

Phytoplankton essential fatty acid and phosphorus content constraints on *Daphnia* somatic growth and reproduction

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Abstract

We performed 12 experiments where the herbivorous zooplankter *Daphnia pulex* was fed three different phytoplankton food types (cyanophytes, chlorophytes, or cryptophytes) at two phosphorus (P) deficiency levels (C:P ≈ 400 or 600) and with two different fatty acid (FA) supplements. Phosphatidylcholine liposome amendments were used to manipulate P availability and/or deliver FA in order to simultaneously test the relative importance of phytoplankton P and FA limitation as constraints on *Daphnia* somatic growth rate and reproduction. We used multiple regression analyses to test the data set for six main effects and three interaction terms. The six main effects tested were phytoplankton taxa, FA supplementation, FA type (EPA or FA-mix), direct (P-D) and indirect (P-ID) phosphorus limitation, and P deficiency level. Food quality was most strongly affected by phytoplankton taxa followed by P-ID, FA supplementation, the interaction between phytoplankton taxa and P-ID, and P-D. *Daphnia* fed cryptophytes grew 0.18 day⁻¹ faster and had an additional 2.6 eggs individual⁻¹ than *Daphnia* fed cyanophytes. Indirect P limitation reduced *Daphnia* somatic growth rates by 0.16 day⁻¹ and egg production by 2.6 eggs individual⁻¹. Direct P limitation reduced *Daphnia* growth rates by 0.04 day⁻¹ and egg production by 1.2 eggs individual⁻¹. FA supplementation improved growth by 0.09 day⁻¹ and egg production by 2.2 eggs individual⁻¹. These results suggest that FA supplementation exerted stronger effects on *Daphnia* somatic growth rate and reproduction than did direct P limitation. Furthermore, these results suggest that phytoplankton taxa and indirect P limitation had the greatest effects on *Daphnia* growth and reproduction.

Phytoplankton phosphorus content and fatty acid composition have been previously identified as factors that may play important roles in herbivorous zooplankton nutrition (Urabe and Watanabe 1992; Müller-Navarra 1995b; Sterner and Schulz 1998). The phosphorus limitation hypothesis is based on the observation that the ratio of carbon (C) to phosphorus (P) in herbivorous zooplankton is nearly constant (Sterner and Hessen 1994), while this ratio in lake seston can vary widely across and within systems. Empirical evidence suggests that for phosphorus rich zooplankton such as *Daphnia* (C:P ≈ 93), the dietary phosphorus limitation threshold occurs in the range of seston C:P > 150–375 (Urabe et al. 1997; DeMott 1998; Brett et al. 2000). In *Daphnia*, a large fraction of the P pool is found in nucleic acids and phospholipids (Vrede et al. 1999). Vrede et al. (2002) demonstrated that the RNA:DNA ratio in *Daphnia galeata* was highly correlated with somatic growth rate and increased as the dietary C:P ratio was decreased down to a threshold of approximately

200. To maintain constant C:P ratios, daphnids are known to make physiological adjustments such as increasing their carbon excretion and respiration rates when food becomes P deficient (Darchambeau et al. 2003). The low food quality of P-limited algae may also be due to morphological changes that cause some phytoplankton to become less digestible when nutrient stressed (Lüring and Van Donk 1997; Van Donk et al. 1997).

The fatty acid limitation hypothesis is based on the observation that phytoplankton food quality is sometimes strongly correlated with the availability of certain polyunsaturated fatty acids (PUFA) (Müller-Navarra 1995b; Müller-Navarra et al. 2000; Wacker and Von Elert 2001). Essential fatty acids (EFA) are necessary in cell membranes and also as precursors for molecules involved in immune responses (Vance and Vance 1985). Fatty acids with double bonds in the n3 and n6 positions of the fatty-acid molecule are considered to be essential because most animals lack the specific desaturases required to synthesize them de novo (Vance and Vance 1985). Controlled laboratory growth experiments also indicate that dietary ω3 PUFA enrichment can have significant effects on *Daphnia* somatic growth rates and egg production (Von Elert 2002; Ravet et al. 2003; Becker and Boersma 2005).

Food quality experiments that have used natural lake seston to compare phytoplankton P and PUFA limitation have reported mixed results. In one study it was found that *Daphnia* growth rates increased when supplementing lake seston from a mesotrophic system with both P and PUFA (Boersma et al. 2001). In another study, DeMott and Tessier (2002) observed only weak responses to both P and PUFA supplementation in six lakes intended to represent a natural gradient in resource quality.

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It has also been shown that herbivorous zooplankton feeding on different phytoplankton taxa experience quite different growth rates due to food quality differences among algal taxa (Infante and Litt 1985; Ahlgren et al. 1990; Lundstedt and Brett 1991). In an effort to tease apart the relative contributions of phytoplankton taxa, EFA composition, and P content to food quality for *Daphnia*, we designed a set of experiments to test the food quality of three phytoplankton taxa (cyanophytes, chlorophytes, or cryptophytes) at two P deficiency levels (C:P = 400 or 600) and with two EFA supplements (EPA or FA-mix). We determined the nutritional importance of these factors by measuring *Daphnia* somatic growth rates and individual clutch sizes at the primiparous instar and tested these responses using multiple regression analyses. We believe that simultaneously testing the relative importance of phytoplankton P and EFA limitation for *Daphnia* nutrition will provide important insights into the nature and complexity of food quality in natural systems.

Methods

Zooplankton culture—All experiments were conducted using a clone of *Daphnia pulex* originally isolated from Clear Lake, California (U.S.A.). *D. pulex* stock cultures were maintained on the green alga *Scenedesmus obliquus* in a growth chamber with a constant temperature of 18°C and a 14:10 light:dark cycle.

Phytoplankton cultures—Eight phytoplankton monocultures, three cyanophytes (*Microcystis aeruginosa* 2063, *Microcystis aeruginosa* 2387, and *Synechococcus elongatus* obtained from the University of Texas culture collection), two chlorophytes (*Scenedesmus obliquus* and *Ankistrodesmus*), and three cryptophytes (*Cryptomonas ovata* 979/44, *Cryptomonas ovata* 979/61, and *Rhodomonas minuta* obtained from the University of Toronto Culture Collection) were maintained on L16 growth medium (Lindström 1983) supplemented with earth extract and B vitamins. These phytoplankton monocultures were used to formulate the cyanophyte, chlorophyte, and cryptophyte spp. mixtures. Each phytoplankton mixture consisted of proportionately equal parts of the respective monocultures with a final concentration of 2.0 mg L⁻¹ phytoplankton dry weight. Phytoplankton biomass in the different monocultures was determined daily using total suspended solids where 250 mL of each culture were filtered onto a pre-weighed glass-fiber filter (Whatman GF/F), dried for 2 h at 105°C, and then weighed.

Experimental design—We conducted a total of 12 flow-through experiments in a 200-L aquarium equipped with 12 partially submerged 120-mL chambers. A peristaltic pump was used to supply phytoplankton mixtures to the *Daphnia* in the chambers at a rate of 1.3 L d⁻¹ per chamber. Each of the three phytoplankton mixtures was tested at moderate (C:P ≈ 400) and severe (C:P ≈ 600) P limitation and with or without amendments of either EPA or a FA mixture. Food treatments were prepared fresh daily.

Figure 1 visually depicts the general experimental design employed in this series of experiments. The phosphorus axis was intended to separate direct (D) phosphorus limitation (i.e., a simple deficit of this nutrient) from indirect (ID) phosphorus limitation (e.g., changes in cell wall morphology) impacts on *Daphnia* nutrition. We employed three treatments of a potential four along this axis. The treatment that had high C:P phytoplankton that was not supplemented with P-rich liposomes had both indirect (ID) and direct (D) phosphorus limitation. The treatment that had low C:P phytoplankton had neither indirect nor direct phosphorus limitation. The treatment that had high C:P phytoplankton supplemented with P-rich liposomes had indirect P limitation but not direct P limitation. We could not complete this matrix because it is impossible to culture or supplement phytoplankton so that they would induce direct P limitation (i.e., have a low P content) without also inducing potential changes in phytoplankton morphology and biochemical composition. Because we are unable to complete this matrix, we are also unable to test for interactions between direct and indirect P limitation impacts on *Daphnia* nutrition.

