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The nutritional quality of P-limited algae for *Daphnia*

Abstract—This paper details results of experiments intended to clarify the relative effects of mineral versus biochemical limitations of algae on the food quality for herbivorous zooplankton. Phosphorus limited algae (*Scenedesmus*) were fed to *Daphnia magna* and the somatic growth rates of the animals were measured. The animals showed an increase in growth rate when phosphorus (P) was added to P-limited algae, especially when the animals were older. The addition of highly unsaturated fatty acids only resulted in an increase in growth rates when the algae had a C : P ratio of less then 300. The main conclusion of this work is that both mineral and biochemical limitations of their food play a role in the growth and population dynamics of zooplankters, but that mineral requirements need to be met first.

It is generally accepted that nutrient limited algae are a food source of low quality for herbivorous zooplankton. However, the reasons for this are less clear. Some authors argue that the mineral content (mostly phosphorus in freshwater systems) directly limits zooplankton growth and reproduction (Hessen 1992; Urabe and Watanabe 1992; Sterner et al. 1993; DeMott et al. 1998), and the recent studies by DeMott (1998) and Urabe et al. (1997) provide direct evidence of this. However, in some field studies the correlation of daphniid growth and the phosphorus content of the seston was low (Hessen 1989; Müller-Navarra 1995*b*), which has lead to alternative explanations for the distinct effect of nutrient limitation in food on the growth of zooplankters in laboratory experiments. Nutrient-limited algae contain a different fatty acid spectrum when compared to non-limited algae (Müller-Navarra 1995*a*; Weers and Gulati 1997*b*), making them potentially less nutritious (Müller-Navarra 1995*b*). Although nutrient-limited algae contain higher absolute amounts of fatty acids, they seem to be lacking in highly unsaturated fatty acids, such as eicosapentaenoic acid (EPA, 20:5ω3), or docosahexaenoic acid (DHA, 22:6ω3) (Reitan et al. 1994; Müller-Navarra 1995*a*), which are essential for animal growth (e.g., Reitan et al. 1997).

The discussion on mineral versus biochemical limitations has triggered a series of studies on the nature of food-quality constraints on zooplankton growth (DeMott and Müller-Navarra 1997; Lürling and van Donk 1997; Sundbom and Vrede 1997; Urabe et al. 1997; Weers and Gulati 1997*b*; Weers and Gulati 1997*a*; DeMott et al. 1998). Thus far, most of the published reports seem to conclude that, direct phosphorus-limitation plays a role, with other unknown differences also of potential importance. One possible difference is that algae also change their morphology under phosphorus limitation (Tillberg et al. 1984; Tillberg and Rowley 1989). The algal cell walls become thicker and harder to digest for zooplankters (van Donk et al. 1997; *but see* DeMott et al. 1998). The aim of this study was to investigate the direct and indirect effects of algal phosphorus limitation on zooplankters.

Two elegant methods exist to differentiate between direct phosphorus limitation and its related effects on nutritional quality. Urabe et al. (1997) showed that *Daphnia magna* are able to take up dissolved phosphorus, and used this to supply daphnids with dissolved phosphate during parts of the day. Rothhaupt (1995) and DeMott (1998) used the fact that phosphorus-limited algae take up dissolved phosphorus very rapidly (Lehman and Sandgren 1982). Preliminary experiments in our laboratory showed that the phosphorus content of the algae increases rapidly with a phosphorus pulse, but that changes in the biochemical composition lag behind. Hence, a difference in growth rate between animals fed phosphorus-limited algae and animals cultured on phosphorus-limited, phosphorus pulsed, algae would indicate a direct effect of phosphorus limitation on growth. Here, I used this method, whereby phosphorus deficient algae were added to *Daphnia* medium, and this medium was subsequently enriched with phosphate. To simultaneously infer the relative contribution of biochemical limitations to the food quality, I crossed the different phosphorus treatments with additions of emulsions containing different fatty acids.

