Effects of dietary phosphorus deficiency on the abundance, phosphorus balance, and growth of *Daphnia cucullata* in three hypereutrophic Dutch lakes

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Abstract

We studied population dynamics, phosphorus balance, and individual growth to evaluate limitation of *Daphnia cucullata* by dietary phosphorus (P) in three interconnected, hypereutrophic Dutch lakes. Consistent with the P-limitation hypothesis, declines in egg production and population density coincided with seasonal increases in the seston C : P ratio from 300 to 400–500 (molar) in all three lakes. Adult *Daphnia* collected from Lake Breukeleveen exhibited a linear increase in specific P content during the first 36 h of feeding on a P-rich diet in the laboratory ($r^2 = 0.99$), which confirms that field *Daphnia* experienced P deficits in their tissues. Laboratory experiments tested the growth of juvenile *D. cucullata* in controls with natural seston and in seston supplemented with phosphate or the P-rich cyanobacterium *Synechococcus*. Growth was also estimated in filtered lake water with *Scenedesmus* (2 mg C L^{-1}) as food. This design allowed us to partition differences between the control and *Scenedesmus* growth rates into limitation by phosphorus, energy and residual factors. In each of 10 experiments, *D. cucullata* exhibited improved growth in response to the phosphate and *Synechococcus* supplements. In agreement with predictions, *Daphnia* exhibited the lowest control growth rate and the strongest response to P addition in seston from the lake with the highest seston C : P ratio. However, animals in seston from two lakes with very similar seston C : P ratios differed in their growth responses. Our results confirm dietary P limitation but show that energy limitation and residual factors also contributed to between-lake differences in *Daphnia* growth.

Zooplankton populations are often described as "food limited." Despite insights from theory and advances from laboratory studies, food limitation in nature is poorly understood (reviews by Sterner and Hessen 1994; Gulati and DeMott 1997; Sterner and Schulz 1998). Mechanisms of food limitation can be placed in three general categories. In the first, termed "energy limitation," carbon assimilation is low because of low food quantity, digestion resistance, interfering particles, or constraints on ingestion due to particle size or shape. In the second category, toxins reduce growth through feeding inhibition and direct toxicity (e.g., DeMott 1999). Finally, even when the quantity of digestible carbon is high, growth can be limited by deficiencies in essential elements (e.g., phosphorus or nitrogen) or biochemical compounds (e.g., essential fatty acids).

Understanding food limitation in nature is complicated by the variety of potential mechanisms and by the possibility that two or more mechanisms may interact. For example, theory predicts an interaction between energy limitation and P limitation (Sterner 1997). When available energy is very low, near the threshold food concentration for individual growth, most assimilated carbon is respired and P-sufficient and P-deficient resources support equivalent, very low growth rates (Sterner and Robinson 1994). Thus, strong energy limitation can preclude a nutritional deficiency. At high levels of energy availability, depression of growth below a maximal rate could be entirely due to P limitation. However, over an intermediate range of energy availability, low-P diets should lead to P limitation, whereas high-P diets should lead to energy limitation. Recent theoretical developments have also suggested interactions between limitation by energy and by certain fatty acids or other essential or semiessential biochemical nutrients (Anderson and Pond 2000).

Laboratory studies have suggested at least two addition interactions between mechanisms of food quality. P-deficient algae are poor-quality resources for daphnids, but whether this is solely or even primarily due to an elemental P deficiency in the animals has been controversial (reviewed by Sterner and Schultz 1999). Because some algae grown under P-deficient conditions exhibit a decrease in essential fatty acids, Müller-Navarra (1995) proposed that P-deficient algae are poor-quality resources because of a deficiency in essential fatty acids. In this example, one kind of nutritional deficiency would be combined with or substituted for another. In a second example, Van Donk and colleagues (Van Donk and Hessen 1993; Van Donk et al. 1997) showed that green algae grown under P deficiency develop thickened cell walls that cause digestion resistance. Here, algae grown under P-deficient conditions would be poor quality foods because of reduced digestibility and energy limitation rather than P deficiency. Recently, Boersma (2000) presented evidence that a factor in addition to elemental limitation is involved in the poor quality of green algae grown under P-deficient conditions for *Daphnia.*

The elemental P-limitation hypothesis has, however, gained strong support from laboratory studies. One experimental test used mixtures of food types known to have low concentrations of one nutrient and high concentrations of another (DeMott 1998). P-limited algae have low concentrations of P and essential fatty acids, whereas the cyanobacterium *Synechococcus* has high amounts of P and only traces of essential fatty acids. Mixing small quantities of *Synecho-*

coccus (5%–20%) with a P-deficient green alga, however, markedly improved the growth of four *Daphnia* species and a littoral daphnid, *Simocephalus vetulus*. This result is contrary to the hypothesis that P-deficient algae are poor foods because of a deficiency in essential fatty acids but supports the P-limitation hypothesis. Contrary to the digestion resistance hypothesis, isotope experiments have shown high P assimilation efficiency by *Daphnia* feeding on P-deficient green algae (DeMott 1998; DeMott et al. 1998). Other laboratory studies have provided further evidence for the elemental limitation hypothesis and/or against the alternative hypotheses (Sterner and Robinson 1994; Sundbom and Vrede 1997; Urabe et al. 1997; Weers and Gulati 1997; DeMott et al. 1998).

Stoichiometric theory can be used to estimate a resource threshold C:P ratio where growth becomes limited by P rather than energy. Such threshold ratios depend on both the stoichiometry of the consumer and the concentration of the resources (Urabe and Watanabe 1992; Sterner and Hessen 1994; Sterner 1997). These theoretical predictions ignore alternative food quality mechanisms and have assumed strict P homeostasis in the consumer. Because daphnids have higher P concentrations in their tissues than do other freshwater zooplankton (Sterner and Hessen 1994), stoichiometric theory predicts that daphnids should be more sensitive to P limitation than should other taxa. For example, bosminids are predicted to be less sensitive than *Daphnia* to P limitation (Urabe and Watanabe 1992), and this prediction has been confirmed in laboratory growth experiments (Schulz and Sterner 1999). Estimates of C:P resource thresholds for *Daphnia* usually range from 200 to 300 (molar units; Sterner and Hessen 1994).