Figure 1 also represents the P and FA level axes used in these experiments. The different P levels represent moderate P limitation (C:P ≈ 400, which is equivalent to the 83rd percentile for natural lake C:P ratios) and strong P limitation (C:P ≈ 600, which is equivalent to the 97th percentile for natural lake C:P ratios). (These percentiles are based on data from Brett et al. 2000, Elser et al. 2001, and Sterner et al. unpubl. data.) The different FA levels represent the fact that Ravet et al. (2003) found EPA additions explained 35% of the food quality difference between cyanobacteria and cryptophyte diets, whereas a mixture of those ω3 PUFAs that were deficient in cyanobacteria but prevalent in cryptophytes explained 55% of the difference.

Because we were unable to vary the intrinsic FA composition of the phytoplankton taxa we used (i.e., the cyanophytes always had very low EFA content, and the cryptophytes always had high EFA content regardless of how we cultured them), the coefficients provided by our regression analyses will systematically understate the impact of our EFA additions and overstate the importance of the phytoplankton taxa used in these experiments. To demonstrate this bias for the EFA addition versus phytoplankton taxa comparison, consider a hypothetical statistical analysis where the unsupplemented cryptophyte and cyanophyte treatments resulted in mean *Daphnia* growth rates of 0.573 ± 0.012 (±1 SD) and 0.354 ± 0.016, respectively. (These were the actual values obtained for these treatments.) If we assume that supplementing EFA rich cryptophytes with EFA will have minimal impacts on their food quality (which was true), then we can assign the cryptophyte plus EFA treatment a mean growth rate of 0.573. We can then hypothetically vary the growth response in the cyanophyte plus EFA treatment to see which coefficients will result. If we also assign the cyanophyte plus EFA treatment a mean growth rate of 0.573, then the most obvious ecological interpretation of this result would be that EFA supplementation ameliorated

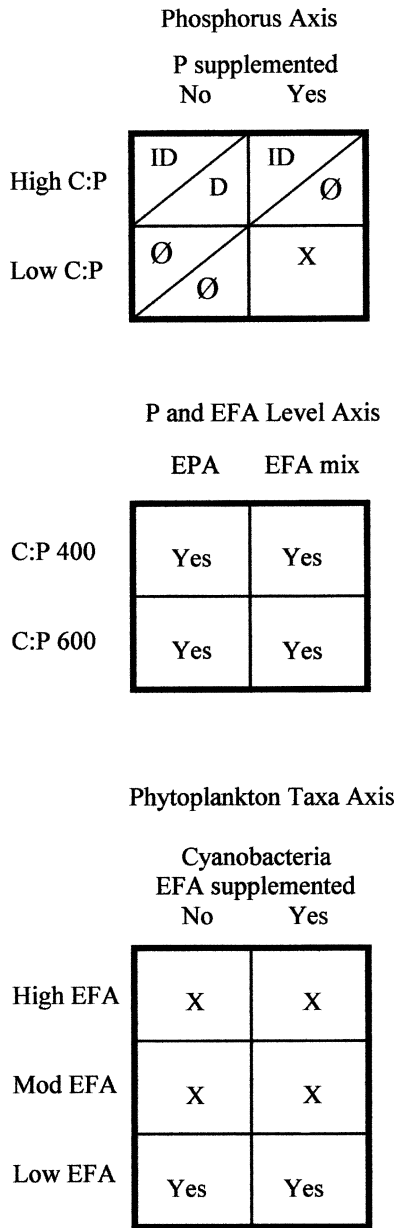


Fig. 1. This figure depicts the experimental design and the treatments that we employed. We were not able to complete the treatment matrix for the P axis because we do not know of a way to culture phytoplankton under P-stressed conditions without inducing changes in morphological and/or biochemical composition. Similarly, we were not able to complete the phytoplankton taxa matrix because we were not able to change the intrinsic FA content of the three phytoplankton taxa. Direct and indirect components are referred to in the figure as D and ID, respectively. The symbol ∅ denotes a null component, and X signifies a treatment parameter that we were not able to test.

all of the low food quality of the cyanobacteria relative to the cryptophyte diet (Ravet et al. 2003). However, in actuality the coefficients provided by the regression analysis in this case would attribute 0.109 of the differences between these treatments to the taxa response and 0.109 to the EFA addition responses even though in this hypothetical case the EFA addition explained all the difference

between the cryptophyte and cyanophyte diets. In the case where the EFA addition accounted for half of the difference between the cryptophyte and cyanophyte treatments (i.e., the cyanophyte plus EFA treatment averaged 0.463), the regression analysis returned coefficient values of 0.164 for the taxa response and 0.055 for the EFA addition response. In both examples the coefficient values for the EFA addition treatment were half of what is intuitively expected on the basis of the total difference between the cryptophyte and cyanophyte treatments that is actually explained by the EFA additions. In this study we corrected for this bias by reporting the *Daphnia* response to the EFA addition treatments as the original coefficient times 2. We similarly corrected the *Daphnia* response to the phytoplankton taxa treatments by subtracting the original coefficient for the EFA addition treatment. For the example above where the EFA addition treatment accounted for half of the difference between the cryptophyte and cyanophyte diets, these corrections would result in coefficients of 0.109 for both treatment responses.

This bias did not occur when assessing the relative importance of direct and indirect P limitation. For example, consider a hypothetical statistical analysis where low C:P green algae and high C:P green algae result in mean *Daphnia* growth rates of 0.465 ± 0.015 and 0.266 ± 0.026 , respectively. If the P addition treatment (i.e., high C:P chlorophytes plus P-rich liposomes) accounted for half of the difference between the low and high C:P treatments, the regression analysis would return coefficient values of 0.100 for the direct P limitation response and 0.100 for the indirect P limitation response. If, on the other hand, the P addition treatment resulted in a mean *Daphnia* growth rate of 0.465, the respective direct P and indirect P limitation coefficients would be 0.199 and 0.000.

Overall, our experimental design includes three phosphorus treatments, three phytoplankton taxa, two FA supplements, and two levels of P and FA limitation. While this design may seem overly complex (especially compared to most studies in the *Daphnia* nutritional physiology literature that typically consider only one constraint at a time), it represents an attempt to reflect the dramatic variation in the P and FA content and species composition commonly encountered in natural phytoplankton assemblages.

Liposome and EFA supplements—Phosphatidylcholine liposomes (Sigma sterile pyrogen free preliposome formulation 5) were formulated and loaded in accordance with the liposome encapsulation methods described by Ravet et al. (2003). Liposomes were used as a carrier to deliver fatty acid amendments and, at concentrations 16–28 times higher, as a P source to manipulate the C:P ratio of the P-amended food treatment. Free-form fatty acids (EPA [20:5 ω 3], DHA [22:6 ω 3], α -linolenic acid [18:3 ω 3], and stearidonic acid [18:4 ω 3]) were purchased from Sigma and stored at -20°C prior to the experiments.

To test for potential positive and/or negative effects of the liposome amendments, we performed a liposome dose-response experiment in which *Daphnia* were fed P-deficient *Scenedesmus* with incrementally increasing liposome concentrations. The percentage of dietary carbon from

liposome amendments was varied from 0% to 35% in this experiment, and *Daphnia* growth rates and egg production responses were measured.

