacterial carbon (Fig. 1). Cholesterol supplementation of a pure *Flavobacterium* sp. diet increased survival of *D. magna*, but cholesterol supplementation did not affect survival on the γ-proteobacteria. In contrast, survival on the γ-proteobacteria increased upon EPA supplementation (Fig. 1).

Somatic growth rates and egg production of *D. magna*

Somatic growth rates of *D. magna* were significantly affected by increasing the proportion of bacteria in their diet. Exchanging 20% of the available carbon with *Flavobacterium* sp. or *E. coli* slightly but significantly increased somatic growth rates as compared to those obtained on a pure *S. obliquus* diet (Fig. 2a, c; Tukey's HSD (a) or Dunnett's T3 (c), $P < 0.05$ following ANOVA: $F_{4,10} = 1506$ (a), $F_{3,8} = 220$ (c), both $P < 0.001$). In general, however, somatic growth rates decreased with increasing proportions of bacterial dietary carbon. With *Flavobacterium* sp. and with *E. coli*, this decrease was significant at proportions ≥80% and with

Table 3 Fatty acid content of the three bacteria *Flavobacterium* sp., *Pseudomonas* sp. and *E. coli*, and of the green alga *Scene desmus obliquus*

	Flavobacterium (% of total FA)	*Pseudomonas* (% of total FA)	*E. coli* (% of total FA)	*S. obliquus* (% of total FA)
14:0	1.39 ± 0.14	0.46 ± 0.12	0.91 ± 0.19	0.98 ± 0.11
15:0	9.33 ± 0.16	n.d.	n.d.	n.d.
i15:0	10.09 ± 0.17	n.d.	n.d.	n.d.
a15:0	1.41 ± 0.00	n.d.	n.d.	n.d.
2 OH 15:0	4.88 ± 0.06	n.d.	n.d.	n.d.
3 OH 15:0	2.06 ± 0.08	n.d.	n.d.	n.d.
15:1n>5	2.16 ± 0.10	n.d.	n.d.	n.d.
15:1n 5	2.12 ± 0.09	n.d.	n.d.	n.d.
16:0	13.14 ± 0.53	32.06 ± 0.48	28.09 ± 0.06	22.62 ± 0.82
i16:0	4.80 ± 0.09	n.d.	n.d.	n.d.
3 OH i16:0	1.88 ± 0.05	n.d.	n.d.	n.d.
3 OH a16:0	3.07 ± 0.08	n.d.	n.d.	n.d.
3 OH 17:0	2.84 ± 0.10	n.d.	n.d.	n.d.
16:1n>7	1.16 ± 0.05	n.d.	n.d.	n.d.
16:1n 7	28.51 ± 0.39	36.68 ± 0.61	1.55 ± 0.04	0.34 ± 0.09
16:1n<7	0.76 ± 0.00	n.d.	0.41 ± 0.04	n.d.
i17:0	1.50 ± 0.01	n.d.	n.d.	n.d.
17:0Δ	2.04 ± 0.26	4.70 ± 0.03	24.77 ± 0.01	n.d.
17:1n 7	3.34 ± 0.05	n.d.	n.d.	n.d.
18:0	2.56 ± 0.48	2.03 ± 0.02	1.79 ± 0.11	3.76 ± 0.27
18:1n 9/n 12	0.96 ± 0.06	24.06 ± 0.04	27.84 ± 0.64	26.5 ± 0.51
19:0Δ	n.d.	n.d.	14.65 ± 0.27	n.d.
18:1n 7	n.d.	n.d.	n.d.	0.41 ± 0.13
18:2n 6	n.d.	n.d.	n.d.	11.63 ± 0.23
18:3n 3	n.d.	n.d.	n.d.	30.60 ± 0.97
18:4n 3	n.d.	n.d.	n.d.	3.16 ± 0.19

Data represent means of two (bacteria) or three (alga) replicates over time ± standard deviation (*n.d.* not detectable, i.e. <20 ng)