In this study, we used three approaches to test and quantify P limitation in the *Daphnia cucullata* populations of three hypereutrophic Dutch lakes. First we studied population dynamics and demographics. If P limitation is important, seasonal increases in the seston C:P ratio to levels about the threshold for P-limited growth should lead to reduced reproductive rates and, eventually, to declines in abundance. Second, we studied P balance in the animals. DeMott et al. (1998) found that *Daphnia magna* suffered a loss in specific P content along a gradient of increasing dietary P deficiency. Moreover, a tight linear relationship was found between *Daphnia's* final P to mass ratio and its growth rate. On the basis of these results, we predicted that P-limited growth should lead to a decline in *Daphnia's* specific P content. Finally and most important, we tested the growth of juvenile *Daphnia* in controls with natural seston and in seston supplemented with phosphate or the P-rich cyanobacterium *Synechococcus*. The growth of each *Daphnia* cohort was also estimated in filtered lake water with a high concentration of a high quality green alga as food. This experimental design allowed us to quantify the roles of P, energy, and residual factors in limiting *Daphnia's* growth rate.

Methods

Background on study site—On the basis of previous studies, multiple mechanisms of food quality limitation of *Daph-*

nia's growth seemed likely at our study site, the hypereutrophic Loosdrecht lakes (DeMott and Gulati 1999 and references cited therein). Moreover, planktivorous fish were abundant and were expected to impact *Daphnia* mortality. The phytoplankton of all three lakes was dominated by cyanobacteria, mainly *Oscillatoria limnetica* and the prochlorophyte *Prochlorothrix hollandica*. Together, these two filamentous forms comprised, on the average, ~90% of the algal mass in suspension and often exceeded 10^5 filaments ml^{-1} (Van Tongeren et al. 1992). About half to two thirds of the seston (<150 μm) consisted of detritus, largely originating from *Oscillatoria* and *Prochlorothrix* (Otten et al. 1992). Filamentous cyanobacteria can be poor-quality foods for a variety of reasons, including toxicity, digestion resistance, difficulties in handling and ingesting filaments, and deficiencies in essential fatty acids (reviews by Lampert 1987*a*; de Bernardi and Giussani 1990; Christoffersen 1996; Brett and Müller-Navarra 1997). More specifically, laboratory experiments with *Oscillatoria* from the Loosdrecht lakes and with detritus derived from the cyanobacterium show that these resources support the growth of *Daphnia galeata* and *D. cucullata* but are poorer in quality than green algae (Repka 1997; Repka et al. 1998).

Despite the potential for limitation by predation and a variety of food quality mechanisms, analysis of a 9-yr data set from the Loosdrecht lakes provides evidence for P limitation of *Daphnia* abundance (DeMott and Gulati 1999). The abundance of *D. cucullata* in lakes Vuntus and Loosdrecht was high (average ~50 ind L^{-1}) during years when the seston P was high (molar C:P ratio, 250–300) but was low (<1 ind L^{-1}) when the seston P was low (C:P ratio, >450; $r = -0.92$ and -0.95, respectively). *Daphnia* abundance in the third lake, Lake Breukeleveen, was consistently higher at a given seston C:P ratio but also appeared to be constrained by seston P. DeMott and Gulati (1999) speculated that fish predation was less intense in Lake Breukeleveen, resulting in higher *Daphnia* densities for a given, food-limited growth rate. They did not, however, exclude interactions between P limitation and other food quality mechanisms. Thus, in addition to testing for *Daphnia* P limitation in each lake, we wanted to understand the differences in *Daphnia* abundance between these three similar lakes.

Field sampling and Daphnia *demographics*—Field sampling was conducted once or twice per week in each lake from late May through July 1999. We collected water samples using a 1.5-m plastic tube with a stopper on the bottom attached to a nylon cord that ran through the length of the tube. At each lake, ~20 samples were collected at a central station from a drifting boat to give a 20-liter composite sample. Because of the shallowness of the lakes (mean depths 1.45–1.85 m) and well-mixed water columns, this method provided representative samples.

The composite samples served three purposes. First, seston passing through a 33-μm screen was analyzed for particulate carbon and particulate phosphorus. Previous studies showed that a 33-μm screen retained most rotifers and copepod nauplii but allowed the passage of nearly all phytoplankton, including the filamentous cyanobacteria. Second, the composite samples were used to estimate *Daphnia* abun-

dance and egg production. Finally, seston passing through a 33-μm screen was used in laboratory *Daphnia* growth assays. Plankton net tows were used to collect *D. cucullata* for P analysis, for growth assays, and to provide additional data on egg production. Secchi depth and water temperature were also measured. In addition, we counted filamentous cyanobacteria in two samples from each lake, using an inverted microscope.

Zooplankton in the composite samples were collected on an 85-μm screen and preserved in 95% ethanol. *Daphnia* from 3–10 liters of lake water were counted under a compound microscope. Animals were characterized as juveniles or adults, depending on the minimum size of egg-bearing females, and the number of eggs was also counted. Males were not observed. Estimates of egg production were supplemented with animals from plankton net tows when <20 adult females were encountered in a composite sample.

We estimated *Daphnia* birth and death rates using the egg ratio method (Paloheimo 1974). Under the assumption of steady-state conditions, the per capita birth rate (b, d^{-1}) can be calculated from the mean number of eggs per female (the egg ratio, E) and the duration of egg development (D, in days)

$$b = \ln(E + 1)/D.$$

An estimate of the death rate (d) is calculated from the difference between the birth rate and the rate of population increase:

$$d = b - r.$$

To estimate egg development times, we regressed data from Elster and Schwoerbel's (1970) temperature-dependent measures for *Daphnia longispina*. Their data are derived from laboratory trials over a wide range of temperatures and are in good agreement with results for other *Daphnia* species (Hebert 1978). Because of the high sampling variability associated with the demographic estimates (DeMott 1980), we present three-point moving averages as well as individual estimates of b, r, and d.

Algal cultures—The green alga *Scenedesmus obliquus* was cultured in chemostats in a medium with 100 μM P, a molar N:P ratio of 20, and a dilution rate of 1.0 d^{-1}. These conditions produced algae with a molar C:P ratio of ~80. The cyanobacterium *Synechococcus elongatus* (UTEX 563) was grown in batch cultures with MBL medium (Guillard and Lorenzen 1972), with 30%–50% of the medium exchanged per day. The C:P ratio of the cyanobacterium was ~50. Both algal species were centrifuged and suspended in filtered lake water from Lake Martseveen before being fed to *Daphnia*. Algal concentrations were determined from absorbance at 750 nm by use of previously determined calibration curves.

