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Environmental Bioassay Techniques and their Application.

A study of phosphate limitation in Lake Maarsseveen: phosphate uptake kinetics versus bioassays

E. Van Donk,[1,*] L.R. Mur[2] & J. Ringelberg[3]
[1] *Provincial Waterboard of Utrecht, Postbox 80300, 3508 TH Utrecht, The Netherlands (* author for correspondence)*; [2] *Laboratory of Microbiology, University of Amsterdam, Nieuwe Achtergracht 127, 1018 WS Amsterdam, The Netherlands*; [3] *Department of Aquatic Ecology, University of Amsterdam, Kruislaan 320, 1098 SM Amsterdam, The Netherlands*

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Abstract

In order to assess possible phosphate limitation for the phytoplankton community of Lake Maarsseveen, two techniques (phosphate uptake experiments and bioassays) were employed simultaneously in February–March 1982. In that period the ambient phosphate concentration of the lake water was less than 0.03 μM P and the diatom *Asterionella formosa* constituted more than 90% of the phytoplankton population. The phosphate uptake experiments showed relatively high uptake capacities and low cell phosphorus contents for the natural phytoplankton community. This suggested phosphate limitation throughout the test period. The growth stimulation of the phytoplankton after enrichment with phosphate, however, only revealed phosphate limitation from the beginning of March and bioassays may therefore be regarded as a less sensitive method.

Introduction

There are three common approaches to the study of nutrient limitation in phytoplankton populations: they are ambient nutrient concentrations of the water, physiological indicators, and the use of bioassays.

The first approach is simply based on measurements of the nutrient content of the water, inferring limitation of those nutrients that are in short supply. However, this method by itself does not yield reliable results if fluxes of nutrients are unknown and if no information is available on the nutrient requirements of the species involved.

The second approach, the use of physiological indicators (e.g. cellular nutrient contents, short-term nutrient uptake kinetics), may give a better insight, especially when physiological characteristics of nutrient-limited reference cultures are available for comparison. In the study of nutrient uptake kinetics, two aspects may be considered. One may examine the steady state specific nutrient uptake rate (q), i.e. nutrient uptake rate proportional to growth rate (μ) (at steady state the amount of nutrient taken up is equal to the amount used to make new cells). This steady state specific nutrient uptake rate (q) can be calculated according to the equation (Droop, 1973)

$$q = \mu \cdot Q, \tag{1}$$

where Q is the amount of internal nutrient per unit population. The other possibility for studying nutrient uptake is to determine the nutrient uptake

curve, by measuring the short-term (initial) nutrient uptake rates (V) at different nutrient concentrations (S) (equation 2):

$$V = V_{max} \cdot \frac{S}{K_{s \cdot u} + S}, \quad (2)$$

where V and V_{max} are the velocity and maximum velocity (uptake capacity) of nutrient uptake; S is the initial nutrient concentration; $K_{s \cdot u}$ is the half-saturation constant for uptake.

Nutrient limitation generally induces the potential for a high uptake capacity (V_{max}) of the limiting nutrient compared with the maximum specific nutrient uptake rate (q_{max}) (e.g. Gotham & Rhee, 1981; Riegman & Mur, 1984a). Therefore short term nutrient uptake experiments may be useful for determining the growth rate limiting nutrient of phytoplankton communities. A lowered cellular content of the limiting nutrient is also a general response to nutrient limitation (e.g. Droop, 1974; Rhee, 1978).

The third approach, the use of bioassays, is based on measuring the *growth* of the phytoplankton after enrichment with nutrients. This technique has been widely applied to assess the nutrient limitation of phytoplankton growth in natural waters. Two major variants to the method can be distinguished: (1) nutrients are added to filtered lake water in which laboratory cultured species are inoculated (e.g. Paasche, 1978; Reynolds & Butterwick, 1979; De Vries, 1983 and 1985) and (2) nutrients are added to lake water, containing the natural phytoplankton community (e.g. Schelske *et al.*, 1974; Frey & Small, 1980; Van der Does & Klapwijk, 1987; Van Donk *et al.*, 1988; Munawar *et al.*, 1988). Variant 2 has been chosen to study the growth limitation of the phytoplankton in Lake Maarsseveen.