P and FA manipulations of the food treatments—To obtain the target C:P ratios in the P-limited food treatments (C:P \approx 400 and C:P \approx 600), we used a modified version of the L16 growth medium (Lindström 1983) in which the K_2HPO_4 component was reduced to limit available P. Since K_2HPO_4 serves as a source of both P and K in this media, we added KNO_3 to compensate for the otherwise accompanying K deficit. Phytoplankton batch cultures were grown in P-deficient media in a growth chamber for 5–10 d until sufficient biomass (>2.0 mg L^{-1} phytoplankton dry weight) was achieved. We carefully maintained algae cultured on P-deficient media in a low-P environment (delivered via peristaltic pump in P-deficient growth media) throughout the experiments. Moderate (target C:P 400) and severe (target C:P 600) P limitation refers to the stoichiometry of the algae.

In food treatments where liposomes were used as a P source to manipulate the C:P ratio, we added specified amounts of the phosphatidylcholine liposome formulation (C:P ratio = 44) to adjust the C:P ratio of the overall food mixture from \approx 400 or 600 down to a target C:P of \approx 150. This required liposome additions ranging from 10 to 30 μ L liposome formulation per liter of phytoplankton food treatment. The C:P ratios of the P amendments were adjusted after the phytoplankton cultures were brought to the target concentration of 2.0 mg L^{-1} phytoplankton dry weight. We limited liposome amendments to $<10\%$ of the total dietary carbon as tested prior to the final experiments (see below).

Liposome fatty acid treatments consisted of individual or combined EFA amendments designed to mimic differences in the EFA content of the cyanophyte and cryptophyte spp. mixtures. See Ravet et al. (2003) for a detailed description of this procedure. In these experiments we tested amendments of EPA alone and also as a combination of four fatty acids (EPA, DHA, α -linolenic acid, and stearidonic acid), which we refer to as the essential fatty acid (EFA) mixture.

Sampling protocol—Twelve hours prior to the start of each experiment, egg-bearing *Daphnia* were separated from stock cultures and placed into individual 20-mL scintillation vials with *S. obliquus* as food. At the beginning of each experiment, neonates from this \approx 6-h-old cohort were randomly selected and transferred to a “rinse” media, which was either the P-deficient or the P-sufficient algal growth media, for approximately 20 min prior to being transferred into the corresponding P-deficient or P-sufficient food treatment chambers. A subsample of approximately 15–20 neonates was simultaneously dried and weighed on a Cahn Microbalance (Model #C33) to provide an initial neonate biomass estimate. Eight neonates were placed in each chamber, and the chambers were provided a constant flow for each of the specific dietary treatments. Each experiment lasted 6 d. At the conclusion of each experiment, *Daphnia* were collected, measured individually for length and clutch size (eggs per individual)

under a microscope, and then dried (24 h at 105°C) and weighed (in groups of four individuals from each replicate) to obtain the average individual weight per replicate. Somatic growth rates (g) were calculated accordingly: $g = (\ln(W_t/W_i))t^{-1}$, where W_i is the initial animal weight, W_t is the final animal weight, and t is the duration of each experiment in days. Mortality was almost always less than 13% in these replicates.

Samples from the phytoplankton food treatments were collected on day 5 of each experiment and prepared for biochemical analyses. Particulate matter was collected in duplicate on precombusted glass-fiber filters (Whatman GF/C). Phytoplankton particulate phosphorus content was determined according to Solórzano and Sharp (1980). Particulate carbon in the food treatments was calculated on the basis of measured dry weights and published carbon mass to dry weight ratios for the three phytoplankton groups. The phytoplankton carbon content for each phytoplankton group, expressed as averaged percentages of dry weight, are 47% for the cyanobacteria spp., 54% for the chlorophytes, and 51% for the cryptophytes (Reynolds 1984).

Fatty acid analysis—Fatty acids were extracted from samples and methylated according to Kattner and Fricke (1986). Fatty acid methyl esters were analyzed with a gas chromatograph (HP6890) equipped with a programmable temperature vaporizer injector, a fused silica capillary column (DB-WAX, J&W Scientific; 30 m \times 0.32 mm with 0.25- μ m film thickness), and a flame ionization detector. We injected 5 μ L of sample and used helium as the carrier gas. The temperature program applied was as follows: 40°C held for 5 min, then heated up at 10°C per minute to 150°C, held for 5 min, then heated up at 1°C per minute to 220°C, where it was kept for 20 min. Individual fatty acids were identified on the basis of the retention times of fatty acid methyl ester standards (Sigma, Supelco, Alltech) dissolved in n-hexane. Quantification was performed with an internal standard (21:0) and quantitative mixes (Alltech) to calculate response factors for each fatty acid analyzed.

Statistical analyses—We used a regression analysis with scored categories to examine these data (Fisher and Yates 1953). In general, we scored the level that we expected to have the lowest food quality 0 and the level we expected to have the highest food quality 1. For example, the treatment axis that represented our EFA treatments was scored 1 when we added EFA and 0 when we did not. We scored our phytoplankton taxa accordingly: cyanobacteria (0), chlorophytes (0.5), and cryptophytes (1). This scoring system for the phytoplankton taxa assumes there would be linear growth and reproductive responses to these combinations of taxa and scores, which was fortuitously approximately true for growth but less so for egg production. We modified the scoring system for phytoplankton taxa in the egg production response to achieve the optimal linear relationship as follows: cyanobacteria (0), chlorophytes (0.2), and cryptophytes (1). This modified scoring system improved the r^2 value of the egg response fit for the overall model from $r^2 = 0.78$ to 0.83. To obtain measures of zooplankton responses to direct and indirect P limitation in these

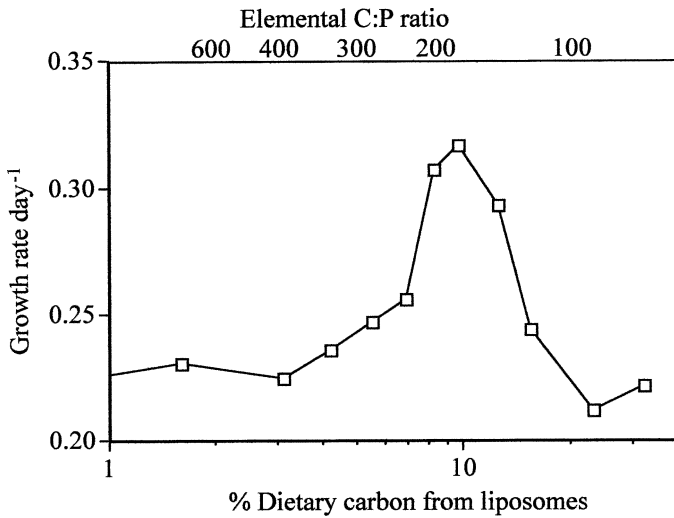


Fig. 2. Results of the liposome dose–response test examining *Daphnia* growth rate in response to increasing proportions of PC liposomes in their diet. Quite similar results were obtained for the reproduction response.

experiments, we scored our three phosphorus treatments accordingly for direct and indirect responses, respectively: high C:P phytoplankton (0, 0), high C:P phytoplankton amended with liposomes (1, 0), and low C:P phytoplankton (1, 1). According to this scoring system, the high C:P treatment was expected to have both direct and indirect P limitation, the high C:P amended with P treatment would not have direct P limitation but would have indirect P limitation, and the low C:P treatment would have neither direct nor indirect P limitation.

Results

Liposome dose–response experiment—Results of the liposome dose–response test showed that both *Daphnia* growth rates (Fig. 2) and egg production significantly increased as the P content of the chlorophyte food treatment was increased using liposome amendments from a C:P ratio ≈ 600 (no liposomes added) to a C:P ratio of ≈ 90 (approximately 12% of total dietary carbon from liposomes). When the percentage of dietary carbon from liposomes was increased above 16%, however, growth rates and egg production declined strongly compared to all other levels.

