

# Effects of nutrient limitation on biochemical constituents of *Ankistrodesmus falcatus*

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## SUMMARY

1. Cell size and volume changed as a function of the type of resource limitation, with nitrogen-limited cells being smaller and less dense and phosphorus-limited cells being larger and more dense than non-limited cells.
2. The major biochemical constituents of the green alga *Ankistrodesmus falcatus* varied as a function of nitrogen or phosphorus limitation (15% of maximum growth rate) compared to cells growing at their maximum rate. Nitrogen-limited cells had much lower protein content and phosphorus-limited cells had higher carbohydrate and lipid contents than cells growing under no limitation.
3. Phosphorus-limited cells had a higher total lipid content than either nitrogen-limited or non-limited cells, but the lipid class composition was similar.
4. The protein : lipid ratio was lowest (0.38) in the nitrogen-limited cells, intermediate in the phosphorus-limited cells (0.44) and highest in the non-limited control cells (1.14).

## Introduction

There is considerable interest in questions of food quality in aquatic foodwebs (Sterner & Hessen, 1994), which has focused attention on the specific aspects of algal food quality that affect the consumers. A general conclusion of previous work is that the physiological condition of the algal cells is a critical factor influencing ingestion, fecundity and population growth rates of zooplankton herbivores. The seston in lakes varies seasonally and among lakes in elemental composition (Sterner, Elser & Hessen, 1992; Hecky, Campbell & Hendzel, 1993; Urabe, 1993) and biochemical composition (Kreeger *et al.*, 1997). Factors that influence these properties include nutrient limitations and intensity of grazing pressure, which can affect the turnover rate of the algae and the recycling of nutrients.

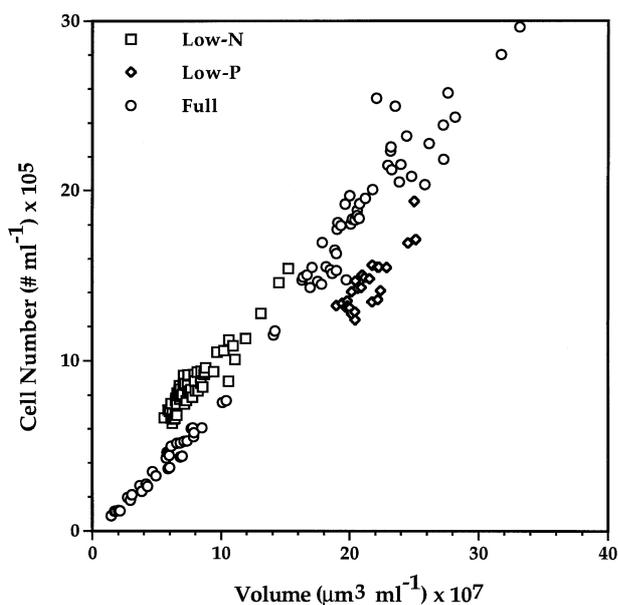
The aspects of physiological condition of algae that can influence grazers include variation in major macromolecules. Algae grown under resource limitation exhibit considerable variation in their biochemical composition, depending on the type of limiting nutri-

ent and degree of limitation (growth rate as percentage of  $\mu_{\max}$ ). Healey & Hendzel (1979) used batch cultures grown under phosphorus (P)- and nitrogen (N)-limitation and compared cellular contents of lipids, carbohydrates and proteins under limited (stationary phase) and non-limited (exponential phase) conditions for a number of algal species. Their results suggested that carbohydrates were the major storage product under P limitation for cryptomonads, and that diatoms and green algae had higher amounts of lipids and carbohydrates than non-limited cells. Nitrogen-limited algae had lower protein contents and higher carbohydrates, but the picture for lipids was variable. N-limited green algae had either somewhat higher or unchanged lipid contents compared to non-limited cells. Shifrin & Chisholm (1981) showed N-limited green algae always had higher lipid contents, but diatoms did not. Light limitation apparently leads to lower lipids (Scott, 1980; Cuhel & Lean, 1987).

Lipids in general [as a percentage of particulate

**Table 1** Cell size and biochemical composition of *Ankistrodesmus falcatus* grown under nutrient limitation at 15%  $\mu_{\max}$  or non-limited. Values are means ( $\pm$ SE).