The objective of the study is to compare the results obtained simultaneously with physiological indicators and natural community bioassay experiments, applied to the phytoplankton community of Lake Maarsseveen.

Methods

Site description

Lake Maarsseveen is situated in the centre of The Netherlands, near the city of Utrecht. It is a man-made lake formed around 1960 by excavation of sand in a peat-bog area. The oligo-mesotrophic, trough-shaped lake (70 ha; 30 m max. depth) is replenished essentially by precipitation and ground water and drained by an outlet. A more comprehensive description of the lake is given by Van Donk (1987). Every year during winter and spring, diatoms are predominant. In the spring of 1982, the diatom *Asterionella formosa* was predominant over a period of more than three months. This alga was able to reach a high abundance (3800 cells/ml), whereas the other species (*Fragilaria crotonensis*, *Stephanodiscus astraea* and *S. hantzschii*) appeared only in small numbers. In previous years (1980–1981) *A. formosa* was heavily infected by a chytrid fungus and the other diatoms were able to bloom. However, in 1982 this fungus was temporarily inhibited in its activity due to low water temperatures (Van Donk & Ringelberg, 1983). It was expected that in 1982 the diatoms were growing under phosphate limitation because the phosphate concentration in the lake was very low (< 0.03 μM P) and nitrate (> 30 μM N) and silicate (> 50 μM Si) were relatively high.

Short-term P-uptake with the natural phytoplankton community

From February 1, 1982, until March 31, 1982, once a week, around nine 'o clock in the morning, composite water samples were collected of the upper 10 metres of the open-water zone of the lake, taken with a 3-litre van Dorn sampler and concentrated through a 55 μm-mesh plankton net. The sample was transported to the laboratory within one hour and filtered over 150 μm mesh plankton net to remove zooplankton. The concentrated sample consisted of more than 90% of *A. formosa*.

The cell phosphorus content was measured according to Menzel & Corwin (1965). To determine the relation between the short term phosphate uptake rate and the phosphate concentration of the water, the sample was divided among six 1-litre Pyrex Erlenmeyer flasks containing 500 ml of sterile medium (Guillard, 1975) with varying concentrations of K_2HPO_4 (0.24–10 μM P). The concentrated sample was divided among the uptake flasks with an initial cell concentration of 1.5×10^5 cells ml^{-1}. Incident light intensity during the experiment was optimal (25 $Watt.m^{-2}$) and the incubation temperature was 10 °C ± 1 °C. Since the cell density was low the incident light intensity was about equal to the intensity experienced by the algae in the flasks. In each flask, orthophosphate was measured immediately after cell addition and subsequently every 15 or 30 minutes onward during the period that the orthophosphate concentration declined as a linear function of time (2–4 hours). Orthophosphate was determined by the Murphy and Riley method (1962), with all the samples filtered through 0.45 μm filters presoaked in distilled water. Spectrophotometric readings were made using 4 cm cuvettes, allowing determinations of as little as 0.03 μM P. The flasks were stirred constantly. For each flask the initial phosphate uptake rate, V, was calculated from the initial disappearance of the phosphate by least squares linear regression analysis of the observed phosphate concentrations. Thus,

$$V = \frac{-\Delta S}{\Delta t} \cdot \frac{1}{n}, \tag{3}$$

where $-\Delta S$ is the decrease in phosphate concentration during the time interval Δt and n is the number of *A. formosa* cells per ml. Calculated values of V were fitted by an iterative, non-linear regression (Hanson *et al.*, 1967) to the Michaelis-Menten equation: (see equation 2).

Short-term P-uptake with reference laboratory cultures of A. formosa

A. formosa was isolated from Lake Maarsseveen using the pipette technique (Guillard, 1973). The cells were cultured in freshwater medium 'WC' (Guillard, 1975) at 10 °C and with 25 $Watt.m^{-2}$ (12 h light – 12 h dark) illumination, provided by cool-white fluorescent tubes. Exponentially growing *A. formosa* cells then transferred to flasks with fresh 'WC' medium without phosphate. KNO_3 was added to supplement potassium, which was low due to the omitted K_2HPO_4. The cells were allowed to grow until they were depleted of phosphorus, as indicated by the culture reaching a stationary phase. To determine the uptake capacity (V_{max}) at various degrees of phosphate limitation, the *A. formosa* cells were preloaded with different amounts of phosphorus.