Pseudomonas sp. somatic growth rates significantly decreased already with 20% of bacterial carbon (ANOVA: $F_{1,4} = 644$, $P < 0.001$; Fig. 2). Growth of *Daphnia* differed significantly in dependence of the fed bacterial phylotype, e.g. at 20% bacterial carbon, *Pseudomonas* sp. fed *Daphnia* grew significantly less than *Flavobacterium* sp. or *E. coli* fed *Daphnia* (Tukey's HSD, $P < 0.05$ following ANOVA: $F_{2,6} = 1041$) and at 50%, *E. coli* fed *Daphnia* grew significantly less than *Flavobacterium* sp. fed *Daphnia* carbon (ANOVA: $F_{1,4} = 27$, $P < 0.01$). When provided as sole food source, all bacteria were highly detrimental for *D. magna*. On a pure *Pseudomonas* sp. diet, none of the animals survived the experimental period and on a pure *E. coli* diet, only animals of one replicate barely survived so that somatic growth rates could not be determined. Growth rates obtained on a pure *Flavobacterium* sp. diet were low, but significantly increased upon cholesterol supplementation up to growth rates obtained on a pure *S. obliquus* diet (Tukey's HSD, $P < 0.05$ following ANOVA: $F_{4,10} = 255$; Fig. 2d). Supplementation of *Flavobacterium* sp. with control liposomes or EPA-containing liposomes did not improve somatic growth rates. Liposome supplementation of *E. coli* slightly improved survival and thus somatic growth rates could be determined. The obtained growth rates, however, were low and not affected by cholesterol or