Parameter	Algal type			ANOVA <i>P</i> -value
	Low-N <i>n</i> = 14	Low-P <i>n</i> = 5	Full <i>n</i> = 18	
POM				
( $\mu\text{g } \mu\text{m}^{-3}$ ) $\times 10^{-7}$	2.57 (0.12)	4.16 (0.24)	3.10 (0.31)	0.013
( $\mu\text{g cell}^{-1}$ ) $\times 10^{-5}$	2.27 (0.09)	6.36 (0.44)	4.10 (0.56)	0.0002
Protein				
( $\mu\text{g } \mu\text{m}^{-3}$ ) $\times 10^{-7}$	0.54 (0.05)	1.05 (0.05)	1.17 (0.05)	<0.0001
( $\mu\text{g cell}^{-1}$ ) $\times 10^{-5}$	0.48 (0.02)	1.61 (0.11)	1.52 (0.12)	<0.0001
Carbohydrate				
( $\mu\text{g } \mu\text{m}^{-3}$ ) $\times 10^{-7}$	0.52 (0.05)	0.67 (0.05)	0.34 (0.03)	0.0001
( $\mu\text{g cell}^{-1}$ ) $\times 10^{-5}$	0.46 (0.04)	1.01 (0.07)	0.44 (0.05)	<0.0001
Lipids				
( $\mu\text{g } \mu\text{m}^{-3}$ ) $\times 10^{-7}$	1.50 (0.10)	2.45 (0.24)	1.59 (0.25)	NS
( $\mu\text{g cell}^{-1}$ ) $\times 10^{-5}$	1.32 (0.08)	3.74 (0.41)	2.15 (0.41)	0.0037
Protein : lipid	0.38 (0.03)	0.44 (0.04)	1.14 (0.22)	0.0064
Protein : carbohydrate	1.19 (0.16)	1.64 (0.18)	3.79 (0.29)	<0.0001
Protein : (carbohydrate + lipid)	0.28 (0.02)	0.34 (0.03)	0.83 (0.12)	0.0006
Cell size ( $\mu\text{m}^3 \text{ cell}^{-1}$ )	91.3 (1.09)	148 (1.68)	125 (2.15)	

**Fig. 1** A comparison of cell numbers and cell volumes for *Ankistrodesmus falcatus* grown without limitation at  $\mu_{\max}$  (circles), or under limitation at 15% of  $\mu_{\max}$  by nitrogen (squares) or phosphorus (diamonds).

organic matter (POM) or as absolute amounts] and particular fatty acids (FA) have been targeted as important variables determining food quality of algae (Ahlgren *et al.*, 1990; Coutteau & Sorgeloos, 1997; Weers & Gulati, 1997a). The characteristic types of FA produced by

diatoms, green algae and/or cryptomonads have been studied by Piorreck, Baasch & Pohl (1984), Cranwell, Creighton & Jaworski (1989) and Ahlgren *et al.* (1990). The general pattern observed is that green algae rarely produce FA in excess of eighteen carbons, while diatoms and cryptophytes make many long-chained (> 18C) polyunsaturated fatty acids (PUFAs). Ahlgren *et al.* (1990) showed that the long-chained PUFAs produced by cryptophytes enhance reproduction in zooplankton, including *Daphnia*.

Many studies of algal biochemistry have been undertaken in the past on cells that were either growing at the maximal rate ( $\mu_{\max}$ ) or at stationary phase. Stationary phase cells are in a variable physiological state that is difficult to replicate from experiment to experiment. Growth rates of algae measured in lakes are frequently found to be at some intermediate growth rate (Lehman & Sandgren, 1985; Sommer, 1989), rather than at stationary phase or  $\mu_{\max}$ . Algae grown in continuous or semi-continuous culture have reduced variation in physiological condition, can be easily replicated, and are more representative of the *in situ* condition.