Chemical analysis—Algal cultures and seston samples were analyzed for particulate carbon and particulate phosphorus. The carbon content of algal cultures and seston was derived from chemical oxidation of filtered samples (GF/F Whatman) by use of a modified procedure that involved digestion of organic matter at 140°C (Gulati et al. 1982). Algal

and seston samples for P determination were concentrated on Whatman GF/F filters, which were dried, ashed at 550°C for 2 h, and transferred to test tubes. Estimates of the specific P content of *Daphnia,* expressed as a percentage of dry mass, used animals collected in plankton net tows. Live adult female *D. cucullata* were sorted under a dissecting microscope within 12 h of collection, placed in small pretared aluminum containers, dried at 60°C, and weighed on an ultrabalance (Mettler ME30) to the nearest microgram. Each sample contained ~100–150 individuals (~250–500 μg).

Methods for determining the P content of the ashed samples of algae, seston, and *Daphnia* followed the method of DeMott et al. (1998). Estimates of particulate C and P in the seston were run in duplicate once or twice weekly for each lake. The mean coefficient of variation (CV, 100 $\times$ SD/mean) for duplicate seston samples was 1.6% for both carbon and phosphorus. The mean CV for triplicate estimates of *D. cucullata's* specific P content was 2.0%.

Phosphorus-balance experiment—To test for a deficit in P balance, adult *D. cucullata* from Lake Breukeleveen were isolated in the laboratory, placed in filtered lake water, and fed a high concentration (2 mg C L^{-1}) of P-sufficient *Scenedesmus* for varying amounts of time. We consider an increase in *Daphnia's* specific P content to be evidence that animals from the field were experiencing a P deficit. The specific P content of the animals was measured initially (time 0) and after 4, 9, 20, 36, and 48 h of feeding on *Scenedesmus.* One 2-liter beaker with ~500 adult *D. cucullata* was used for each time step. The experiment was run in two parts, with animals for time 0 and 36 h collected and isolated on one day and animals for 4, 9, and 48 h collected and isolated 2 d later. Specific P content was determined in triplicate for each time step.

Daphnia growth assays—Growth assays tested *Daphnia's* ability to growth on natural seston or natural seston supplemented with phosphate or with the P-rich cyanobacterium *Synechococcus*. In addition, we measured the growth of each cohort with a high concentration (2 mg C L^{-1}) of the green alga *Scenedesmus* in filtered (GF/F) water from Lake Breukeleveen. The 4-d growth assays were run in beakers, with food and medium exchanged after 2 d. On the basis of clearance estimates for animals feeding on 6-μm particles in seston from Lake Breukeleveen (DeMott et al. 2001), we estimate that grazing caused a decline of ~10% in the seston concentration of the experimental beakers over the last 2 d of each experiment. We did not have a laboratory culture of *D. cucullata.* Moreover, *D. cucullata* rarely produced more than one egg per female in the field, making it difficult to isolate sufficient numbers of young from gravid field-collected females. Therefore, we collected *D. cucullata* from Lake Breukeleveen and isolated cohorts of field-collected early instar juveniles under a dissecting microscope using a pipette. To avoid including any larger, older individuals in the experiments, the animals were again checked under a dissecting microscope before being placed in experimental treatments. Isolating, sorting, and checking sufficient numbers of early instar juveniles for an experiment took 8–12 h. *D. cucullata* were collected from Lake Breukeleveen in the

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morning, and the growth assays were begun in the evening of the same day.

Three samples were used to estimate the initial dry mass, with each sample containing 10–15 *D. cucullata*. Mean initial mass ranged from 0.7 to 0.9 µg per individual. Each beaker contained 150 ml of medium and 10–12 *D. cucullata,* and each experimental treatment included three replicate beakers. Precautions were taken to avoid biases in sorting the animals among the experimental treatments. The number of survivors and their lengths and number of eggs were recorded at the end of each 4-d experiment. All individuals from a single beaker were transferred to a single, pretared aluminum container. Samples were dried at 60°C overnight and weighed to the nearest microgram.

The *Daphnia* growth experiments included four treatments: (1) natural seston, (2) natural seston + 100 µg P L^{-1} as phosphate, (3) natural seston + 2 mg C L^{-1} of *Synechococcus* and (4) filtered lake water with 2 mg C L^{-1} of *Scenedesmus*. An initial experiment showed that >95% of 100 µg P L^{-1} as phosphate was taken up by the natural seston (particulate fraction <33 µm) within 30 min. The added *Synechococcus* also contained ~100 µg P L^{-1}. On the basis of initial estimates of seston C and the seston C:P ratio, the phosphate and *Synechococcus* treatments were both designed to reduce the seston C:P ratio to <200, below the threshold for P limitation in *Daphnia* (Sterner and Hessen 1994).

Growth rates for each treatment were calculated from the equation

$$g = [\ln(M_4) - \ln(M_o)]/t$$

where *g* is the growth rate and M_o and M_4 are the mean individual masses initially and after 4 d, respectively. M_4 is the dry mass of survivors/the number of survivors. Only a few animals died or were lost (<10% for each experiment). Because *g* is an exponential rate, like the birth- and death-rate estimates, growth responses were quantified as differences between treatments. The difference in growth between natural seston supplemented with phosphate and natural seston alone provides a measure of P limitation. The addition of 2 mg C L^{-1} of *Synechococcus* represented ~20%–30% of the seston carbon concentration in the Loosdrecht lakes. Because the phosphate and *Synechococcus* treatments had the same nonlimiting C:P levels, we consider the difference between the *Synechococcus* and phosphate treatments a measure of energy limitation once P requirements are satisfied. We also assume that growth was near maximal in the treatment with filtered water and *Scenedesmus*. The difference in growth between the *Scenedesmus* and *Synechococcus* treatments is termed "residual limitation." Note that the *Synechococcus* addition would not necessarily relieve all energy limitation. It should increase the average digestibility of the diet, thus overcoming at least a part of energy limitation due to digestion resistance. Energy limitation due to feeding inhibition by filaments should not be overcome by energy supplementation and should appear primarily in the "residual" term. Thus, residual limitation could result from a variety of mechanisms, including poor digestibility of natural seston, toxicity or feeding interference by natural particles, and deficiency of essential fatty acids.
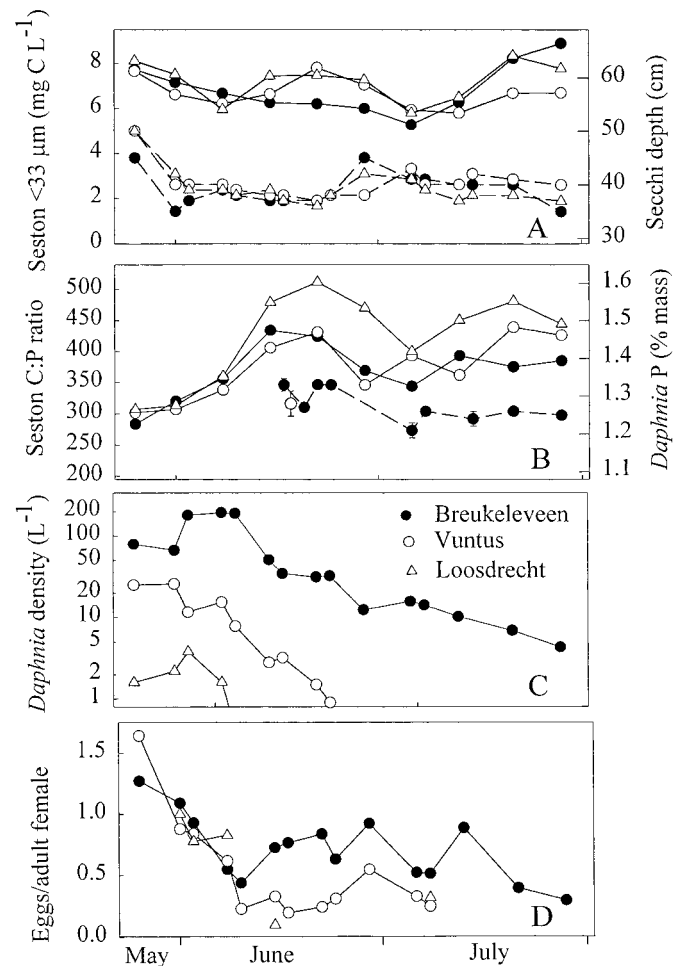