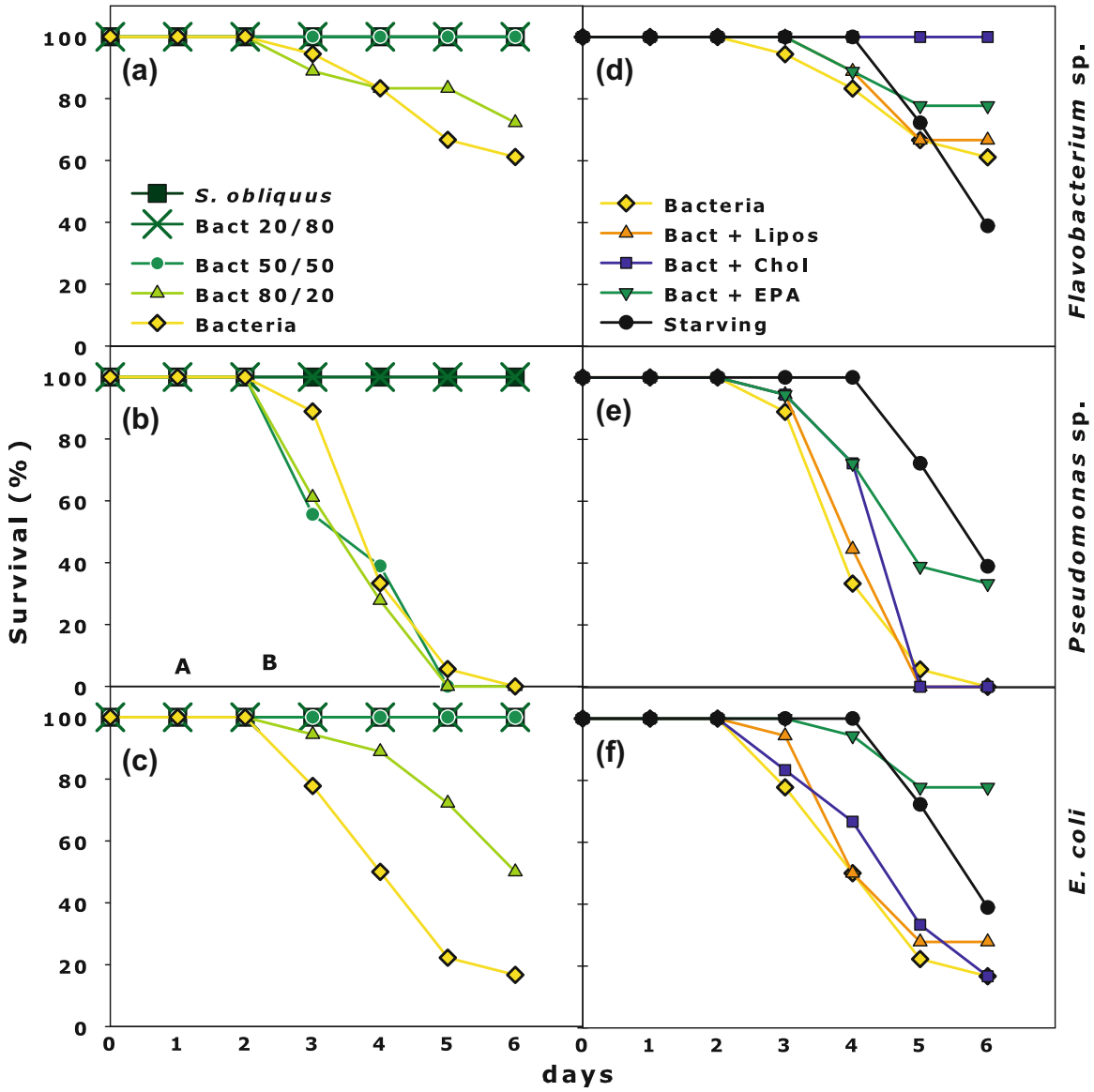

Fig. 1 Survival of juvenile *D. magna* exposed to different bacteria/*Scenedesmus obliquus* mixtures (**a** *Flavobacterium* sp., **b** *Pseudomonas* sp., and **c** *E. coli*) as well as to bacteria supplemented with cholesterol or EPA containing liposomes or control liposomes (Lipos) without added EPA or cholesterol (**d** *Flavobacterium* sp., **e** *Pseudomonas* sp., and **f** *E. coli*) in comparison to starved animals. Data were calculated from the numbers of individuals which survived the experimental period of 6 days (means of n 3 jars)

EPA supplementation. Liposome supplementation could also not improve somatic growth of *D. magna* on a pure *Pseudomonas* sp. diet (Fig. 2).

As observed for somatic growth, the detrimental effect of bacterial carbon on *Daphnia* egg production increased with decreasing proportions of dietary *S. obliquus* (Fig. 3). Animals raised on a diet consisting of ≥80% *Flavobacterium* sp., did not produce eggs within the experimental period and egg production was significantly reduced on a diet consisting of ≥50% *E. coli* (Tukey's HSD, $P < 0.05$ following ANOVA: $F_{2,6} = 20$). Animals exposed to *Pseudomonas* sp. did not produce eggs, even at the lowest dietary concentration. Animals raised on pure *Flavobacterium* sp. produced eggs upon cholesterol supplementation but less than produced on a pure *S. obliquus* diet.

Discussion

It has been recognized that heterotrophic bacteria are of poor food quality for *Daphnia* (Martin-Creuzburg