P content of food treatments—As previously stated, the target C:P ratios for the low C:P and P-amended treatments and moderately high C:P and high C:P treatments were 150, 400, and 600, respectively. In fact, these treatments ended with the following average C:P ratios: 162 ± 24 , 409 ± 37 , and 586 ± 24 , respectively. To place these ratios into a broader context, they were compared to C:P ratios observed in natural lakes as reported in a few large-scale surveys (Brett et al. 2000; Elser et al. 2001; Sterner et al. unpubl. data). For the approximately 615 unique cases reported in these lake surveys, C:P ratios of 162, 409, and 586, respectively, correspond to the 27th, 85th, and 95th percentiles.

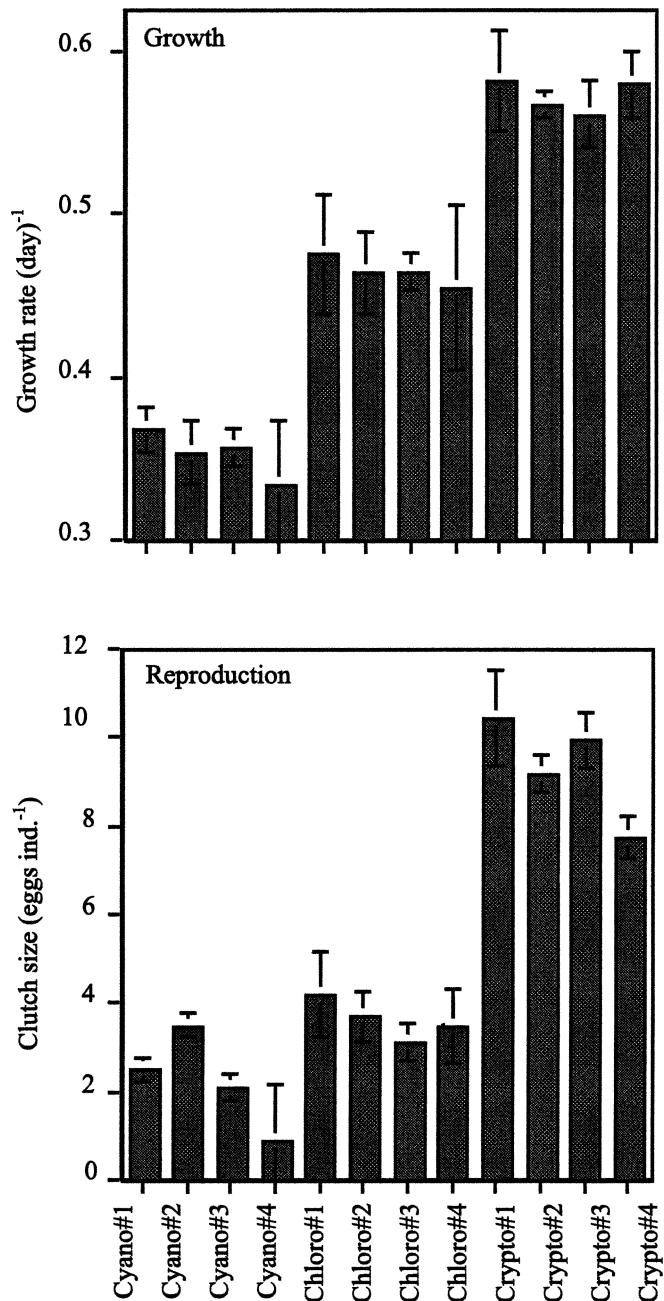


Fig. 3. *Daphnia* growth rate and clutch sizes of the low C:P/no FA treatment in each of the 12 experiments. This comparison was made in order to investigate the potential for maternal preconditioning of the *Daphnia* among the 12 experiments.

Maternal preconditioning test—Because we ran 12 separate experiments to complete the overall experimental matrix, it is possible that some of the differences between experiments were due to unintended differences in neonate preconditioning, that is, “maternal effects” (see Brett 1993b) or other experimental artifacts. To test for maternal effects between experiments, we compared *Daphnia* somatic growth rates and egg production for the low C:P phytoplankton without fatty acids treatment across all 12 experiments (Fig. 3). This food treatment served as an

Table 1. The results of a single-factor analysis of variance performed on the low C:P/no FA treatment from each of the 12 experiments. This shows that maternal preconditioning only explained 1% of the total sum of squares for the growth rate responses and 7% of the total variation for the egg production responses.

Source	df	SS	F-test	p-value
Growth rate				
Between groups	2	0.096	402.00	0.0001
Within groups	9	0.001		
Total	11	0.097		
Clutch size				
Between groups	2	113	62.00	0.0001
Within groups	9	8.25		
Total	11	121		

internal standard in the overall experimental design matrix because it was applied consistently in all experiments. The results of a single-factor analysis of variance performed on these observations (Table 1) indicated that maternal preconditioning explained only 1% of the total sum of squares for the growth rate responses and 7% of the total variation for the egg production responses.

Regression model performance—To test the dietary impact of phytoplankton taxa, essential fatty acid, and phosphorus content on *Daphnia* growth and reproduction, we used an overall multiple regression analysis (Fisher and Yates 1953) for all 12 experiments (Tables 2 and 3). Both multiple regression models explained a large proportion of the total variation in the data set; for the growth rate model, $r^2 = 0.95$, and for the egg production model, $r^2 = 0.83$. The root-mean-square error for the growth rate model was $\pm 0.03 \text{ d}^{-1}$ and for the egg production model $\pm 1.4 \text{ eggs individual}^{-1}$.

Coefficients for the food quality components tested—The effect of phytoplankton taxa on *Daphnia* nutritional responses was quite strong in both regression analyses. The corrected regression coefficients indicate that *Daphnia* fed cryptophytes grew 0.18 d^{-1} faster and had an additional 2.6 eggs individual^{-1} than *Daphnia* fed cyanophytes (uncorrected coefficients are reported in Tables 2 and 3). In fact, the results of our maternal effects analyses (Fig. 3) show that in the absence of P limitation, *Daphnia* consuming cryptophytes grew 0.23 d^{-1} faster and produced seven eggs more per individual than did *Daphnia* consuming cyanophytes. Figures 4 and 5 depict the experimental results in terms of phytoplankton taxa \times P axis and phytoplankton taxa \times FA axis, respectively.

Fatty acid supplementation had a significant effect on both *Daphnia* growth rate and egg production. Supplementation with FA improved the corrected growth rates by 0.09 d^{-1} and egg production by 2.2 eggs individual^{-1} (uncorrected coefficients are reported in Tables 2 and 3). The type of fatty acid supplement (EPA vs. FA-mix) was not significant at $\alpha = 0.05$ for the growth rate response, but it did have a small significant impact on egg production.

Table 2. Results of the overall multiple regression analysis performed on the somatic growth rate data. The “taxa” and “EFA addition” coefficients reported in this table are the original uncorrected values.

Analysis of variance					
Source	df	SS	r^2	F-test	p-value
Model	9	2.17	0.95	275.96	0.0001
Residual	134	0.12			
Beta coefficients					
Variable	Coefficient	SE	t-test	p-value	
Intercept	0.16				
Taxa	0.23	0.009	24.74	0.0001	
EFA addition	0.05	0.006	7.69	0.0001	
EFA type	0.00	0.005	0.83	0.4081	
P-D	0.04	0.007	5.91	0.0001	
P-ID	0.16	0.007	21.90	0.0001	
P level	0.02	0.005	4.33	0.0001	
EFA add \times taxa	-0.03	0.010	3.10	0.0024	
P-D \times taxa	0.05	0.011	4.56	0.0001	
P-ID \times taxa	-0.09	0.013	6.68	0.0001	

Conversely, the interaction between the fatty acid supplements and phytoplankton taxa was significant and strong for the growth rate response but not significant for the egg production response (Fig. 5).