One day before each uptake experiment, cells from the stationary phase where inoculated in sterile medium with a particular amount of phosphate. By the time the cells were used for the uptake experiments, all added phosphorus had been taken up by the cells. No free phosphorus could be measured in the medium. Before incubation the cell phosphorus content was measured according to Menzel and Corwin (1965). By varying the added amount of phosphate it was possible to perform uptake experiments with cells having different internal phosphorus contents. The uptake experiments were done according to the procedure described for the natural phytoplankton community.

In order to compare the maximum value of the steady state specific nutrient uptake rate (q) (equation 1) with the uptake capacity (V_{max}), q_{max} ($= \mu_{max} \cdot Q_{max}$) was calculated using the internal storage model of Droop (1973) (equations 1, 4 and 5). The Droop model describes the relationship between growth rate and internal phosphorus content, during steady state conditions. It is probable that the Droop model can also be used for non steady state conditions:

$$\mu = \mu'_{max} \cdot \frac{Q - Q_o}{Q}, \tag{4}$$

where Q_o is the minimum cell quota and μ'_{max} refers to 'infinite' internal phosphorus content. μ'_{max} is higher than the true μ_{max} value (Droop, 1973).

$$\mu_{max} = \mu'_{max}\left(1 - \frac{Q_o}{Q_{max}}\right). \quad (5)$$

For μ_{max}, Q_o and Q_{max} (the maximum cell quota) we used the values measured by Van Donk (1983) and Van Donk and Kilham (1989) at 10 °C ($\mu_{max} = 0.58\ d^{-1}$, $Q_o = 6 \times 10^{-9}\ \mu M$ P cell^{-1} and $Q_{max} = 124 \times 10^{-9}\ \mu M$ P cell^{-1}). To get an upper limit for q (q_{max}), μ'_{max} instead of μ was used in equation 1.

Bioassays

Mixed water samples of the upper seven metres of Lake Maarsseveen, taken with a 3-litre van Dorn sampler, were brought back to the laboratory within one hour after sampling. The samples were filtered through 150 μm gauze to remove the crustacean zooplankton. Once a week from February 17 until March 31 the natural phytoplankton cells were incubated in three 1-litre Pyrex Erlenmeyer flasks and placed in the laboratory under optimal light conditions (25 Watt. m^{-2}, 12 h light – 12 h dark) at 5 °C (ambient lake temperature) and 10 °C (standard temperature). The flasks were manually shaken twice a day. The nutrient combinations tested were 'All', 'All-P' and 'LW'. 'All' indicates the lake water (LW) enriched with all nutrients listed in Table 1 and 'All-P' all nutrients added except phosphorus. The growth of the different algal species in the flasks was followed for seven days by counting the number of cells each day. The growth rates were calculated by a linear least squares regression of log transformed data.

Results

Table 2 gives the percentages of the phytoplankton community consisting of *A. formosa* during the spring of 1982. Also included in the table are the ambient concentrations of silicon, phosphate and nitrate. The details about the abundance and succession of the diatom species and nutrient concentrations in Lake Maarsseveen have been published elsewhere (Van Donk & Ringelberg, 1983; Van Donk *et al.*, 1988).

Figure 1 illustrates the relationship between the cell phosphorus content (Q) and the kinetic parameter (V_{max}) of the natural phytoplankton community as well as the laboratory cultures of *A. formosa*. Also depicted in the graph is the relationship between Q and q_{max}, i.e. the maximum specific uptake rate of *A. formosa*, calculated according to equations 1, 4 and 5.

From the experiments with the laboratory cul-

Table 1. Range and concentration of nutrients added to the bioassay enclosures.