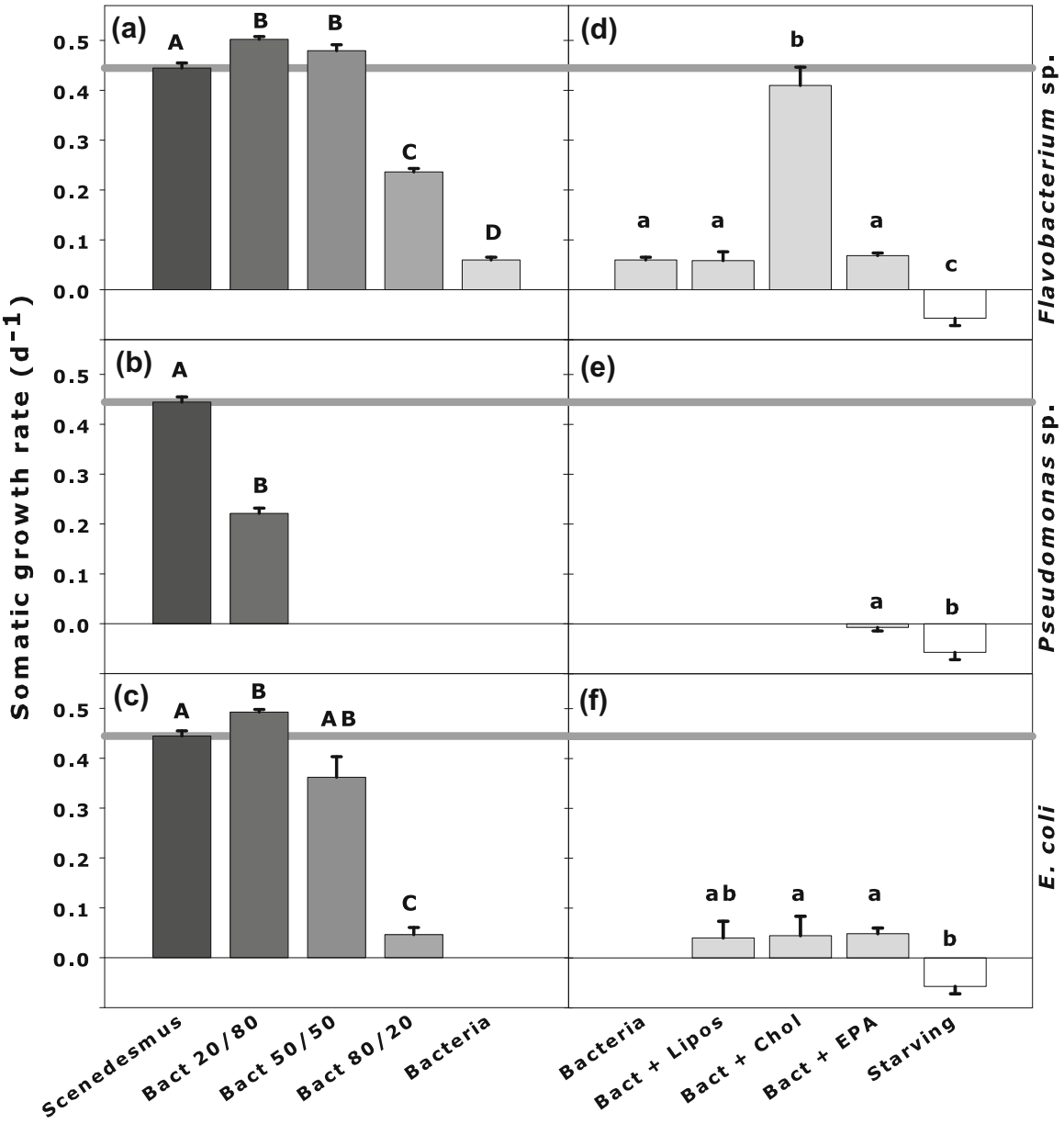

Fig. 2 Somatic growth rates of juvenile *D. magna* on different diets in comparison to starved animals. *Daphnia* were fed different bacteria/*Scenedesmus obliquus* mixtures (**a** *Flavobacterium* sp., **b** *Pseudomonas* sp., and **c** *E. coli*) as well as bacteria supplemented with cholesterol or EPA containing liposomes or control liposomes (Lipos) without added EPA or cholesterol (**d** *Flavobacterium* sp., **e** *Pseudomonas* sp., and **f** *E. coli*). Growth rates of animals fed 100% *E. coli* were excluded since only few animals of one replicate survived. The horizontal gray bar indicates growth rates of *D. magna* fed *S. obliquus*. Data are means of three replicates per treatment; *error bars* indicate SD. Bars labelled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$ following ANOVA)