The objective of this study was to alter the biochemical composition of the green alga *Ankistrodesmus falcatus* (Braun) by growing it at 15% of maximum growth rate under limitation by nitrogen or phos-

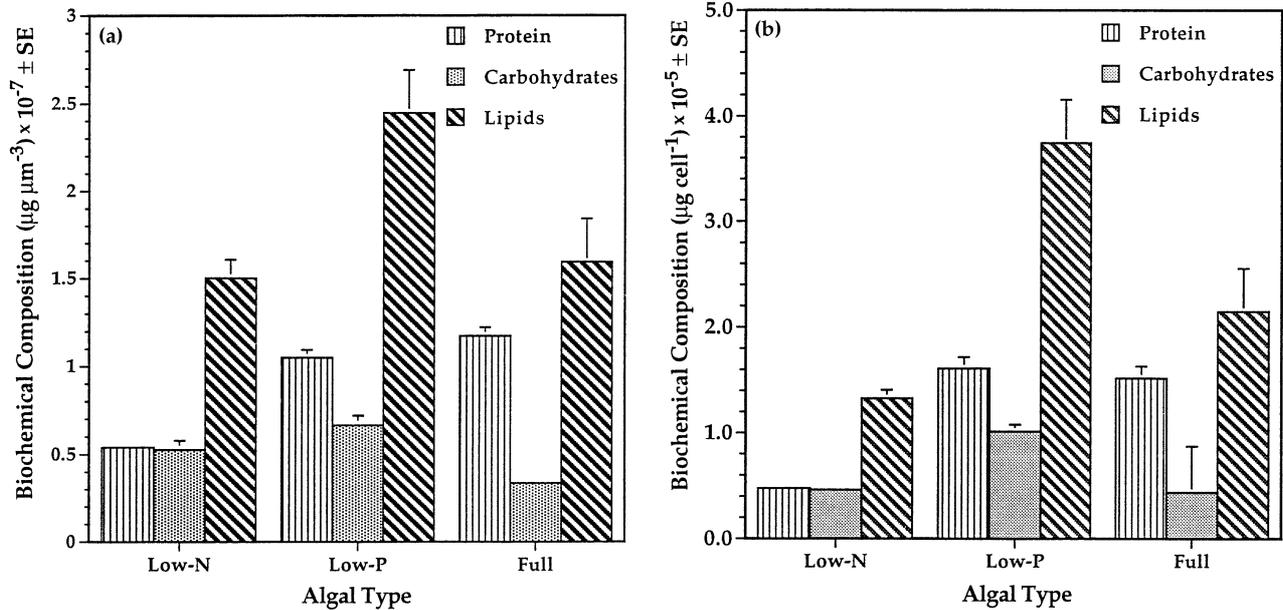


Fig. 2 The biochemical composition (per volume, on left, and per cell, on right) of protein, lipids and carbohydrates for *Ankistrodesmus falcatus* cells growing without limitation at  $\mu_{max}$  or under limitation by phosphorus or nitrogen.

Table 2 Phosphorous and carbon content of *Ankistrodesmus falcatus* grown under nutrient limitation at 15%  $\mu_{max}$  or non-limited. Values are means ( $\pm$ SE).

Parameter	Algal type			ANOVA <i>P</i> -value
	Low-N <i>n</i> = 14	Low-P <i>n</i> = 5	Full <i>n</i> = 18	
P				
( $\mu\text{g } \mu\text{m}^{-3}$ ) $\times 10^{-10}$	2.29 (0.19)	0.23 (0.004)	1.42 (0.06)	<0.0001
( $\mu\text{g cell}^{-1}$ ) $\times 10^{-8}$	2.02 (0.15)	0.34 (0.005)	1.69 (0.10)	<0.0001
( $\mu\text{g } \mu\text{m}^{-3}$ ) $\times 10^{-9}$	7.16 (0.58)	0.70 (0.01)	4.44 (0.20)	<0.0001
( $\mu\text{g cell}^{-1}$ ) $\times 10^{-7}$	6.31 (0.47)	1.07 (0.01)	5.27 (0.32)	<0.0001
C*: P (mass)	17.5 (1.51)	297 (13.5)	30.9 (1.65)	<0.0001
C*: P (atomic)	45.6 (3.93)	772 (35.0)	80.4 (4.29)	<0.0001

\*Carbon concentration was assumed to be equal to 50% of POM

phorus, or non-limited and growing at  $\mu_{max}$ , and to compare resulting compositions of the major biochemical constituents: proteins, carbohydrates, lipids and lipid classes. The relative food value of these algae for *Daphnia pulex* (Forbes) was assessed in experiments reported elsewhere (Kilham *et al.*, 1997).