Fig. 1. Seasonal variation in seston and *D. cucullata* in the Loosdrecht lakes during 1999. Data include (A) seston concentration (solid lines) and Secchi transparency (dashed lines); (B) seston C: P ratio (solid lines) and *Daphnia* P content (dashed line); (C) *D. cucullata* density; and (D) *D. cucullata* adult female egg ratio. Data for *Daphnia's* P content are means ± SE for three replicate determinations.

Results

Field data and demographic analysis—Seston concentration and water transparency showed little variation between lakes and over time. Seston concentration was very high and water transparency was very low in all three lakes (Fig. 1A). Periodic observations with an inverted microscope showed that filamentous prokaryotes dominated the phytoplankton throughout the study period. The concentration of filamentous prokaryotes was estimated for two coarsely filtered (33 µm) samples from each lake taken during July. Estimates ranged from 1.2 to 2.2 × 10^5 filaments ml^{-1}. Both visual observations and microscope counts confirmed that the cyanobacteria filaments (diameter 1–3 µm; mean length 80 µm) readily passed through the 33-µm screen.

The seston C:P ratio was lowest at the beginning of the study in late May, reached a peak in late June, and remained high for the remainder of the study (Fig. 1B). The seston C:
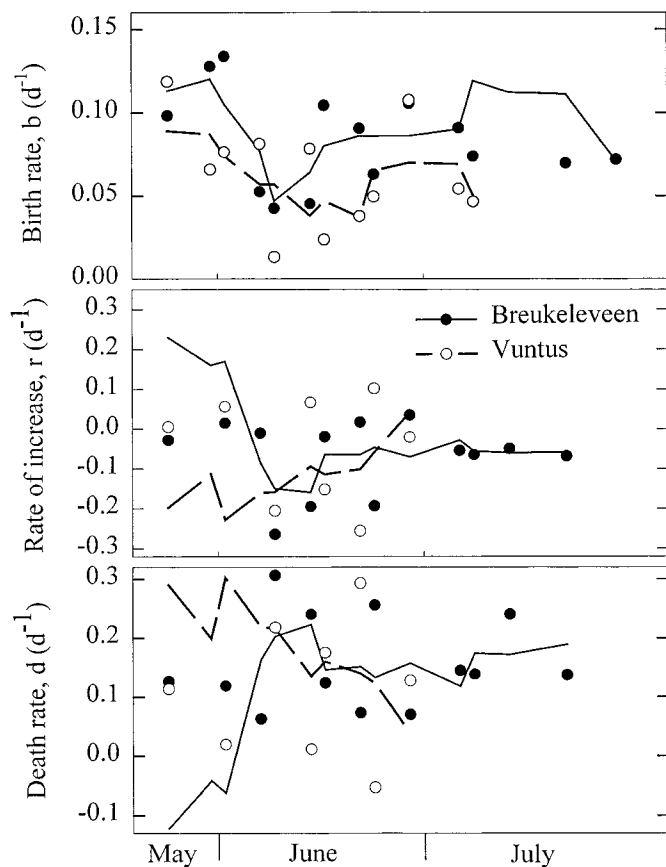
Fig. 2. Seasonal variation in rates of birth (*b*), increase (*r*), and death (*d*) for *D. cucullata* in lakes Breukeleveen and Vuntus. Circles show estimates for each time interval, and lines show three-point moving averages. Five individual estimates are off scale.

P ratio was highest in Lake Loosdrecht, whereas lakes Breukeleveen and Vuntus exhibited nearly identical values. From mid-June through the end of the study, mean seston C:P ratios were Loosdrecht, 465; Breukeleveen, 391; and Vuntus, 398. Water temperature ranged from 15.7°C to 23.5°C, with variation between lakes on a given date <0.5°C (data not shown).

Estimates of the specific P content of adult *Daphnia* from Lake Breukeleveen ranged from 1.21% to 1.33% of dry mass, with a small decline over time (Fig. 1B). A single estimate of the P content of *Daphnia* from Lake Vuntus was similar to the value from Lake Breukeleveen on the same date. Additional estimates of the P content of *Daphnia* from lakes Vuntus and Loosdrecht were precluded by low population densities (see below). Unfortunately, no data on *Daphnia* P content were collected for late May, before the increase in the seston C:P ratio.