Algal characteristics—Scenedesmus obliquus was available from semi-continuous cultures. Phosphorus sufficient (P+) algae were cultured in Z/4 Medium (Zehnder and Gorham 1960), which contains 45 μM P. The same medium with a reduced phosphorus concentration (2.7 μM) was used to culture the phosphorus deficient (P−) algae. For the pulsed phosphorus (P*) treatment, phosphorus deficient algae were added to the artificial phosphorus-free *Daphnia* medium (Klüttgen et al. 1994), and this medium was subsequently enriched with 45 μM of phosphate. As the media were changed daily, this gave a mean exposure time to the phosphorus enrichment of 12 h. Algal-media were kept in the dark to avoid photosynthesis and algal growth in the food media. Algal carbon and phosphorus contents were measured daily, and the molar C : P ratio of the P+ algae averaged 275 (standard error = 21), whereas the C : P ratio of the P− algae was 971 (SE = 131). The P* algae took up the dissolved phosphorus rapidly, and showed a C : P value after 24 h of 245 (SE = 1). Fatty acid-content of *S. obliquus* under the different regimes was analyzed using methods described by Wiltshire et al. (in press). The total amount of fatty acids present per mg C was 208 μg and 178 μg for the P− and P* algae, respectively, whereas the P+ algae contained only 43 μg mg^{-1} C. The fatty acid spectra of the P− and P* algae were also very similar (Table 1). Hence, with respect to the fatty acids, the P* algae were still phosphorus limited.

Fatty acid emulsions—The use of emulsions to supply consumers with additional fatty acids was originally developed in the aquaculture, and has proven to be highly successful in increasing the yields of commercially important

Table 1. Fatty acid composition of *S. obliquus* grown under P-sufficient (P+) and P-deficient (P−) conditions, and the fatty acid content of algae grown under P-limited conditions, and pulsed with phosphorus, before feeding them to the daphnids (P*). Values are μg mg^{-1} C (with standard errors in brackets), and as a percentage of the total fatty acid content.

P−	%	P+	%	%	P*	%
C10:0	0.01(0.01)	0.01	0.05(0.03)	0.12	0.07(0.07)	0.04
C12:0	0.44(0.20)	0.21	0.84(0.27)	1.96	0.75(0.63)	0.42
C14:0	1.30(0.29)	0.62	0.97(0.19)	2.27	1.48(0.40)	0.83
C14:ln9	0.15(0.15)	0.07	0	0	0	0
C16:0	63.39(10.69)	30.41	26.70(4.48)	62.68	64.17(14.92)	36.03
C16:ln9	0.17(0.03)	0.08	0.21(0.05)	0.49	0.37(0.13)	0.21
C16:2n4	0	0	0	0	0	0
C16:3n4	0	0	0.16(0.17)	0.38	0	0
C18:0	7.41(1.54)	3.55	3.78(0.97)	8.86	8.12(2.29)	4.56
C18:ln9	102.73(24.36)	49.28	3.36(1.50)	7.88	74.36(28.97)	41.75
C18:ln7	1.38(0.56)	0.66	1.16(0.46)	.71	2.13(1.01)	1.20
C18:2n6	14.57(5.28)	6.99	0.89(0.52)	2.09	6.86(6.24)	3.85
C18:3n6	1.53(1.37)	0.74	0.24(0.06)	0.55	0.10(0.10)	0.05
C18:3n4	0	0	0.03(0.03)	0.07	0	0
C18:3n3	9.45(3.16)	4.54	0.80(0.22)	1.88	6.86(0.55)	3.85
C18:4n3	2.73(0.84)	1.31	0.07(0.03)	0.16	2.04(0.26)	1.15
C20:0	0.49(0.16)	0.23	0.26(0.09)	0.60	1.62(0.38)	0.91
C20:ln9	0.74(0.43)	0.36	0.53(0.34)	1.24	5.87(2.39)	3.30
C20:ln7	0.92(0.35)	0.44	0.43(0.22)	1.01	1.71(0.63)	0.96
C20:3n6	0.25(0.17)	0.12	0.23(0.12)	0.53	0	0
C20:4n6	0	0	0.05(0.04)	0.11	0	0
C2:3n3	0.12(0.07)	0.06	0.20(0.10)	0.47	0	0
C20:4n3	0.05(0.05)	0.02	0	0	0	0
C20:5n3	0	0	0	0	0	0
C22:0	0.16(0.07)	0.08	0.11(0.08)	0.27	0.09(0.09)	0.05
C22:ln9	0.05(0.05)	0.02	0	0	0	0
C22:2n6	0.09(0.04)	0.04	0.24(0.09)	0.56	0.49(0.22)	0.28
C22:4n6	0.03(0.03)	0.02	0.14(0.08)	0.32	0.00(0.00)	0.0
C22:5n3	0.19(0.11)	0.09	0.77(0.36)	1.82	1.00(0.13)	0.56
C22:6n3	0.03(0.04)	0.02	0.34(0.21)	0.79	0	0
C24:0	0.06(0.07)	0.03	0.08(0.08)	0.19	0	
Sum Sat	73.26(12.36)		32.78(5.73)		76.30(9.30)	
Sum Unsat	135.18(32.65)		9.82(3.96)		101.79(15.80)	
MUFA	106.13(24.74)		5.68(2.53)		84.44(12.40)	
PUFA	29.05(8.86)		4.14(1.48)		17.35(3.40)	
Sumn3	12.57(3.90)		2.18(0.76)		9.90(0.34)	
Sumn6	16.48(5.03)		1.77(0.68)		7.45(3.06)	