**Materials and methods**

Algal cultures were maintained in a chamber with a 14 : 10 h light : dark cycle at 20 °C  $\pm$  1 °C. COMBO medium (S.S. Kilham, D.A. Kreeger, C.E. Goulden and L. Herrera, personal communication) was used in

all experiments and was modified for low-nutrient experiments. This medium can be used for growing both algae and cladocerans.

The green alga *A. falcatus* (clone from Academy of Natural Sciences; Goulden, Henry & Tessier, 1982) was used in all experiments. Experimental cultures were continually aerated and light saturated ( $> 100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Cultures usually consisted of 1 l of medium in 2-l glass flasks. Control cultures were grown at  $\mu_{max}$  in full COMBO and diluted once per day. Nutrient-limited cultures were maintained at 15% of maximum growth rate ( $\mu_{max} = 1.34 \text{ day}^{-1}$ ) using semi-continuous dilutions

(180 ml day<sup>-1</sup>) once each day (Killham, 1978). The nutrient manipulations were as follows: full COMBO had  $1 \times 10^3 \mu\text{M N}$ ,  $50 \mu\text{M P}$ , giving N : P = 20 : 1; low-N COMBO had  $50 \mu\text{M N}$ ,  $50 \mu\text{M P}$ , giving N : P = 1 : 1; low-P COMBO had  $1 \times 10^3 \mu\text{M N}$ ,  $5 \mu\text{M P}$ , giving N : P = 200 : 1. Cultures were at steady state (constant fluorescence and cell volume) for at least a week before they were used in animal experiments (Killham *et al.*, 1997). Samples for analyses of cellular constituents (protein, lipids, lipid classes, carbohydrates, C, N, P, and ash-free dry weight) were taken at this time (see Kreeger *et al.*, 1997 for analytical methods). Cell counts and cell volumes were monitored using a Coulter Counter with Channelyser.

Biochemical differences among the three algal foods were compared statistically with one-way ANOVA procedures (Sokal & Rohlf, 1969) and the LSD multiple range analyses (Statgraphics v. 6.0) at the  $\alpha = 0.05$  level.

## Results

The relationship between cell number ( $n \text{ ml}^{-1}$ ) and cell volume ( $\mu\text{m}^3 \text{ ml}^{-1}$ ) of the *A. falcatus* (Fig. 1; Table 1) changed when grown under N limitation (smaller;  $y = 0.81x + 2.23$ ,  $r^2 = 0.89$ ) or P limitation (larger;  $y = 0.82x - 3.07$ ,  $r^2 = 0.73$ ) compared to the relationship for cells growing with no limitation ( $y = 0.95x - 1.17$ ,  $r^2 = 0.98$ ). The weights and densities of nutrient-limited cells were significantly different from control cultures and from each other, with P-limited cells being heavier/denser and N-limited cells being lighter/less dense than control cultures (Table 1).

The biochemical composition of *A. falcatus* growing at  $\mu_{\text{max}}$  or growing at 15% of  $\mu_{\text{max}}$  under limitation by N or P is shown in Fig. 2 and Table 1. Nitrogen-limited cells had a low protein content, and a lipid content similar to the control cells growing at  $\mu_{\text{max}}$ . The protein : lipid ratio was 0.38 (Table 1). Phosphorus-limited cells had significantly higher amounts of all constituents than N-limited cells and a higher ratio of protein : lipid (0.44). Carbohydrates and especially total lipids were higher and protein content slightly lower in P-limited cells than in control cells. The control cells growing at  $\mu_{\text{max}}$  had a high protein : lipid ratio (1.14). The directly measured ash-free dry weight and the particulate organic matter (POM) calculated as the sum of total protein, lipid and carbohydrate were not significantly different for any of the algal treatments.