Direct phosphorus limitation had a significant effect on both growth rates and egg production. Direct P limitation improved *Daphnia* growth rates 0.04 d^{-1} and egg production by 1.2 eggs individual^{-1} . The interaction between direct phosphorus and phytoplankton taxa was significant for the growth rate model but not on the egg production model. Indirect phosphorus limitation (P-ID) was strong and highly significant for both growth rate and egg production. Indirect P limitation reduced *Daphnia* somatic growth rates by 0.16 d^{-1} and egg production by 2.6 eggs individual^{-1} in these experiments. The interaction between indirect P limitation and phytoplankton taxa was also significant and strong for both the growth rate and the egg production responses (Fig. 4). The P deficiency level (400 or 600) was a significant contributor to the growth rate response. This term suggests that both indirect and direct P limitation was more severe at higher C:P ratios. Figure 6 illustrates a comparison of the P axis and FA axis for these experiments.

Since several authors have previously suggested the FA and mineral P limitation hypotheses may not be independent because P stress may affect the FA content of phytoplankton (see discussion), we used a two-factor analysis of variance to test for potential interdependence between fatty acid composition and C:P level in the phytoplankton food treatments. This was done using the fatty acid composition data for the phytoplankton treatments that did not receive P or FA amendments (Tables 6 and 7). This analysis showed that phytoplankton taxa explained the vast majority of fatty acid composition differences in the food treatments and that the P treatment levels did not have a significant effect on fatty acid

Table 3. Results of the overall multiple regression analysis performed on the egg count data. The “taxa” and “EFA addition” coefficients reported in this table are the original uncorrected values.

Analysis of variance					
Source	df	SS	r ²	F-test	p-value
Model	9	1182	0.83	70.11	0.0001
Residual	134	251			
Beta coefficients					
Variable		Coefficient	SE	t-test	p-value
Intercept		-1.3			
Taxa		3.7	0.502	7.28	0.0001
EFA addition		1.1	0.278	3.92	0.0001
EFA type		-0.5	0.228	2.22	0.0280
P-D		1.2	0.339	3.44	0.0008
P-ID		2.6	0.342	7.44	0.0001
P level		1.3	0.228	5.84	0.0001
EFA add×taxa		-0.7	0.476	1.51	0.1348
P-D×taxa		0.1	0.578	0.17	0.8623
P-ID×taxa		2.6	0.593	4.30	0.0001

composition. Interestingly, the interaction between phytoplankton taxa and P level was significant for the $\omega 3:\omega 6$ fatty acid ratio. When cyanobacteria were not P stressed, their $\omega 3:\omega 6$ ratio averaged 2.3 ± 1.1 , and when they were P stressed, their $\omega 3:\omega 6$ ratio averaged 0.9 ± 0.5 . Conversely, when cryptophytes were P stressed, their $\omega 3:\omega 6$ actually increased from an average of 3.0 ± 0.1 (for P sufficient algae) to 3.5 ± 0.2 (for P stressed algae). However, the $\omega 3:\omega 6$ ratio of the green algae was unaffected by the P treatments and averaged 0.9 ± 0.1 .

Discussion

The objective of this study was to conduct a simultaneous test of the mineral P and FA limitation hypotheses for *Daphnia* food quality. We performed a series of 12 experiments using three groups of phytoplankton with a wide range of food quality. *Daphnia* somatic growth rates and reproduction were measured in response to mineral P and FA manipulations for three phytoplankton groups. We found that mineral P limitation effects on phytoplankton food quality consist of two components that affect *Daphnia* nutrition directly and indirectly and that mineral P and FA limitation have independent effects on *Daphnia* somatic growth and reproduction. Overall, phytoplankton food quality was most strongly influenced by the phytoplankton taxa used in the experiments followed by indirect P limitation and then FA limitation.

Before making inferences about the relative importance of the phytoplankton P and EFA limitation observed in this study, it is necessary to address the efficacy and bioavailability of the phosphatidylcholine (PC) liposome carriers used to manipulate dietary C:P ratios and deliver FAs. The composition and structure of PC liposomes are generally quite similar to a biologic cell membrane (Lasic 1993). Liposomes have been used extensively as chemical or pharmaceutical delivery agents (Bally et al. 1988; Gregor-

iadis 1993) and have been demonstrated to be useful for delivering $\omega 3$ PUFAs in a variety of applications (Jenski et al. 1995; McEvoy et al. 1996). The specific liposome carrier that we have been using in our studies (Sigma preliposome formulation 5) consists of polar phosphatidylcholine molecules integrated with distearoyl functional groups (C18:0). To our knowledge, the only study to use liposome supplementation for *Daphnia* diets and to report a control treatment (empty liposome added to algal food treatment) is Ravet et al. (2003). Liposome supplementations alone did not confer any nutritional benefits to *Daphnia* in these experiments aside from serving as a source of particulate P. At the present time, we do not know if EFA supplements provided to *Daphnia* via liposome carriers are as bioavailable as EFA found within natural phytoplankton cells. However, it has been shown that PC liposome carriers are useful for delivering FAs because they confer a high degree of oxidative stability (Nara et al. 1998) and incorporate FAs in a way that is compatible with digestive enzymes in the gut (Diez et al. 1994; Ozkizilcik and Chu 1994). Ozkizilcik and Chu (1994) demonstrated that *Artemia* nauplii fed ¹⁴C-labeled PC liposomes incorporated 85% of the radiolabeled material after 24 h. Similarly, Tonheim et al. (2000) reported that pure PC liposomes (>99% PC by weight) were two to three times more effective (assimilation efficiency) at delivering the free amino acid methionine to *Artemia* than an emulsion amendment with the same methionine concentration. In a study dealing directly with the ability of PC liposomes to serve as a carrier for EPA, Tago and Teshima (2002) reported that the bioavailability of ¹³C-labeled EPA in the plasma of Japanese flounder increased when delivered via PC liposomes relative to EPA delivered in an ether ester form. Phosphorus in the form of phosphate, provided via phosphatidylcholine liposomes, is likely metabolized by *Daphnia* via a three-step process in which (1) phospholipase A and B remove fatty acids from the molecule; (2) phospholipase C and D break phospho-

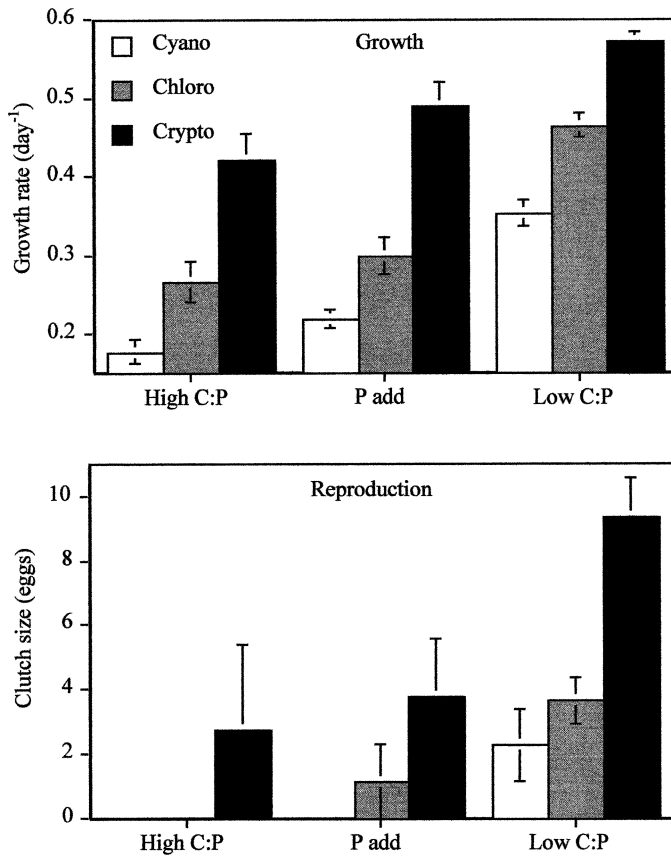


Fig. 4. *Daphnia* growth rate and clutch size responses for all three phytoplankton taxa at high C:P, low C:P, and high C:P with P supplements (phytoplankton taxa \times P axis). This figure does not include the data for cases where EFA was supplemented in the *Daphnia* diets.