species or their food (Coutteau and Sorgeloos 1997). Only fairly recently, this technique has been applied by researchers in aquatic sciences (DeMott and Müller-Navarra 1997; Weers and Gulati 1997*a*; Goulden et al. 1999), first using fish-oils, but now mostly using commercially available products. In this study, I used two of the emulsions originally developed and standardized by the International Council for the Exploration of the Sea (ICES)-working group on mass rearing juvenile fish: ICES 30/0.6/C, and ICES 0/-/C, supplied by INVE technologies Belgium (Coutteau et al. 1996). Both emulsions contain triglycerides with different fatty acids, and emulsifiers and antioxidants. They differ in their fatty acid composition. ICES 30/0.6/C is rich in long-chained polyunsaturated fatty acids, especially EPA and DHA, whereas ICES 0/-/C consists mainly of saturated and monounsaturated fatty acids (Table 2). Fresh emulsions were prepared daily by mixing 0.5 mg wet weight of emulsion (0.4 mg C) per liter of *Daphnia* feeding suspension and subsequent mixing. Analysis of the particle size showed that the emulsion particles were in the ranger of 1–2 μm, but that they were readily taken up by daphnids (*see also* Weers and Gulati 1997*a*).

Daphnia *growth experiment*—Three algal treatments: phosphorus sufficient algae (P+), phosphorus deficient algae (P−), and phosphorus deficient algae with phosphorus pulse (P*) were crossed in a full factorial design with three emulsion treatments: HUFA-rich, HUFA-poor, and no emulsions added, yielding nine different treatments. The inclusion of a no-emulsion treatment allowed for the investigation of the effect of supplying extra energy to the daphnids, by contrasting the no-emulsion with the HUFA-poor treatment. The comparison of the HUFA-poor and the HUFA-rich treatment enabled the assessment of the effect of addition of polyunsaturated fatty acids to the food suspensions. The carbon content of the algal cultures was checked daily before preparing the feeding suspensions: 5–10 ml were filtered through precombusted GF/C filters, and analyzed for carbon

Table 2. Fatty acid composition of the emulsions used in this study. Values are μg mg^{-1} dry weight (with standard errors in brackets), and as a percentage of the total fatty acid content.