Daphnia density at the beginning of the study varied markedly among the three lakes, with the highest initial value in Lake Breukeleveen and the lowest in Lake Loosdrecht (Fig. 1C). *Daphnia* abundance declined in all three lakes throughout most of the study. *Daphnia* remained common in Lake Breukeleveen throughout the study but declined to densities too low to be sampled quantitatively in the other two lakes ($\ll 1$ L^{-1}). *Daphnia* were, however, found in plank-
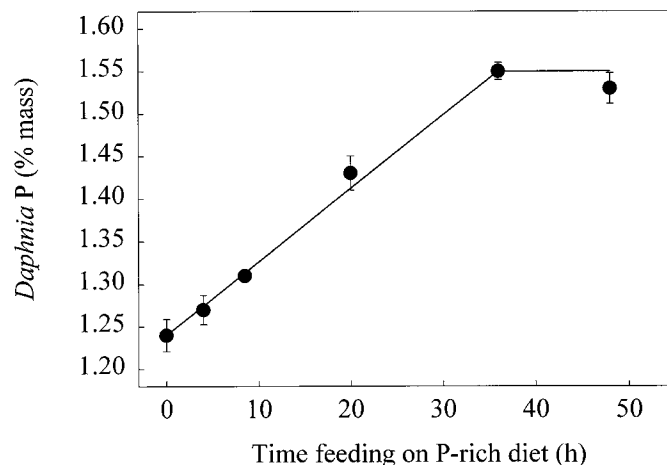

Fig. 3. Effect of time feeding on P-rich *Scenedesmus* on the specific P content of adult *D. cucullata* from Lake Breukeleveen. The data for time 0–36 h are fitted to a least-squares linear regression ($r^2 = 0.99$).

ton net tows in all three lakes throughout the study period. In all three lakes, the initial declines in *Daphnia* abundance coincided with increases in the seston C:P ratio and followed declines in the number of eggs per adult female (Fig. 1B–D). From mid-June through mid-July, egg production per adult female remained low in all three lakes but was consistently higher in Lake Breukeleveen than in the other two lakes (Fig. 1D). Samples from net tows were used in estimating egg production when population densities were low.

Daphnia's birth and death rates were estimated for Lake Breukeleveen for the entire study period and for Lake Vuntus for the first 30 d, when population density exceeded 1.0 L^{-1}. The *Daphnia* population density in Lake Loosdrecht was too low for useful estimates. Consistent with the estimates of eggs per adult female (Fig. 1D), the birth rate tended to be higher in Lake Breukeleveen than in Lake Vuntus (Fig. 2, top panel). In late May and early June, *Daphnia* in Lake Breukeleveen experienced a higher rate of increase and a lower death rate than in Lake Vuntus (Fig. 2, middle and lower panels). By mid-June, however, the growth rates of the two populations converged, and both populations exhibited steady declines.

Daphnia *phosphorus-balance experiment*—A P-balance experiment was begun on 14 July, when the seston C:P ratio in Lake Breukeleveen was 390. Adult *Daphnia* from Lake Breukeleveen exhibited a linear increase in specific P content over the first 36 h of feeding on P-rich *Scenedesmus* in the laboratory ($r^2 = 0.99$, $P < 0.001$; Fig. 3). No further increase was observed between 36 and 48 h. Because *Daphnia's* gut-filling time is ~15 min and certainly <1 h, the linear increase in *Daphnia* P over 36 h cannot be explained by the replacement of low P seston by P-rich *Scenedesmus* in the gut. The mean specific P content for 36 and 48 h was 1.54%. Comparing the initial, time 0 value (1.24%) with the mean after 36 and 48 h, we estimate that adult *D. cucullata* in Lake Breukeleveen experienced a P deficit of ~20%.

Table 1. The growth (per day) of *D. cucullata* in seston from the three Loosdrecht lakes and in seston supplemented with phosphate (+P) or *Synechococcus* (+Syn). Data are means ± SE for three replicate beakers. The 4-d growth assays were begun on 15 June (series 1), 22 June (series 2), 4 July (series 3), and 12 July (series 4). One-way ANOVA tested for differences between treatments for each experiment. Treatments not connected by lines are significantly different (Tukey test, $P < 0.05$).

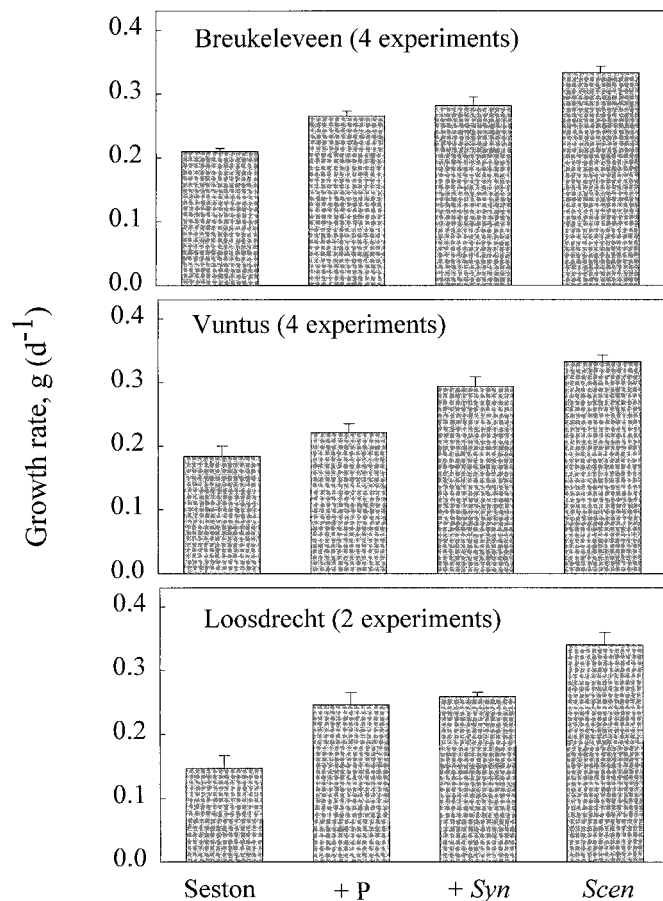| Series	Seston	+P	+Syn	F	p
Breukeleveen					
1	0.21±0.01	0.27±0.01	0.31±0.02	14.1	0.005
2	0.20±0.01	0.25±0.01	0.24±0.02	6.7	0.03
3	0.21±0.02	0.26±0.03	0.30±0.01	14.1	0.005
4	0.22±0.02	0.28±0.02	0.28±0.01	7.5	0.02
Vuntus					
1	0.16±0.04	0.20±0.03	0.30±0.04	10.2	0.02
2	0.17±0.02	0.20±0.01	0.25±0.02	18.5	0.003
3	0.17±0.02	0.23±0.02	0.31±0.02	47.1	0.001
4	0.23±0.02	0.26±0.02	0.32±0.03	8.5	0.02
Loosdrecht					
3	0.13±0.02	0.23±0.02	0.27±0.02	36.8	0.001
4	0.17±0.02	0.27±0.03	0.25±0.02	11.9	0.01

Fig. 4. The growth of *D. cucullata* in seston from the three Loosdrecht lakes, seston supplemented with phosphate (+P) or *Synechococcus* (+Syn) and in filtered lake water with *Scenedesmus* (*Scen*). Data show overall means ± SE for four experiments with seston from lakes Breukeleveen and Vuntus and two experiments with seston from Lake Loosdrecht.