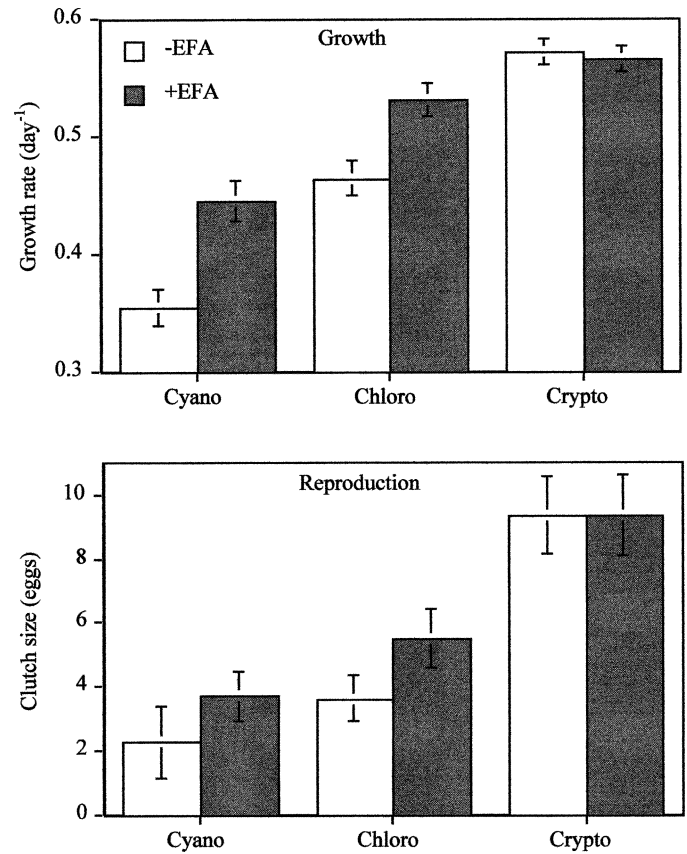


Fig. 5. *Daphnia* growth rate and clutch size responses for all three phytoplankton taxa grown at low C:P and with or without essential fatty acid (EFA) supplements (phytoplankton taxa \times FA add). This figure does not include data for high C:P or P supplemented treatments.

tidylcholine into diglyceride, choline phosphate, and phosphatidic acid components; and then (3) alkaline phosphatase releases phosphatase phosphate from choline phosphate and phosphatidic acid (Gurr and Harwood 1991). Because we also used a highly pure PC liposome carrier (>99% PC by weight) and because of evidence that the lipase enzyme activity (phospholipid metabolism) of *Artemia franciscana* and *Daphnia magna* are quite similar (Berges and Ballantyne 1991), our C:P supplementation method provides P in a form that should be highly bioavailable for *Daphnia*.

The results observed in these experiments indicate that phytoplankton taxa effects had the greatest effect on *Daphnia* growth rate and egg production responses. We purposely selected phytoplankton taxa that span a wide range of known phytoplankton food quality for the purpose of putting mineral P and FA limitation into a broad context. Our results suggest that *Daphnia* responses to P and EFA manipulation vary considerably across the phytoplankton taxa we tested (Figs. 4 and 5, respectively). It is likely that multiple factors combine to produce the strong taxa effects that we observed in these experiments. The phytoplankton used in these experiments had a wide range of FA content that was probably an important

component of their food quality differences. In fact, several studies have already presented direct empirical evidence that phytoplankton EFA content has a significant contribution in food quality (Von Elert 2002; Ravet et al. 2003). Sterols, like EFAs, are an essential nutrient for arthropods (Von Elert et al. 2003; Martin-Creuzburg et al. 2005) and may also play a significant role in the food quality of the phytoplankton taxa used in these experiments.

FA limitation effects were significant for both *Daphnia* growth rate and egg production. These results suggest that EFAs are an independent component of *Daphnia* nutritional requirements and not a secondary artifact of mineral limitation as previous studies have suggested (Boersma et al. 2001). When taking into account both the type and the amount of phytoplankton FA supplementation, these results suggest that FAs play a strong role in the reproductive physiology and somatic growth rate of *Daphnia*.

Direct P effects were significant for somatic growth rate and egg production. These results support the hypothesis that mineral P limitation affects phytoplankton food quality for *Daphnia*. However, direct P limitation appears to be only one of several components involved in *Daphnia*

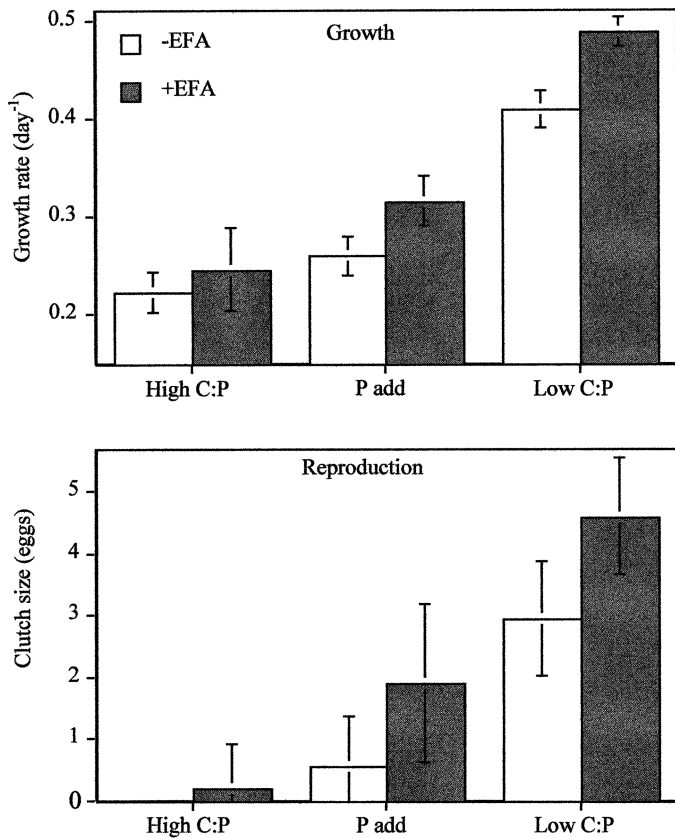


Fig. 6. *Daphnia* growth rate and clutch size responses for the combined cyanophyte and chlorophyte food treatments at high and low C:P and with or without EFA supplements (FA add \times P axis). The cyanophyte and chlorophyte food treatments were "folded" to a single value by taking the mean difference between these treatments. This transformation resulted in an overall standard deviation (SD) for the pooled data that was equal to the average SD for the individual treatments.

nutrition. It is important to note that these results also indicate that mineral P and FA limitation had separate and mutually independent impacts on phytoplankton food quality (Fig. 6).

One important conclusion of these experiments is that indirect effects of P limitation on phytoplankton food quality were quite strong for both *Daphnia* somatic growth rate and egg production. Indirect effects of P limitation may include factors such as changes in phytoplankton cell physical properties and/or biochemical composition (Brett 1993a). For example, several types of green algae have been shown to exhibit morphological changes, including increased cell wall thickness, when grown in P-limited medium (Lüring and Van Donk 1997; Van Donk et al. 1997). These studies have shown that P-limited cells passed mostly intact and viable through the daphnid gut and that *Daphnia pulex* feeding on P-limited algae had lower growth rates compared to the nonlimited algal diet. The authors propose that such morphological changes may be considered as an algal defense mechanism to reduce grazing pressure when growth rates are low because of nutrient limitation. In our study, the significant interaction term

Table 4. A review of Urabe et al. (1997) and DeMott (1998), who examined *Daphnia* growth responses to P-limited phytoplankton. We extracted the data from these publications and applied our statistical analysis approach by creating pseudodata with the same means and standard deviations as reported in the original figures and tables.