	HUFA- poor (ICES/0/−/C)	%	HUFA- rich (ICES/30/0.6/C)	%
C8:0	2.34(0.69)	0.32	0	0
C10:0	17.57(2.65)	2.38	0	0
C12:0	554.34(16.93)	75.13	2.15(0.31)	0.79
C14:0	61.25(2.17)	8.30	17.52(1.06)	6.45
C16:0	36.55(1.47)	4.95	40.58(2.71)	14.94
C16:1	0	0	18.33(1.27)	6.75
C16:2	0	0	2.78(0.13)	1.02
C16:3	0	0	1.36(1.24)	0.50
C18:0	11.51(0.43)	1.56	11.86(1.12)	4.36
C18:ln9	30.09(1.17)	4.08	51.57(6.56)	18.98
C18:ln7	0	0	9.50(0.47)	3.50
C18:2n6	22.11(0.92)	3.00	19.42(3.98)	7.15
C18:3n3	2.07(0.09)	0.28	4.66(0.71)	1.72
C18:4n3	0	0	8.90(1.34)	3.27
C20:3n3	0	0	2.04(0.98)	0.75
C20:5n3	0	0	40.06(5.23)	14.75
C22:5n3	0	0	6.96(1.16)	2.56
C22:6n3	0	0	33.97(4.63)	12.51

using a LECO-carbon analyzer. The algal carbon contents of the media supplied to the daphnids was set to 1 mg C L^{-1}. All treatments were carried out with 10 replicates.

The *D. magna* clone used was originally collected from a pond in Frankfurt, Germany, and has been kept in the laboratory for many years. Juvenile animals were collected from a stock culture, placed individually in 200-ml containers, fed a suspension of P-sufficient *S. obliquus* at 20°C, and a day–night rhythm of 16 : 8 h. Third brood juveniles of these animals were collected within 12 h of birth and placed in 120-ml flow-through chambers, with a flow rate of 1 L d^{-1}. Initial weight of the animals was established by taking four juveniles from each clutch, drying them for 24 h at 60°C, and subsequent weighing to the nearest 0.1 μg using an electronic microbalance. Dry weights of the animals harvested after 3 and 6 days were established on individual animals. Somatic growth rates were computed for the complete period (day 0 to day 6), as these values have the highest correlation with the intrinsic rate of population increase *r* (Lampert and Trubetskova 1996). The data were analyzed in a two-way analysis of variance, with algal phosphorus content and emulsion treatment as the independent (fixed) variables and the somatic growth rate as the dependent variable. Post-hoc comparisons were carried out using Duncan's multiple range tests.

The ANOVA table shows that the somatic growth rate of the animals under study was significantly influenced by both the mineral limitation of the algae and the emulsion treatment (Table 3; Fig. 1).

Effect of P-addition—Although post-hoc comparisons show that all of the P-treatments were significantly different from each other, the actual differences between the growth rates of animals fed with P-limited and P* algae were small. In contrast, the growth rates of animals fed with P-sufficient

Table 3. Summary table of the analysis of variance with emulsion type, phosphorus as independent factors, and the somatic growth rate as the dependent factor.

Effect	MS	df	*F*	*P*
Emulsion type	0.0837	2	27.9	<0.001
P-treatment	0.5931	2	197.9	<0.001
Emulsion × P	0.0272	4	9.1	<0.001
Error	0.0029	71		

Scenedesmus were higher in all cases. This could lead to the conclusion that the direct effect of phosphorus limitation is not large, which would be in contrast to the findings of Urabe et al. (1997) and DeMott (1998). These authors concluded that a substantial part of the decreased growth rates of animals on P-limited algae could be attributed to direct phosphorus limitation. In contrast to the experiments of Urabe et al. (1997), the phosphorus was not supplied to the daphnids in dissolved form, but rather was taken up by the algae first. Hence, although these algae were no longer P-limited, as they showed the same C : P ratios as the phosphorus sufficient algae, the changes in morphology were most likely small. Tillberg et. al. (1984) observed that some organelles of P-limited *Scenedesmus* cells changed rapidly when re-supplied with phosphorus, but cell wall changes were slower, especially under conditions when no photosynthesis takes place. As van Donk et. al. (1997) showed that the altered cell wall morphology as a result of nutrient depletion was the most important factor explaining reduced digestibility of nutrient limited algae, this could indicate that the morphological features of nutrient-limited algae cause difficulties in digestion (*but see* DeMott et al. 1998). Using a similar approach to the one presented here, DeMott (1998) observed that somatic growth rates of daphnids on P* and