Daphnia *growth assays*—We ran a total of 10 growth assays with *D. cucullata* in four series of experiments. The first two series included seston from Lakes Breukeleveen and Vuntus, whereas the third and fourth series included seston from all four lakes. In each of the 10 assays, *Daphnia* exhibited increased growth in response to supplementation with phosphate and with *Synechococcus* (Table 1). Figure 4 shows the overall means for all 10 experiments, including the treatment with *Scenedesmus* in filtered lake water. The strength of *Daphnia's* response to the two supplement treatments appears to differ between lakes. *D. cucullata* seemed to exhibit a stronger response to P addition in seston from Lake Loosdrecht, whereas responses seemed stronger to *Synechococcus* addition in seston from Lake Vuntus (Table 1, Fig. 4). In 8 of 10 experiments, growth in the *Synechococcus* treatment equaled or exceeded growth in the phosphate treatment. Moreover, growth in the phosphate treatment was never significantly greater than growth in the *Synechococcus* treatment (Tukey tests, $P > 0.05$).

The growth responses were quantified by subtracting the values of successive treatments to give growth differentials. Figure 5 summarizes the growth differentials for each treatment in each lake. One limitation of this approach is that each experiment provides only a single estimate of each growth differential. The growth differentials varied between lakes (two-way ANOVA, lake × treatment interaction, $F = 6.6$; $P < 0.001$). Pairwise comparisons showed that the energy differential was higher for Lake Vuntus than for the other two lakes and that the P differential was higher for Lake Loosdrecht than for Lake Vuntus (Tukey tests, each $P < 0.05$).

Growth in the control treatment is of interest in comparisons between lakes. The overall means (SE) in the controls for each lake were Breukeleveen, 0.21 (0.005); Vuntus, 0.18 (0.016); and Loosdrecht, 0.15 (0.021). A one-way ANOVA blocked by time revealed differences in control growth lakes between lakes ($F = 19.0$; $P < 0.001$; Tukey tests, each lake significantly different, $P < 0.05$).

Because of the short duration of the growth assays, many *D. cucullata* failed to reach maturity before the end of an
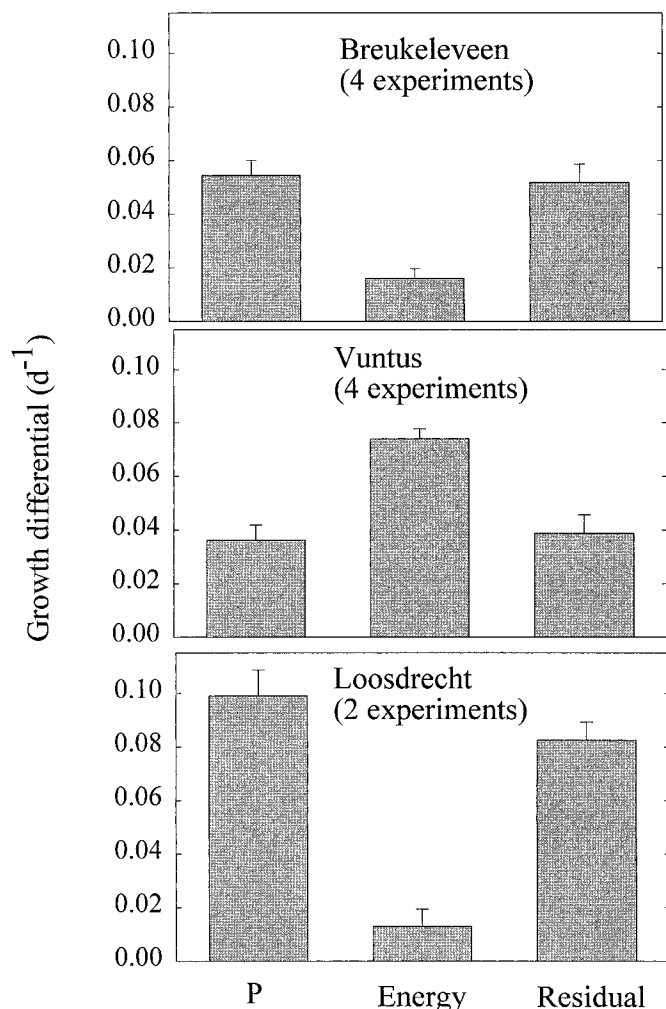
Fig. 5. Growth differentials for *D. cucullata* feeding in seston from the three Loosdrecht lakes. The phosphorus differential (P) is the difference in growth between the phosphate and seston (control) treatments; the energy differential is the difference between the *Synechococcus* and phosphate treatments, whereas the residual is the difference between the *Scenedesmus* and *Synechococcus* treatments. Data show overall means ± SE for four experiments with seston from lakes Breukeleveen and Vuntus and two experiments with seston from Lake Loosdrecht.
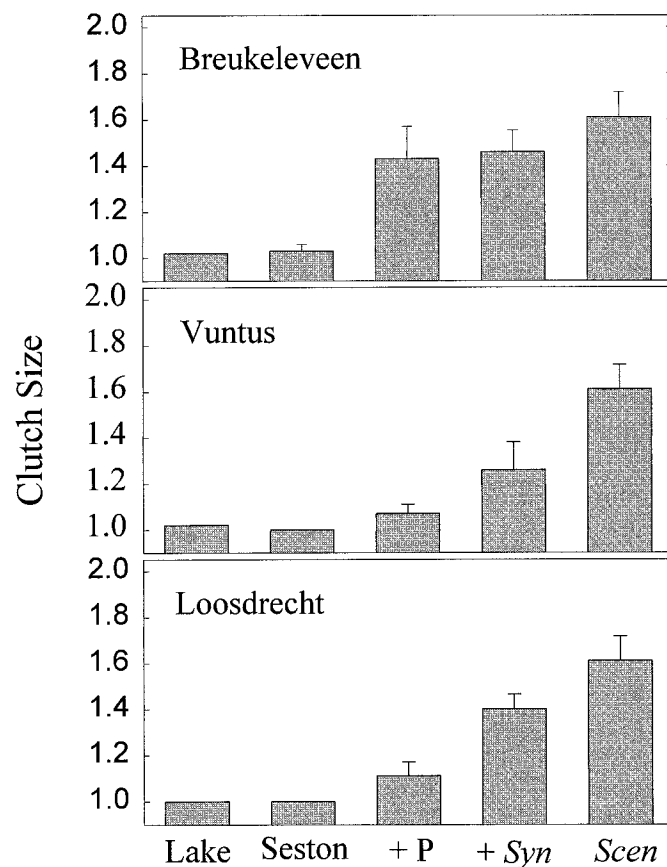