et al., 2011; Wenzel et al., 2012; Taipale et al., 2012). In a previous study, we have shown that this poor food quality is partially due to a dietary deficiency in sterols, as indicated by a growth-enhancing effect upon sterol supplementation of different bacterial diets (Martin-Creuzburg et al., 2011). However, bacteria are not only characterized by a deficiency in sterols, they usually are also deficient in PUFAs (Russell & Nichols, 1999; Okuyama et al., 2007), suggesting a co-limitation by sterols and PUFAs as has been shown for cyanobacterial diets (Martin-Creuzburg et al., 2009). Moreover, a number of bacterial strains isolated from aquatic habitats have been shown to produce toxic secondary metabolites that are active

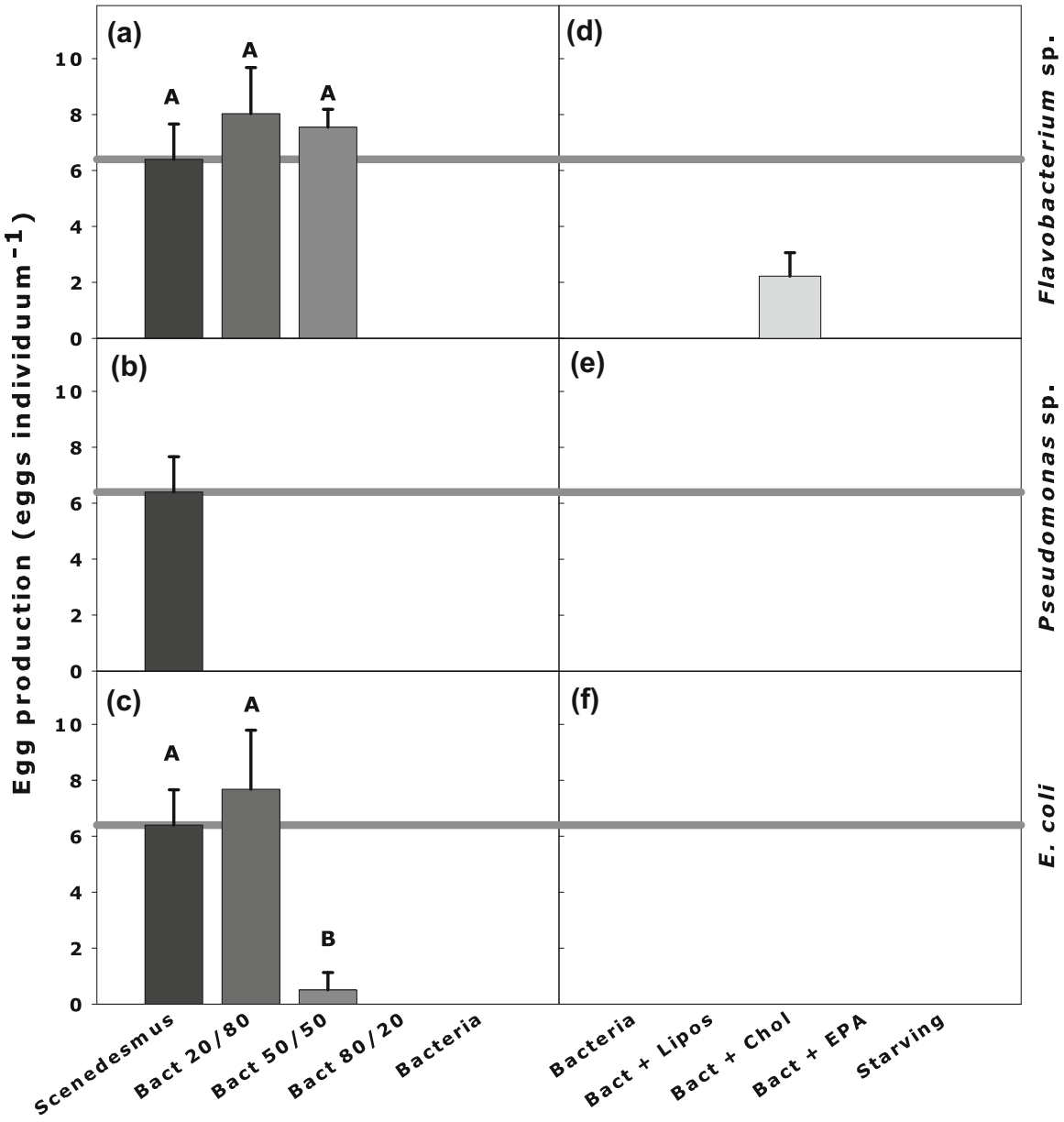

Fig. 3 Egg production of *D. magna* on different diets in comparison to starved animals. *Daphnia* were fed different bacteria/*Scenedesmus obliquus* mixtures (**a** *Flavobacterium* sp., **b** *Pseudomonas* sp., and **c** *E. coli*) as well as bacteria supplemented with cholesterol or EPA containing liposomes or control liposomes (Lipos) without added EPA or cholesterol (**d** *Flavobacterium* sp., **e** *Pseudomonas* sp., and **f** *E. coli*). The horizontal gray bar indicates number of eggs of *D. magna* fed *S. obliquus*. Data are means of three replicates per treatment; *error bars* indicate SD. Bars labelled with the same letters are not significantly different (Tukey's HSD, $P < 0.05$ following ANOVA)

towards protozoans and metazoan grazers, among them *D. magna* (Matz & Kjelleberg, 2005; Matz et al., 2008; Deines et al., 2009). High bacterial toxicity was also observed in our previous study, in which *D. magna* were exposed to a *Hydrogenophaga* sp. strain or a *Pseudomonas* sp. strain, which were previously isolated from the digestive tract of *D. magna* (Martin-Creuzburg et al., 2011). The high toxicity of this *Pseudomonas* sp. strain was corroborated in this study. When provided in combination with *S. obliquus*, growth and egg production of *D. magna* were significantly impaired even at low dietary concentrations of *Pseudomonas* sp. (20% of dietary carbon). When *Pseudomonas* sp. was provided in higher concentrations, none of the animals survived the experimental period of 6 days. *Pseudomonas* spp. are known to