The lipid class components for the three algal types are shown in Fig. 3, both as amounts per cell volume and as percentages of total lipids. Phosphorus-limited cells had a higher triglyceride content than N-limited or non-limited cells (Fig. 3a). Both types of nutrient limitation resulted in a higher percentage of triglycerides than non-limited cells, and non-limited cells had a higher percentage of phospholipids and sterols (Fig. 3b). In all other aspects the lipid classes were similar in the three algal types.

Phosphorus contents of the algae grown under limitation varied as expected, with the P content of P-limited cells being much lower than for N-limited or non-limited cells (Table 2). The P contents were significantly different in all three algal types. The C : P ratio (atomic), calculated from C as 50% of POM, is low for the non-limited cells (80.4) and N-limited cells (45.6), but high for the P-limited cells (772).

## Discussion

The cell size of *A. falcatus* changed under N limitation and P limitation, with N-limited cells being smaller and less dense than cells growing at  $\mu_{\text{max}}$ , and P-limited cells being larger and denser than the control cells (Figs 1; Table 1). This may be related to the lower protein content in the N-limited algae and the higher carbohydrate and lipid content of the P-limited algae. Rhee (1978) found that volume per cell of P-limited cells of the green alga *Scenedesmus* sp. was higher than for N-limited cells growing at the same growth rate (44% of  $\mu_{\text{max}}$ ), and cell carbon was higher in P-limited cells.

There were major differences in the cellular biochemical constituents of *A. falcatus* grown at 15%  $\mu_{\text{max}}$  under N or P limitation (Fig. 2; Table 1). P-limited algae (low P) had significantly higher protein contents than N-limited algae (low N), which was also observed for *Scenedesmus* sp. by Rhee (1978). Harrison, Thompson & Calderwood (1990) also found a lower protein content in a variety of N-limited algae than in non-limited control cells. In contrast, Sterner & Robinson (1994) found no difference in the protein content of *Scenedesmus* cultured under moderate N limitation or severe P limitation; rather, they found P-limited algae to contain a greater proportion of carbohydrate than N-limited algae. The P-limited *A. falcatus* in the present study had significantly higher carbohydrate content per cell, but not per volume, than the N-limited cells.

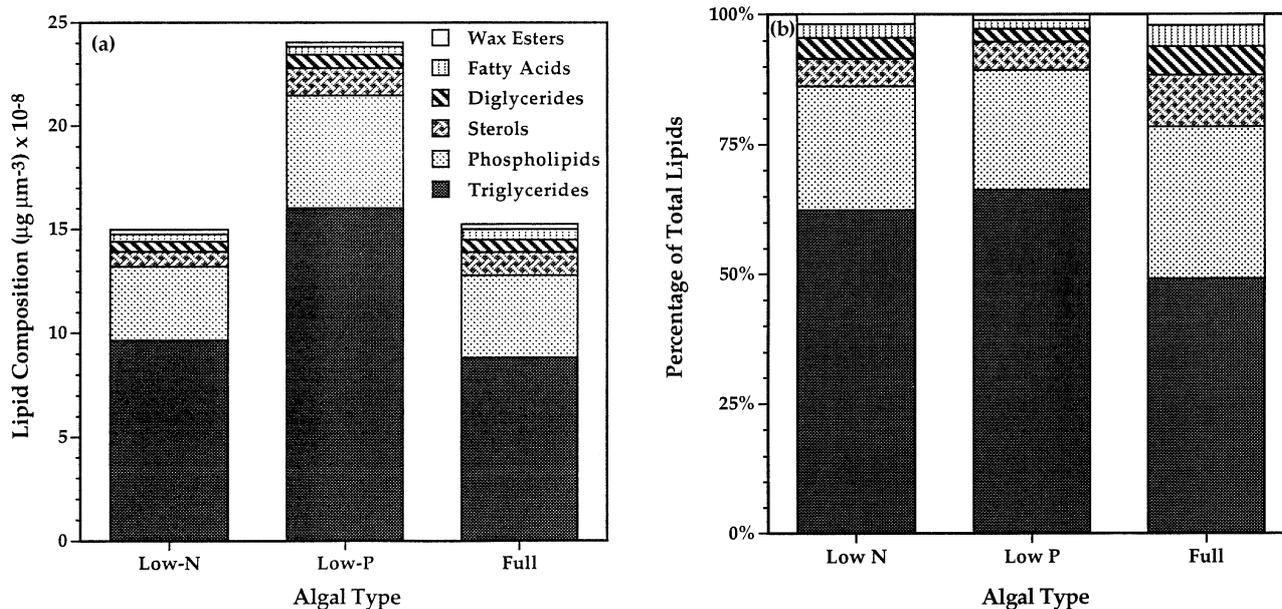


Fig. 3 The composition of the major lipid classes (per cell volume, on left, and percentage of total lipids, on right) for *Ankistrodesmus falcatus* growing without limitation at  $\mu_{\text{max}}$  or under limitation by nitrogen or phosphorus.