Fig. 6. Effects of experimental treatments on the clutch size of egg-bearing females in the growth assays. The first bar shows mean values for lake populations for the same time interval as the growth assays. Data show overall means ± SE for four experiments with seston from lakes Breukeleveen and Vuntus and two experiments with seston from Lake Loosdrecht.

experiment, even in the *Scenedesmus* treatment. We therefore based comparisons of egg production on the clutch size of egg-bearing females rather than the number of eggs per adult female. Figure 6 summarizes the clutch size data for the growth assays and for the lake populations during the same interval. The maximum observed clutch size was two eggs. Adult females carrying two eggs constituted only ~2% of egg bearers in the control (seston) treatment and in the natural populations sampled over the same interval (June 14–July 27). The proportion of females carrying two eggs increased in the phosphate and *Synechococcus* treatments in seston from each lake and reached a maximum in the *Scenedesmus* treatment. As in the growth assays, animals feeding in seston from Lake Breukeleveen responded more strongly to P addition, whereas animals in seston from Lake

Vuntus responded more to *Synechococcus* addition. The mean clutch sizes in the *Synechococcus* treatment were similar to those observed in the late-May lake samples (Breukeleveen, 1.31; Vuntus, 1.30; and Loosdrecht, 1.2).

Discussion

We used three methods to test and quantify the effects of dietary P deficiency on *Daphnia* in the Loosdrecht lakes. Here we evaluate the evidence for P limitation from each method and consider the broader implications of our study.

Evidence from field data on Daphnia *population dynamics and demographics*—Declines in egg production, birth rates, and *Daphnia* abundance coincided with increases in the seston C:P ratio in each lake. Moreover, the declines occurred as the seston C:P ratio increased to 350–500, values above the threshold of ~200–300 for P limitation in *Daphnia* (Sterner and Hessen 1994). Thus, temporal dynamics in all three lakes were consistent with the P-limitation hypothesis. Differences among lakes in their seston C:P ratios, however, were not consistent predictors of *Daphnia's* success. Lakes Breukeleveen and Vuntus showed very similar seston C:P

ratios, but *Daphnia* in Lake Breukeleveen exhibited higher egg production throughout the period of decline. This suggests that food quality was higher in Lake Breukeleveen for a given seston C:P ratio.

Daphnia abundance and population growth rates differed markedly between the lakes at the beginning of our study. Thus, our data cannot clarify the strong absolute differences in *Daphnia* abundance between lakes. Growth rates in natural seston from lakes Vuntus and Breukeleveen (means of 0.18 and 0.21 d^{-1}, respectively) were considerably higher than birth rates in the field during the same period (0.06 and 0.11, respectively). Because water temperature and clutch sizes were very similar in the field and in the growth assays, the difference between these two approaches suggests a low proportion of mature adults in the field populations. Strong predation on adult stages might explain the deficit in egg-bearing adults in the natural populations. However, although fish predation is expected to be strong in the Loosdrecht lakes (Lammens et al. 1992), differences in food quality seem to account for much of the between year and between lake variation in *Daphnia* abundance. As discussed in DeMott and Gulati (1999) this result may reflect consistently high predatory mortality coupled with variation in food-limited growth rates.

Differences among the lakes, although not predicted by the seston C:P ratio, are highly consistent with population data collected between 1983 and 1990 in all three lakes (DeMott and Gulati 1999). During all 8 yr, the abundance of *Daphnia* was higher for a given seston C:P ratio in Lake Breukeleveen than in the other two lakes. Thus, the persistence of *D. cucullata* in Lake Breukeleveen and its decline to nearly undetectable levels in the other two lakes agrees with historical patterns. However, analysis of long-term phytoplankton and seston data (Von Tongeren et al. 1992) provides no obvious hypotheses for better food quality in Lake Breukeleveen for a given seston C:P ratio. A tendency was noted for higher concentrations of filamentous cyanobacteria in Lake Breukeleveen, but this seems likely to be a negative factor.

Total food concentration, expressed as particulate carbon passing through a 33-μm screen, ranged from 5.3 to 8.9 mg L^{-1}, with little or no seasonal trend or difference between lakes. *Daphnia* typically exhibits maximal growth, with ~0.4 mg C L^{-1} of high-quality algae, such as *Scenedesmus* (Lampert 1987*b*). However, high food quantity does not rule out energy limitation, because of poor digestibility or particle interference. Indeed, the differences between lakes suggest that food quality factors other than P limitation were also operating. Previous studies have shown that the phytoplankton of the Loosdrecht lakes is dominated by filamentous prokaryotes year round (De Kloet et al. 1984). Our estimates of filament densities are in the range of historical estimates.

Evidence from Daphnia's *P balance*—Nine estimates of the P content of *Daphnia* from Lake Breukeleveen ranged from 1.21% to 1.33% of mass, with a single estimate of 1.28% from Lake Vuntus. These values are clearly below the expected range from most previous studies of ~1.5%–1.6% for *Daphnia* spp. feeding on P-sufficient resources (re-

viewed by Sterner and Hessen 1994). The data were collected after the seasonal increase in the seston C:P ratio and show little or no temporal variation. Thus, although the precision of our estimates was very good, our field data on *Daphnia's* P content provide less than convincing evidence for P deficit. Our P-balance experiment, however, showed that *Daphnia* collected from the field exhibited a linear increase in P content from 1.24% to 1.54% when fed a P-rich diet. This agrees well with a 2-d laboratory experiment in which the final P content of *D. magna* varied from 1.14% to 1.54% over a similar range in the C:P ratio of *Scenedesmus* (~475–80). Moreover, the maximum P content that we observed after 36–48 h of feeding on a P-rich diet, 1.54%, agrees with a mean of 1.58% for seven samples of *D. cucullata* collected from two Norwegian lakes (Hessen and Lyche 1991). Thus, our P-balance experiment, together with field estimates, provides strong evidence that field *Daphnia* experienced a P deficit in their tissues.