produce a variety of toxic secondary metabolites (Gross & Loper, 2009), suggesting that the negative effects of the isolated *Pseudomonas* sp. strain on the performance of *D. magna* we observed here and in our previous study were also caused by toxic secondary metabolites. Numerous *Pseudomonas* species/strains have been shown to act as pathogens in a wide range of invertebrates (Padmanabhan et al., 2005; e.g. Hilbi et al., 2007); detrimental effects on *Daphnia* have been reported for *P. aeruginosa* and *P. entomophila* (Le Coadic et al., 2012). However, Wenzel et al. (2012) reported that *Daphnia* can survive on diets containing high proportions of another *Pseudomonas* strain, indicating that *Pseudomonas* strains are not necessarily detrimental to consumers.

A somewhat lower mortality was observed on diets containing *E. coli*. Although *E. coli* is mostly classified as commensal, the potential of some strains to cause diseases in humans and other mammals has long been recognized. Less is known about pathogenicity of *E. coli* for invertebrates. However, *E. coli* strains were found to act as opportunistic pathogens in stressed and immunocompromised invertebrates, i.e. in old individuals and in individuals exposed to other pathogens [Millet & Ewbank, 2004 (nematode); Broderick et al., 2006 (gypsy moth larvae)]. Since higher concentrations of *E. coli* than *Pseudomonas* sp. were required to cause the death of *Daphnia*, one may speculate that high amounts of a low quality food which likely reduced fitness of the animals increased *Daphnia* susceptibility to the pathogens. On the other hand, most *E. coli* strains from aquatic environments are non-pathogenic (e.g. Hamelin et al., 2007). The detrimental effect of higher proportions of *E. coli* may not be caused by a possible pathogenicity (or toxicity) but by a more pronounced response of *Daphnia* to their restricted food quality, since the effect of food quality increase with food quantity (Sterner, 1997).