The lipid content of low-P cells was significantly higher than for non-limited control cells or low-N cells (Table 1). Rhee (1978) observed higher lipid contents in P-limited cells than in N-limited cells of another green alga. Sterner & Hessen (1994) suggest that there may be a general pattern of higher lipids at low relative growth rates of algae, but the low-N cells in the present study did not show this pattern. The protein : lipid ratio was significantly lower in nutrient-limited algae, with low-N cells being lowest. The carbohydrate content of P-limited cells was significantly higher than for the other treatments, as has been frequently reported for most algae (Healey & Hendzel, 1979). The pattern of biochemical constituents observed by Harrison *et al.* (1990) for N starvation vs. no limitation of three species of marine algae was the same as the present results: the percentage lipid stayed the same, the percentage carbohydrate increased and the percentage protein decreased. These observations are consistent with the suggestion by Lizotte & Sullivan (1992) that protein : carbohydrate ratios less than 1.2 indicate that the algae are nutrient stressed. Protein : CHO ratios in the low-N, low-P and non-limited algae in the present study were 1.19, 1.64 and 3.79, respectively. The results contrast with Shifrin & Chisholm (1981) who showed that N limitation always led to increased lipid content in ten species of green algae.

The phosphorus content (Table 2) was much lower

in the P-limited cells ( $3 \times 10^{-9} \mu\text{mol cell}^{-1}$ ) than in N-limited or non-limited cells ( $c. 2 \times 10^{-8} \mu\text{mol cell}^{-1}$ ) as expected. The phosphorus contents per cell of three species of freshwater diatoms grown under P limitation were slightly higher ( $1-2 \times 10^{-8} \mu\text{mol cell}^{-1}$ ) than for *A. falcatus* (Van Donk & Kilham, 1990). The C : P ratio was very high in the P-limited cells (Table 2), and very low in the other cases.

The two major lipid classes, triglycerides (TG) and phospholipids (PL; Fig. 3), showed significantly higher levels per cell (but not per volume) of both components under P limitation than under N limitation or for non-limited cells. The TG : PL ratio tended to be a little higher for nutrient-limited cells (3 vs. 1.6), but the differences were not significant. The TG : PL ratio indicates the relative importance of the storage lipids (TG) and the structural lipids (PL).

For the green alga *A. falcatus*, the biochemical composition varied with the type (N-limited, P-limited, non-limited) and degree (15%  $\mu_{\text{max}}$  or  $\mu_{\text{max}}$ ) of resource limitation. N limitation reduced cellular protein contents twofold. In contrast, lipid and carbohydrate contents were enriched in P-limited cells. The protein : lipid, protein : carbohydrate, and protein : (carbohydrate + lipid) ratios were significantly depressed under conditions of nutrient limitation. Any of these factors have the potential to decrease the value of this species as food for their consumers.