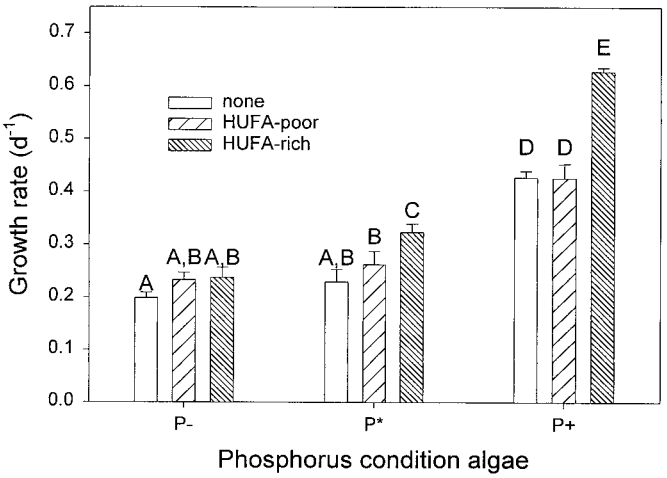

Fig. 1. Growth rates of *Daphnia magna* cultured with three types of *Scenedesmus obliquus* (P− phosphorus limited; P+ phosphorus sufficient; and P* phosphorus-limited and given a pulse of phosphorus, just before feeding), and three different emulsion treatments (none, HUFA-poor, HUFA-rich). Error bars indicate standard errors, data points marked with identical characters are not significantly different (Duncan's multiple range test).

P+ algae were not significantly different. The most important difference in experimental set-up is that DeMott used animals cultured on P-sufficient algae for one day rather than newborn daphnids transferred directly into experimental treatments. This difference in developmental stage and pretreatment of experimental animals could be significant because I observed that the growth rate differences between animals fed with P* and P+ algae was almost exclusively attributable to the first three days of the experiment (0.19 d^{-1} for P* animals versus 0.54 d^{-1} for animals fed P+ algae; $F_{1,17} = 42.7$; $P < 0.001$). In the age-interval from days 3 to 6, I observed no significant differences between the growth rates of animals fed with P* and P+ algae (0.23 d^{-1} for P* animals versus 0.31 d^{-1} for animals fed P+ algae; $F_{1,15} = 3.7$; $P = 0.07$).

In short, the phosphorus content of the algae directly affects the somatic growth rates of the daphnids, especially when the animals are older. The present results and those of DeMott (1998) suggest that especially young daphnids have problems with aspects of P-limited algae other than phosphorus content.

Effect of the addition of fatty acids—The effect of adding fatty acid emulsions to the P− algae was a small (but significant in a contrast analysis with no-addition versus HUFA-rich and HUFA-poor added; $F_{1,25} = 4.42$; $P = 0.04$) increase in growth rates. There was no significant difference between the two emulsions. For the P* and P+ algae, I observed a significant increase in growth rates only when the HUFA-rich emulsions were added (Fig. 1). Even though the algal concentrations supplied to the daphnids were clearly above the incipient limiting level, most likely the reduced digestibility of P-limited algae lowered the effective food concentration. Since there was no significant difference between the HUFA-rich and the HUFA-poor emulsions, the increase in growth rate caused by the fatty acid emulsions to the P− algae was most likely a result of additional energy uptake. An alternative explanation could come from the observation that fatty acid emulsions also contained 1ng phosphorus per gram. Thus, 0.5 μg P L^{-1} was added with the emulsions. This may seem little, but total phosphorus content of the phosphorus-limited algae was only around 2.5 μg L^{-1}. The added phosphorus could have increased the growth rates of the daphnids, which could explain the lack of a significant difference between the control and HUFA-poor treatment in the P* algae.

HUFA-rich emulsions only had an effect when enough phosphorus was present in the food. The HUFA-rich emulsions increased daphnid growth rate by 30% in the P* treatment and 45% in the P+ treatment. Hence, only when the C : P ratio of the algae was lower than the proposed threshold of 300 (atomic ratio) (Sterner 1993) an HUFA effect was observed. This fits well with the results of Sundbom and Vrede (1997), who observed a stronger effect of HUFA addition to phosphorus sufficient algae than to phosphorus-limited algae. These findings could also explain why Müller-Navarra (1995*b*) observed a stronger correlation of daphnid somatic growth rates with HUFAs than with phosphorus content of the seston, as the C : P ratios in her studies were mostly below the proposed threshold (Sterner 1993).