The mortality of *D. magna* raised on diets containing *Flavobacterium* sp. was far less pronounced and presumably caused by nutritional challenges rather than toxicity. This at least was suggested by the growth-enhancing effect of sterol supplementation, indicating a sterol limitation of *D. magna*. In fact, somatic growth rates of *D. magna* on a pure *Flavobacterium* sp. diet increased upon sterol supplementation up to the growth rates obtained on a pure *S. obliquus* diet, showing that the absence of sterols is the major food quality constraint daphnids are confronted with while feeding on this bacterium. We propose that the negative effects associated with a dietary deficiency in sterols are on the tested γ-proteobacteria *Pseudomonas* sp. and *E. coli* masked by the toxicity of these bacteria.

It has been shown that daphnids feeding on cyanobacteria are simultaneously limited by sterols and PUFAs (Martin-Creuzburg et al., 2009; Sperfeld et al., 2012). In a previous study, we did not find clear evidence for such a co-limitation of *Daphnia* while feeding on heterotrophic bacteria (Martin-Creuzburg et al., 2011). In this study, supplementation of heterotrophic bacteria with EPA did not increase somatic growth rates or egg production of *D. magna*, suggesting that the absence of dietary PUFAs did not constrain the performance of the animals. This is somehow supported by the finding that somatic growth rates of *D. magna* on a pure *Flavobacterium* sp. diet, which contained neither EPA nor other PUFAs, increased upon sterol supplementation up to the level obtained on a pure algal (*S. obliquus*) diet. However, dietary PUFAs are indispensable for proper growth and reproduction of *Daphnia*, as has been shown by numerous studies, and thus it is rather unlikely that the performance of animals feeding on heterotrophic bacteria is not affected by the absence of dietary PUFAs. One might argue that the experimental design we used here, i.e. short-term growth experiments, are unsuitable to detect potential consequences associated with a dietary PUFA deficiency, because the animals may still rely on maternal PUFA reserves. However, the maternal PUFA supply was presumably low, because the animals were pre-raised on *S. obliquus*, a green alga deficient in long chain PUFAs. Although, these PUFAs can be produced and retained from shorter algal PUFAs by *Daphnia* (Kainz et al., 2004, Taipale et al., 2011), previous studies have repeatedly shown that even short-term feeding on PUFA deficient diets results in a limitation by PUFAs, provided that at least small amounts of dietary sterols are available (Martin-Creuzburg et al., 2009; Sperfeld et al., 2012). In our previous study, however, simultaneous supplementation of *Flavobacterium* sp. with cholesterol and EPA did also not reveal clear evidence for a PUFA limitation once sterol requirements were met (Martin-Creuzburg et al., 2011). Overall, we did not find clear evidence for a limitation by EPA on bacterial diets within our 6 day lasting growth experiments.

However, it remains to be tested whether a potential limitation by EPA can be detected when more than one reproduction cycle is considered, i.e. when potential PUFA reserves are exhausted (cf. Martin-Creuzburg et al., 2009). Interestingly, EPA supplementation of the γ-proteobacteria increased the survival of *D. magna*, suggesting that the detrimental effects mediated by these bacteria may have been alleviated by dietary EPA. We conclude that further (long-term) supplementation experiments are required before we are able to assess the role of PUFAs in determining the food quality of heterotrophic bacteria for *Daphnia*.