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### References

- Ahlgren G., Lundstedt L., Brett M & Forsberg C. (1990) Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. *Journal of Plankton Research*, **12**, 809–818.
- Coutteau P. & Sorgeloos P. (1997) Manipulation of dietary lipids, fatty acids and vitamins in zooplankton cultures. *Freshwater Biology*, **38**, 501–512.
- Cranwell P.A., Creighton M.E. & Jaworski G.H.M. (1989) Lipids of four species of freshwater chrysophytes. *Phytochemistry*, **27**, 1053–1059.
- Cuhel R.L. & Lean D.R.S. (1987) Influence of light intensity, light quality, temperature and daylength on uptake and assimilation of carbon dioxide and sulfate by lake plankton. *Canadian Journal of Fisheries and Aquatic Sciences*, **44**, 2118–2132.
- Goulden C.E., Henry L.L. & Tessier A.J. (1982) Body size, energy reserves, and competitive ability in three species of Cladocera. *Ecology*, **63**, 1780–1789.
- Harrison P.J., Thompson P.A. & Calderwood G.S. (1990) Effects of nutrient and light limitation on the biochemical composition of phytoplankton. *Journal of Applied Phycology*, **2**, 45–56.
- Healey F.P. & Hendzel L.L. (1979) Indicators of phosphorus and nitrogen deficiency in five algae in culture. *Journal of the Fisheries Research Board of Canada*, **36**, 1364–1369.
- Hecky R.E., Campbell P. & Hendzel L.L. (1993) The stoichiometry of carbon, nitrogen, and phosphorus in particulate matter of lakes and oceans. *Limnology and Oceanography*, **39**, 709–724.
- Kilham S.S. (1978) Nutrient kinetics of freshwater planktonic algae using batch and semicontinuous methods. *Mitteilungen Internationale Vereinigung für Theoretische und Angewandte Limnologie*, **21**, 147–157.
- Kilham S.S., Kreeger D.A., Goulden C.E. & Lynn S.G. (1997) Effect of algal food quality on fecundity and population growth rates of *Daphnia*. *Freshwater Biology*, **38**, 639–647.
- Kreeger D.A., Goulden C.E., Kilham S.S. & Lynn S.G. (1997) Seasonal changes in the biochemistry of lake seston. *Freshwater Biology*, **38**, 539–554.
- Lehman J.T. & Sandgren C.D. (1985) Species specific rates of growth and grazing loss among freshwater algae. *Limnology and Oceanography*, **30**, 34–46.
- Lizotte M.P. & Sullivan C.W. (1992) Biochemical composition and photosynthate distribution in sea ice microalgae of McMurdo Sound, Antarctica: evidence for nutrient stress during the spring bloom. *Antarctic Science*, **4**, 23–30.
- Piorreck C.C., Baasch K.-H. & Pohl P. (1984) Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. *Phytochemistry*, **23**, 207–216.
- Rhee G.-Y. (1978) Effects of N : P atomic ratios and nitrate limitation on algal growth, cell composition, and nitrate uptake. *Limnology and Oceanography*, **23**, 10–25.
- Scott J.M. (1980) Effect of growth rate of the food alga on the growth/ingestion efficiency of a marine herbivore. *Journal of the Marine Biological Association UK*, **60**, 681–702.
- Shifrin N.S. & Chisholm S.W. (1981) Phytoplankton lipids: Interspecific differences and effects of nitrate, silicate and light-dark cycles. *Journal of Phycology*, **17**, 374–384.
- Sokal R.R. & Rohlf F.J. (1969) *Biometry*. W.H. Freeman, San Francisco.
- Sommer U. (1989) Nutrient status and nutrient competition in a shallow, hypertrophic lake. *Limnology and Oceanography*, **34**, 1162–1173.
- Sterner R.W. & Hessen D.O. (1994) Algal nutrient limitation and the nutrition of aquatic herbivores. *Annual Review of Ecology and Systematics*, **25**, 1–29.
- Sterner R.W. & Robinson J.L. (1994) Thresholds for growth in *Daphnia magna* with high and low phosphorus diets. *Limnology and Oceanography*, **39**, 1228–1232.
- Sterner R.W., Elser J.J. & Hessen D.O. (1992) Stoichiometric relationships among producers, consumers and nutrient cycling in pelagic ecosystems. *Biogeochemistry*, **17**, 49–67.
- Urabe J. (1993) Seston stoichiometry and nutrient deficiency in a shallow eutrophic pond. *Archiv für Hydrobiologie*, **126**, 417–428.
- Van Donk E. & Kilham S.S. (1990) Temperature effects on silicon- and phosphorus-limited growth and competitive interactions among three diatoms. *Journal of Phycology*, **26**, 40–50.
- Weers P.M.M. & Gulati R.M. (1997a) Effect of addition of polyunsaturated fatty acids to the diet on growth and fecundity of *Daphnia galeata*. *Freshwater Biology*, **38**, 721–729.