Nutrients	Compound	Concentration
P	K_2HPO_4	3.20 (μM)
N	$NaNO_3$	71.00
Si	$Na_3SiO_3 \cdot 9H_2O$	35.70
B	H_3BO_3	0.10
Vitamin mix		
Biotin		0.05 (μg l^{-1})
B_{12}	Cyanocobalamin	5.0
B_1	Thiamine · HCl	100.0
Trace metals		
Cu	$CuSO_4 \cdot 5H_2O$	0.039 (μM)
Zn	$ZnSO_4 \cdot 7H_2O$	0.077
Co	$CoCl_2 \cdot 6H_2O$	0.042
Mn	$MnCl_2 \cdot 4H_2O$	0.914
Mo	$Na_2MoO_4 \cdot 2H_2O$	0.026
Fe	$FeCl_3 \cdot 6H_2O$	16.00
EDTA	$Na_2EDTA \cdot 2H_2O$	13.00

Table 2. Percentage of the phytoplankton community consisted of *A. formosa* and the ambient concentration of PO_4-P, NO_3-N and SiO_2-Si in early spring of 1982.

Date	*A. formosa* (%)	PO_4-P (μM)	NO_3-N (μM)	SiO_2-Si (μM)
30–01–82	60	0.05	35	63
28–02–82	90	<0.03	31	58
31–03–82	99	<0.03	32	57
30–04–82	20	<0.03	31	52

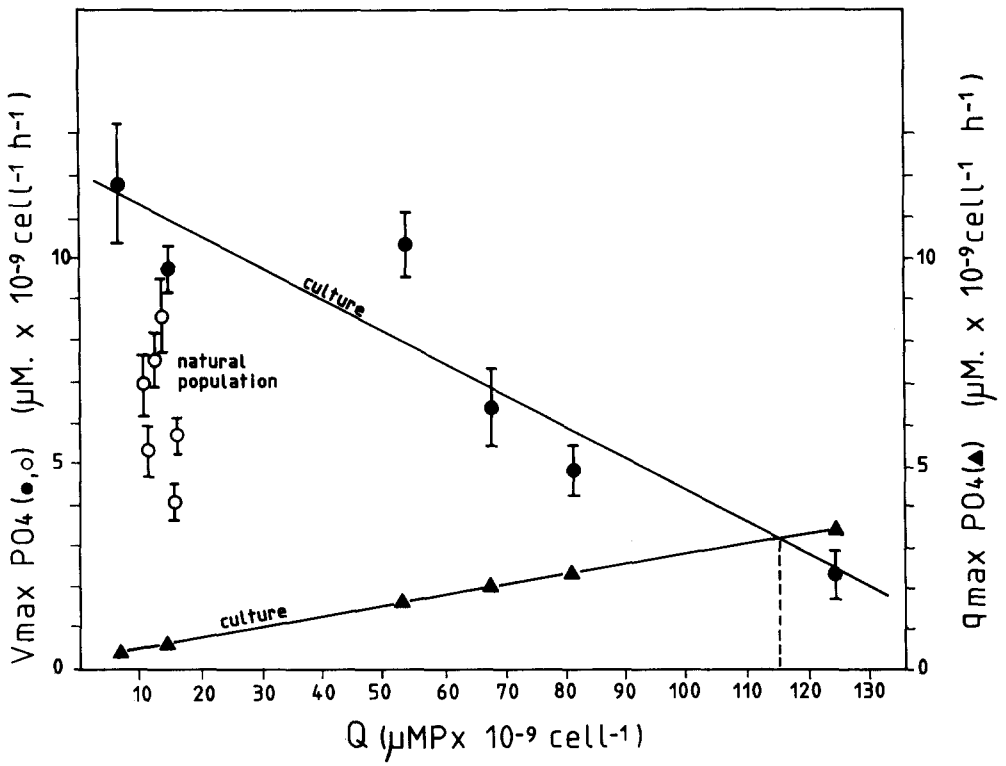

Fig. 1. Maximum specific uptake rate (q_{max} PO_4, ▲) and uptake capacity for phosphate (V_{max} PO_4, ○, ●) as function of the cell phosphorus content (Q) of the cultured *A. formosa* population (closed symbols) and of the natural phytoplankton community (open symbols). The 95% confidence intervals are given for the uptake capacity.

tures of *A. formosa*, a linear declining slope ($r^2 = 0.87$) between V_{max} and Q was observed. High V_{max} values ($> 9 \times 10^{-9}$ µM P cell^{-1} h^{-1}), were measured for cells with internal P-contents ranging from 6 to 53×10^{-9} µM P cell^{-1}. A low V_{max} of 2.2×10^{-9} µM P cell^{-1} h^{-1} was found for cells with a Q-value of 124×10^{-9} µM P cell^{-1} (Q_{max} at 10 °C). For Q-values higher than 115×10^{-9} µM P cell^{-1}, q_{max} exceeded V_{max}; these *A. formosa* cells were not P-limited. Cells with Q-values lower than 115×10^{-9} µM P cell^{-1} were growing under P-limitation.