In the field, *Daphnia* do not feed solely on heterotrophic bacteria, and thus it is important to investigate at which dietary proportions bacteria become detrimental. By feeding *D. magna* with different combination of bacteria and the green alga *S. obliquus*, we show here that growth and egg production of *D. magna* are constraint when 80 20%, respectively, of the available carbon are represented by bacteria. This corroborates previous findings by Wenzel et al. (2012), who tested the food quality of a *Pseudomonas* strain in different combinations with *Rhodomonas*. They found that a 20% share of *Rhodomonas* in the food allowed survival of *Daphnia* and that a 50% share enabled *Daphnia* to reproduce. Taipale et al. (2012) reported that high proportions of a type one methanotroph provided in different combinations with *Cryptomonas* resulted in high reproduction of *Daphnia* over 2 weeks. At limiting quantities of phytoplankton, MOB could even partially support *Daphnia* growth (Deines & Fink, 2011). Thus, it appears that the dietary proportions at which bacteria become detrimental for *Daphnia* strongly depend on the bacterial phylotype and presumably also on the biochemical composition of the predominant algae.

Our results show that low proportions of heterotrophic bacteria in the food suspension can even increase somatic growth rates of *Daphnia*, as compared to a pure *S. obliquus* diet, suggesting that bacteria can provide essential nutrients not available in the green alga. Bacteria are often characterized by high P:C ratios and the bacteria in our experiment, especially *Flavobacterium* sp., had higher P:C ratios than *S. obliquus* (cf. Vadstein, 2000). However, the P:C-ratio of *S. obliquus* was already far above limiting levels (cf. DeMott, 1998; Persson et al., 2011) and thus a limitation of *D. magna* by phosphorus was rather unlikely in particular because daphnids seem to incorporate phosphorus from bacteria and algae with similar efficiencies (Wenzel et al., 2012). Nevertheless, low proportions of *Flavobacterium* sp. and *E. coli* increased somatic growth rates of *D. magna*, suggesting that other bacteria-derived nutrients were responsible for the observed upgrading of the *S. obliquus* diet. Vitamins, for instance, which can be produced by many bacteria, including members of the Flavobacteria and Enterobacteriaceae (Donderski & Nowacka, 1992), are potentially important for *Daphnia* and it has been suggested already that vitamin addition can improve the food quality of *S. obliquus* for *Daphnia* (D'Agostino & Provasoli, 1970; Mehdipour et al., 2011). The role of vitamins in determining food quality for *Daphnia* certainly requires further research.

Bacteria can comprise the major fraction of suspended organic matter in particular in oligo- to mesotrophic lakes (cf. Simon et al., 1992), but are quantitatively important also in eutrophic waters. High or increasing bacteria:phytoplankton ratios may constrain growth and reproduction of *Daphnia* even under field conditions. However, low proportions of algae may be enough to compensate for the nutritional deficiency of the bacteria and the bacteria algae mixtures may support *Daphnia* at least for a defined period of time. Especially members of Bacteroidetes, as well as of Actinobacteria (Taipale et al., 2012), even sustain *Daphnia* growth at higher bacterial proportions. Both bacterial groups are often numerically dominant in freshwater habitats, persist over seasons and seem to play an important role in the degradation of complex organic matter (Eiler & Bertilsson, 2007; Newton et al., 2011; Parveen et al., 2011). Consequently, they potentially gain in importance for *Daphnia* nutrition at the end or between phytoplankton blooms (i.e. at higher bacteria:phytoplankton proportions).

Overall, our study highlight that feeding on heterotrophic bacteria can be associated with multiple challenges *Daphnia* have to cope with. Bacteria per se had detrimental effects on *Daphnia* because of their nutritional restraints or potential toxicity. Depending on the bacterial phylotype, however, *Daphnia* may be able to grow and reproduce even at high dietary proportions of bacteria, i.e. when provided in combination with eukaryotic phytoplankton. Moreover, low bacterial proportions may even upgrade the nutritional value of phytoplankton-dominated food. Thus, we propose that the relative contribution of bacteria and

phytoplankton to total dietary carbon as well as their phylogenetic composition will strongly affect growth and survival of *Daphnia* and potentially other filter-feeding zooplankton under field conditions.

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