On the basis of earlier work, including both laboratory and field data, Hessen and his colleagues concluded that *Daphnia* and other zooplankton exhibit no significant intraspecific variation in their specific P content in response to food conditions (reviewed by Sterner and Hessen 1994). We suggest that their conclusion is based on a lack of data for P-limited zooplankton. In retrospect, the range in dietary C:P that Hessen (1990) tested in the laboratory was too narrow (C:P ratio 65–164) and was probably not above the threshold for P limitation. Andersen and Hessen (1991) found no relationship between the P content of *D. longispina* and the seston C:P ratio of small lake over a range of seston C:P from about ~150 to 500 (molar). However, they also presented anecdotal evidence that the *D. longispina* population was limited by energy, not by P. Although Hessen and Lyche (1991) studied seasonal variation in the P content of *D. longispina* and *D. cucullata* in four lakes, the highest molar seston C:P ratio was only 170, a value below the expected threshold for P limitation. Thus, although work by Hessen et al. provides evidence that energy limitation alone does not cause a decline in *Daphnia's* P content, their studies were done under conditions in which P-limited growth was not demonstrated or even expected. Thus, we believe that our results showing a reduced P content in *Daphnia* growing on P-deficient diets are consistent with earlier work showing high, constant P levels with varying energy limitation.

Hessen (1990) noted that *Daphnia* P tended to decline with age. This ontogenetic was minimized by restricting our measurements of *Daphnia* P to adult females. Moreover, the trend that we observed in the P-balance experiment, an increase in P over time, runs counter to the expected decline in *Daphnia* P with age.

The interpretation of *Daphnia* P balance is more straightforward in shallow, unstratified lakes, such as the Loosdrecht lakes, than in stratified lakes. In stratified lakes, *Daphnia* might ameliorate or avoid P limitation by feeding in deeper waters with lower seston C:P ratios. Thus, in stratified lakes, one might need to take into account variation in the seston C:P ratio with depth and diel migration in *Daphnia*. Our current study, along with the results of DeMott et al. (1998), suggests that a deficit in *Daphnia's* P balance may be an indicator P-limited growth. On the basis of work by Hessen

and colleagues (Sterner and Hessen 1994), it also seems likely that the lack of a P deficit in zooplankton tissues means that growth is not P-limited even when the resource C : P ratio exceeds the threshold for P limitation. These possibilities need further testing in laboratory and field studies.

The major P pools in *Daphnia* include nucleic acids, especially ribosomal RNA, membrane-bound phospholipids, and phosphate in the carapace (Vrede et al. 1999). If the phosphorus deficits in field animals are associated with reduced concentrations of RNA, this would provide a direct biochemical mechanism for poor growth on P deficient diets. Because strongly P-limited *Daphnia* can have difficulty molting (Sterner 1993), declines in carapace P may also have significant costs.

Evidence from growth assays—Laboratory experiments have shown that adding phosphate to P-deficient green algae rapidly improves their food quality for *Daphnia* (DeMott 1998; but see Boersma 2000). In part because of concerns about potential artifacts, we used additions of phosphate and *Synechococcus* as alternative means for reducing the C : P ratio of the total diet to the same P-sufficient level. Experiments with laboratory algal cultures show that P-deficient algae can exhibit reductions in essential fatty acids (Müller-Navarra 1995) and thickened cell walls that inhibit digestion (Van Donk and Hessen 1993; Van Donk et al. 1997). Thus, the phosphate additions could have caused changes in the biochemistry or morphology of algae in the natural seston over the 2 d between exchanges of natural seston. *Synechococcus* is rich in both P and energy but contains only traces of essential omega-3 fatty acids (DeMott and Müller-Navarra 1997). Thus, if essential fatty acids were enhanced by the phosphate additions and if essential fatty acids limited *Daphnia's* growth, one would expect higher growth in the phosphate treatment than in the *Synechococcus* treatment. However, contrary to this scenario, the growth of *D. cucullata* in the *Synechococcus* treatment equaled or exceeded that in the phosphate treatment. The potential effects of P deficiencies on digestion resistance are difficult to evaluate, because such effects could be subtle. However, most evidence for digestion resistance induced by P deficiency comes from experiments with green algae, whereas the seston in the Loosdrecht lakes is dominated by detritus and cyanobacteria. Thus, our results and previous studies support the assumption that the P growth differential is a measure of P-limited growth, whereas the difference between the *Synechococcus* and phosphate treatments is a measure of energy limitation once P requirements are satisfied.

Limitation by essential fatty acids could be included in the residual limitation, although strong responses to the *Synechococcus* additions seem contrary to this possibility. Increases in long-chain omega-3 fatty acids might also increase the growth rate above that for *Scenedesmus,* which may be deficient in eicosapentaenoic acid (Weers and Gulati 1997). However, our conclusions differ from those of Müller-Navarra et al. (2000), who argue that a deficiency in essential fatty acids was the primary cause of poor growth of *D. magna* during summer in a lake dominated by *Oscillatoria.*

Our growth assays showed consistent differences among lakes, with very low variation between replicates or between repeated experiments. Thus, use of early instar *Daphnia* isolated from the field provided consistent results. Because the field-collected animals already experienced a P deficit in their tissues, this method could produce stronger and more realistic data on P-limited growth than experiments initiated with animals grown in the laboratory on P-sufficient diets.

The growth assays showed that dietary P deficiency constrained the growth of *Daphnia* in all three lakes. Moreover, in agreement with stoichiometric theory, the lake with the strongest P deficiency (Lake Loosdrecht) showed a tendency for stronger P limitation than the other two lakes. Lake Loosdrecht also showed a tendency for stronger residual limitation than the other two lakes. However, comparisons between lakes Breukeleveen and Vuntus, lakes with very similar seston C : P ratios, showed similar responses to P addition but significant differences in control growth rates and in their energy differentials. Taken together, these results clearly show that focusing on one food quality mechanism while ignoring others would provide incomplete or even misleading conclusions about food limitation in nature. Theoretical estimates of threshold C : P ratio predict a sharp boundary from energy limitation to P limitation at relatively low energy levels (e.g., Sterner 1997). These models assume strict P homeostasis, an assumption that may need to be relaxed. The high seston carbon concentrations in the Loosdrecht Lake emphasize that energy limitation is not equivalent to low food quantity. Indeed, our results suggest that animals in nature may often find themselves near transitions between P limitation and energy limitation. More theoretical and empirical research is needed on interactions between nutritional deficiencies and energy limitation.

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