Urabe et al.					
Source	df	SS	r^2	F-test	p-value
Model	2	0.197	0.96	229.62	0.0001
Residual	19	0.008			
Beta coefficients					
Variable	Coefficient	SE	t-test	p-value	
Intercept	0.167				
D-P	0.052	0.011	4.70	0.0002	
ID-P	0.166	0.011	15.48	0.0001	
DeMott: <i>Daphnia pulicaria</i>					
Source	df	SS	r^2	F-test	p-value
Model	2	0.081	0.98	139.45	0.0001
Residual	6	0.002			
Beta coefficients					
Variable	Coefficient	SE	t-test	p-value	
Intercept	0.17				
D-P	0.14	0.014	10.09	0.0001	
ID-P	0.09	0.014	6.48	0.0006	
DeMott: <i>Daphnia magna</i>					
Source	df	SS	r^2	F-test	p-value
Model	2	0.063	0.97	109.34	0.0001
Residual	6	0.002			
Beta coefficients					
Variable	Coefficient	SE	t-test	p-value	
Intercept	0.17				
D-P	0.14	0.014	10.09	0.0001	
ID-P	0.06	0.014	4.32	0.0050	
DeMott: <i>Daphnia pulex</i>					
Source	df	SS	r^2	F-test	p-value
Model	2	0.095	0.92	33.78	0.0001
Residual	6	0.008			
Beta coefficients					
Variable	Coefficient	SE	t-test	p-value	
Intercept	0.140				
D-P	0.150	0.031	4.90	0.0027	
ID-P	0.100	0.031	3.27	0.0171	
DeMott: <i>Daphnia galeata</i>					
Source	df	SS	r^2	F-test	p-value
Model	2	0.080	0.98	138.06	0.0001
Residual	6	0.002			
Beta coefficients					
Variable	Coefficient	SE	t-test	p-value	
Intercept	0.11				
D-P	0.17	0.014	12.25	0.0001	
ID-P	0.05	0.014	3.60	0.0113	

Table 5. A review of Boersma (2000), who measured *Daphnia* growth response to P-limited algae as well as saturated fatty acid (SAFA) and PUFA supplements. We extracted the data from this publication and applied our statistical analysis approach by creating pseudodata with the same means and standard deviations as reported in the original figures and tables.

Analysis of variance: Main effects only					
Source	df	SS	r^2	F-test	p-value
Model	4	1.31	0.80	76.88	0.0001
Residual	76	0.32			
Beta coefficients					
Variable	Coefficient	SE	t-test	p-value	
Intercept	0.178				
SAFA addition	0.021	0.018	1.20	0.2356	
PUFA addition	0.089	0.018	5.00	0.0001	
D-P	0.049	0.018	2.74	0.0078	
ID-P	0.222	0.018	12.51	0.0001	
Analysis of variance: With interactions					
Source	df	SS	r^2	F-test	p-value
Model	8	1.41	0.87	58.03	0.0001
Residual	72	0.22			
Beta coefficients					
Variable	Coefficient	SE	t-test	p-value	
Intercept	0.199				
SAFA addition	0.031	0.026	1.19	0.2371	
PUFA addition	0.006	0.026	0.22	0.8281	
D-P	0.028	0.026	1.09	0.2795	
ID-P	0.199	0.026	7.65	0.0001	
D-P × SAFA	0.003	0.037	0.09	0.9280	
ID-P × SAFA	-0.036	0.037	0.98	0.3309	
D-P × PUFA	0.054	0.037	1.47	0.1464	
ID-P × PUFA	0.141	0.037	3.84	0.0003	

between indirect P limitation and phytoplankton taxa also suggests that the indirect impacts of P limitation on phytoplankton food quality varies substantially between phytoplankton groups. Figure 5 illustrates that percent direct P limitation was relatively low (i.e., 24–17%, respectively) for the cyanophytes and chlorophytes but clearly higher for cryptophytes (i.e., 45%). This could be due to the fact that cryptomonads lack rigid cell walls and are therefore less prone to becoming digestion resistant when nutrient stressed. A more recent study comparing seston food quality of six lakes also supports the digestive resistance hypothesis (DeMott and Tessier 2002). These studies indicate that phytoplankton defenses against grazing may present an important caveat to the stoichiometric P limitation hypothesis.

The homogeneity of the food treatments tested in these experiments must also be considered. Compared to natural lake seston, the mixtures of phytoplankton taxa used in these experiments were more homogeneous in their biochemical composition. One recent study has suggested that some types of herbivorous zooplankton may benefit from stoichiometric (C:P) heterogeneity in their diets (Acharya et al. 2004). In that context it is quite possible, then, that this study may overestimate the negative effects of feeding *Daphnia* P-starved phytoplankton. On the other hand, DeMott (2003) has suggested that short-term growth assays, such as this one, may underestimate the negative effects of P-deficient resources. Kilham et al. (1997)

demonstrated that short-term negative effects of P limitation on *Daphnia* fecundity can be compensated by increasing the amount of P-limited food, while long-term negative effects on *Daphnia* population growth cannot be compensated for by simply increasing food supply. Jensen and Verschoor (2004) observed that the rotifer *Brachionus calyciflorus* fed on *Scenedesmus* responded to both P limitation and EFA limitation in their food supply, suggesting that these types of food quality constraints may extend to other types of zooplankton.

One of the main objectives of this study was to determine the extent to which the previously noted poor food quality of nutrient stressed phytoplankton was due to direct mineral P deficiency or alternatively to physiological changes in cell morphology and/or biochemical composition caused by the nutrient stress (Brett 1993a). We were able to address this issue directly because our means of manipulating dietary C:P ratio delivered P in a highly bioavailable form, with minimal likelihood of affecting the physiological state of the P-stressed phytoplankton. We were also able to statistically untangle the relative importance of direct and indirect P limitation because we applied a novel statistical approach to this problem.

As previously noted, in our experiments indirect P limitation explained the majority of the decline in phytoplankton food quality in our high C:P phytoplankton treatments. In fact, direct P limitation explained only

Table 6. Fatty acid composition ($\mu\text{g mg}^{-1}$ phytoplankton dry weight) and atomic C:P ratios of the phytoplankton for the high and low C:P treatments.

	Cyanophytes			Greens			Cryptophytes		
	High C:P	Low C:P	High C:P	Low C:P	High C:P	Low C:P	High C:P	Low C:P	
	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	
C:P	546 \pm 135	161 \pm 24	521 \pm 81	175 \pm 30	466 \pm 109	155 \pm 24			
Sum FA	108 \pm 4	106 \pm 4	222 \pm 5	218 \pm 5	219 \pm 8	221 \pm 5			
SAFA	70.3 \pm 3.3	67.6 \pm 1.7	112 \pm 3	107 \pm 2	139 \pm 3	138 \pm 5			
MUFA	29.1 \pm 1.9	28.2 \pm 4.3	31.2 \pm 1.7	31.3 \pm 3.3	20.8 \pm 1.3	19 \pm 1			
18:3 ω 3	0.8 \pm 0.3	1.3 \pm 0.4	26.1 \pm 3.8	26.5 \pm 3.5	9.2 \pm 0.3	10.5 \pm 1.2			
18:4 ω 3	1.6 \pm 0.3	3.2 \pm 1.2	10.9 \pm 0.8	10.1 \pm 2.0	20.7 \pm 3.3	21.1 \pm 2.3			
20:5 ω 3	1.8 \pm 0.4	2.2 \pm 0.7	0 \pm 0	0.3 \pm 0.4	13.9 \pm 1.2	14.0 \pm 1.6			
22:6 ω 3	0 \pm 0	0 \pm 0	0 \pm 0	0.1 \pm 0.2	2.4 \pm 0.1	2.3 \pm 0.4			
18C ω 6 PUFA	0.3 \pm 0.3	0.3 \pm 0.4	43.2 \pm 2.0	43.3 \pm 1.5	13.3 \pm 1.2	16.0 \pm 1.7			
20:4 ω 6	4.4 \pm 1.6	3.0 \pm 1.0	0 \pm 0	0 \pm 0	0 \pm 0	0 \pm 0			
ω 3: ω 6	1.0 \pm 0.5	2.3 \pm 1.1	0.9 \pm 0.1	0.9 \pm 0.1	3.5 \pm 0.2	3.0 \pm 0.1			

Table 7. Analysis of variance results for a comparison of phytoplankton taxa and nutrient regime impacts on phytoplankton fatty acid composition.