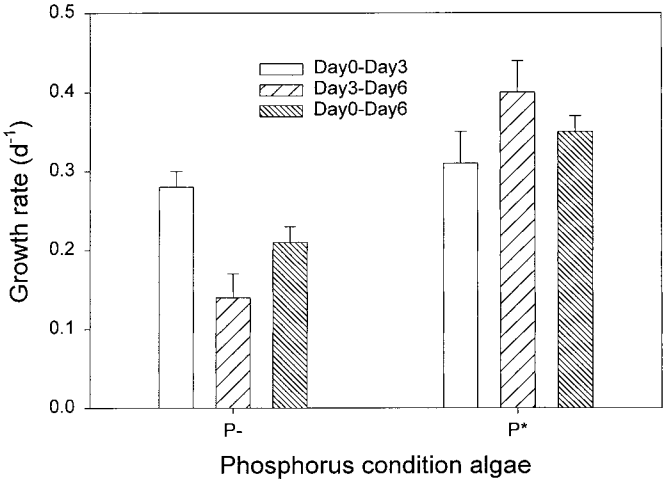

Fig. 2. Growth rates of *D. magna* cultured with two types of *C. erosa* (P− phosphorus limited; and P* phosphorus-limited and given a pulse of phosphorus, just before feeding). Growth rates of two different periods (day 0 to day 3, day 3 to day 6 and total growth rates are shown). Error bars indicate standard errors.

Mineral versus biochemical limitations—In this study I incorporated both mineral and biochemical limitations in one experimental design to investigate somatic growth of daphnids. Neither the addition of fairly high concentrations of HUFA-rich emulsions, the addition of HUFA-poor emulsions (energy), nor the addition of phosphorus to the P-limited algae could adequately explain the difference in growth rates of animals grown under P-limited and P-sufficient conditions. Therefore, other factors still must play a role. On the one hand, it could be that the morphology of the algae is such that actual assimilation of ingested algae by the daphnids is low (van Donk et al. 1997), or the other the not-measured aspects of the P-limited algae cauld cause low food quality.

To test the effect of the changes in morphology, I carried out an almost identical experiment as that described above with the cryptophyte *Cryptomonas erosa*, feeding daphnids with P− and P* algae (0.2 mg C L^{-1}), and established growth rates for 0–3 and 3–6 days. Cr*yptomonas* is not enclosed by a cell wall, but by a periplast (Van Den Hoek et al. 1995), and morphological changes under phosphorus limitations seem to be small (Lürling and van Donk 1997). Hence, differences in growth rates of animals fed with P-limited (C : P ratio of 560, SE 30) and P-pulsed (C : P ratio 51, SE 1) *Cryptomonas* would be a direct effect of the phosphorus content of the algae. Indeed, I observed that the effect of the P-pulse on daphnid growth rates was highly significant for the whole growth period (Fig. 2; $F_{1,16} = 31.6$; $P < 0.001$), but that, as for *Scenedesmus*, the growth differences mainly occurred in the second period. This could imply that digestion resistance of nutrient limited cells did not limit uptake of these cells, as suggested by DeMott et al. (1998), but that especially young daphnids have problems with other aspects of nutrient-limited algae than those studied here. Alternatively, *Cryptomonas* cells could also change under nutrient limitation, limiting uptake by younger daphnids. In any case, my experiments show that direct phosphorus content

of algal cells can influence *Daphnia* growth rates considerably.

In conclusion, my results and those of others (Urabe et al. 1997; DeMott 1998; DeMott et al. 1998) suggest that direct P-limitation of food can indeed directly affect growth of daphnids, especially when the animals are slightly older (*see also* DeMott 1998). Highly unsaturated fatty acids are also of importance, but only when the C : P ratio of the algae is low enough, and the algae contain enough phosphorus.

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