Total fatty acids					
Source	df	SS	F-test	p-value	% of SS
Phyto. taxa	2	6,8502.5	1,220.47	0.0001	99.2%
P level	1	16.0	0.57	0.4599	0.0%
Interaction	2	45.8	0.82	0.4581	0.1%
Error	18	505.2			0.7%
Saturated fatty acids					
Phyto. taxa	2	1,9485.3	927.26	0.0001	98.7%
P level	1	44.3	4.22	0.0549	0.2%
Interaction	2	14.5	0.69	0.5154	0.1%
Error	18	189.1			1.0%
Monounsaturated fatty acids					
Phyto. taxa	2	538.5	41.69	0.0001	81.5%
P level	1	4.4	0.68	0.4189	0.7%
Interaction	2	1.8	0.14	0.8721	0.3%
Error	18	116.2			17.6%
18:3 ω 3					
Phyto. taxa	2	2,624.8	273.46	0.0001	96.7%
P level	1	3.4	0.70	0.4127	0.1%
Interaction	2	0.9	0.10	0.9100	0.0%
Error	18	86.4			3.2%
18:4 ω 3					
Phyto. taxa	2	1,375.9	182.27	0.0001	94.8%
P level	1	1.1	0.30	0.5915	0.1%
Interaction	2	5.9	0.78	0.4731	0.4%
Error	18	67.9			4.7%
20:5 ω 3					
Phyto. taxa	2	891.2	567.36	0.0001	98.4%
P level	1	0.5	0.65	0.4307	0.1%
Interaction	2	0.1	0.08	0.9233	0.0%
Error	18	14.1			1.6%
22:6 ω 3					
Phyto. taxa	2	28.4	558.15	0.0001	98.4%
P level	1	0.0	0.02	0.8995	0.0%
Interaction	2	0.0	0.31	0.7362	0.1%
Error	18	0.5			1.6%
18C ω 6					
Phyto. taxa	2	7,645.7	2,143.67	0.0001	99.4%
P level	1	5.4	3.04	0.0985	0.1%
Interaction	2	9.5	2.66	0.0969	0.1%
Error	18	32.1			0.4%
20:4 ω 6					
Phyto. taxa	2	72.0	63.12	0.0001	83.3%
P level	1	1.4	2.46	0.1344	1.6%
Interaction	2	2.8	2.46	0.1139	3.2%
Error	18	10.3			11.9%
ω 3: ω 6					
Phyto. taxa	2	23.3	44.32	0.0001	72.1%
P level	1	0.5	2.00	0.1745	1.6%
Interaction	2	3.7	7.12	0.0053	11.6%
Error	18	4.7			14.7%

21–31% of the reduced food quality in these treatments. To compare our results to those of prior studies that examined *Daphnia* growth responses to P-limited phytoplankton (Urabe et al. 1997; DeMott 1998; Boersma 2000), we applied our statistical approach to their reported results. When we did this for Urabe et al.'s experiments, we found that both direct and indirect P limitation significantly

reduced phytoplankton food quality but that direct P limitation explained only 24% of the reduced food quality for their P-stressed *Scenedesmus* (Table 4). Similarly, if we focus on just the main effects in Boersma's (2000) study (i.e., direct P, indirect P, plus saturated fatty acids, and plus PUFA), we calculate that direct P limitation explained 18% of the reduced *Scenedesmus* food quality due to P stress

(Table 5). In contrast, when we analyzed the results that DeMott (1998) presented for four different *Daphnia* species (and used his P_{short} treatment to represent P-amended conditions), we found that direct limitation was strongest and explained on average 67% of the reduced food quality noted for P-stressed *Scenedesmus*.

The results for Boersma's (2000) experiment are somewhat more complicated than presented above because he used a more complex experimental design, which made it possible to examine four main effects and even more interactions. If we analyzed his data while focusing on the four main effects mentioned above, we find that direct P limitation reduced *Scenedesmus* food quality by 0.05 d^{-1} , indirect P limitation reduced growth by 0.22 d^{-1} , PUFA additions improved growth by 0.09 d^{-1} , and saturated fatty acid additions did not have a statistically significant impact on growth. However, if we simultaneously consider the four main effects as well as the interactions between direct and indirect P limitation and the saturated fatty acid and PUFA additions (i.e., D-P \times SAFA, ID-P \times SAFA, D-P \times PUFA, and ID-P \times PUFA), the term for indirect P limitation was significant and strong (at 0.20 d^{-1}), while the terms for direct P limitation and the PUFA additions were no longer significant (Table 5). However, the interaction between indirect P limitation and the PUFA additions was significant and strong (at 0.14 d^{-1}). In other words, these results suggested that in Boersma's experiment, indirect P limitation had a strong effect on *Scenedesmus* food quality and that PUFA additions strongly increased growth rates but primarily when *Scenedesmus* was not P stressed.

One of the main topics in the food quality debate is the nature of cause-and-effect relationships vis-à-vis mineral P and essential fatty acid regulation of phytoplankton food quality. Some researchers have pointed out that since the EFA content of phytoplankton may be altered when they are P stressed, these variables may covary, making it difficult to separate the two hypotheses (Ahlgren et al. 1997). Müller-Navarra (1995a) hypothesized that phytoplankton EPA content may exert primary control over phytoplankton food quality, whereas Elser et al. (2001) hypothesized that mineral P limitation exerts the greatest direct constraint on daphnid growth. Boersma et al. (2001) hypothesized that when phytoplankton are P stressed, direct P limitation will take precedence over EFA limitation, but that when phytoplankton are not P stressed, phytoplankton EFA content will determine food quality. An alternative to these scenarios is Park et al.'s (2002) recent observation that both phytoplankton EPA and C:P were independently correlated with *Daphnia* growth but that the EPA correlation with growth was much stronger. Our analyses mostly agree with Park et al.'s (2002) findings that EFA and mineral P impacts on phytoplankton food quality are largely independent. However, because we did find that the $\omega 3:\omega 6$ ratio of P-stressed cyanobacteria declined 61% and low $\omega 3:\omega 6$ ratios are thought to adversely affect fish nutrition (Olsen 1999; Sargent et al. 1999), it is plausible that for cyanobacteria the mineral P and EFA limitation hypotheses are not completely independent.

These results provide an important direct comparison of the roles of mineral P and EFA in phytoplankton food quality. We found that dietary P limitation had a direct effect on *Daphnia* somatic growth rate and, to a lesser extent, on reproduction but that a large majority of the food quality effects associated with P limitation are due to indirect effects that are probably due to physiological changes in the physical properties and biochemical composition of P-starved phytoplankton. This finding presents an important caveat in the P limitation hypothesis of phytoplankton food quality and suggests that more research is needed to better understand how phytoplankton physiological responses to P-stressed conditions affect food quality. We also found that EFA supplements had a strong effect on *Daphnia* reproduction and somatic growth rates. *Daphnia* responses to the EFA supplements were distinct from the effects exerted by P limitation. In a broader context, however, direct effects of P limitation and EFA supplementation were relatively small compared to the effects attributed to phytoplankton taxa. The broad range of food quality for the phytoplankton taxa we used may be due to the varying physiological responses to P stress, differences in their innate digestibility or biochemical composition (e.g., sterol content), as well as large differences in their EFA composition. Nevertheless, our results emphasize that phytoplankton food quality impacts on *Daphnia* nutrition are far more complex than single constituent hypotheses (i.e., EFA or P) would otherwise suggest.

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