Table 3 presents the P-uptake kinetics V_{max} and $K_{s.u}$ obtained from experiments with the

Table 3. Phosphate uptake data of the natural *Asterionella formosa* population. 95% confidence intervals are given in parentheses. Also given are the percentage of cells infected by *Zygorhizidium planktonicum* (% I) at the start of the experiment.

Date	Q (µM P × 10^{-9} cell^{-1})	V_{max} (µM P × 10^{-9} cell^{-1} h^{-1})	$K_{s.u}$ (µM)	% I
01-02-82	23.0	–	–	–
05-02-82	19.0	–	–	–
17-02-82	12.5	7.54 (6.99–8.10)	1.48 (1.03–1.92)	15
24-02-82	–	7.12 (6.57–7.66)	0.73 (0.47–1.00)	16
03-03-82	12.6	8.52 (7.47–9.56)	1.28 (0.76–1.70)	8
10-03-82	10.6	6.90 (6.07–7.73)	0.64 (0.41–0.88)	14
17-03-82	15.4	5.71 (5.38–6.05)	0.65 (0.54–0.76)	32
24-03-82	10.9	5.40 (4.96–5.83)	0.47 (0.35–0.60)	71
31-03-82	14.8	4.07 (3.74–4.39)	0.57 (0.39–0.75)	88

natural phytoplankton community; also compiled in the table are the cell phosphorus contents and the percentage of cells infected by the fungus *Zygorhizidium planktonicum*.

Before March 10, V_{max} was high ($>7 \times 10^{-9}$ μM P cell^{-1} h^{-1}; about the same magnitude of P-uptake was observed for *A. formosa* cultured under P-limitation) and the cell phosphorus content was low, decreasing from 23×10^{-9} μM P cell^{-1} (February 1) to 10.6×10^{-9} μM P cell^{-1} (March 10). After March 10, the number of cells, infected by *Z. planktonicum*, increased up to a percentage of 88 (March 31) and V_{max} decreased to 4.1 (March 31). V_{max} of the natural phytoplankton community was consistently higher than q_{max} (Fig. 1) – an indication suggesting that the phytoplankton was growing under phosphate limitation from the start of the experiments. The decrease in V_{max} after March 10, probably caused by fungal parasitism, lowered the P-uptake rate of the phosphate limited cells.

Table 4 presents the growth rates of the three dominant diatom species, i.e. *A. formosa*, *S. hantzschii* and *S. astraea* in the bioassays, performed at 5 °C and 10 °C. The growth rates at 10 °C were consistently higher than those observed at 5 °C and the rates in 'All' higher than those recorded in 'All-P' and 'LW'. According to the growth responses *A. formosa* appeared to be not significantly phosphorus limited before March 10, while both *Stephanodiscus* species showed signs of phosphorus limitation. It was in the middle of March the three diatom species revealed the largest growth rate discrepancies between 'All' and 'All-P' treatments. At the end of March the growth rate of *A. formosa* in 'All' was very low as more cells became infected by the fungus *Z. planktonicum* (Table 3).

Discussion

P-uptake capacity (V_{max})

In many studies it has been found that phytoplankton growth, limited by essential growth ele-

Table 4. The growth rates (μ) of three dominant species from the lake at 5 °C and 10 °C, as response to nutrient addition. LW = pure lake water; All = addition of all nutrients (see Table 1); All-P = addition of all nutrients except phosphorus. The 95% confidence intervals are in parentheses.

Date	Species	LW μ(d^{-1})		All-P μ(d^{-1})		All μ(d^{-1})	
		5 °C	10 °C	5 °C	10 °C	5 °C	10 °C
17–2	*A. formosa*	0.23(±0.06)	0.45(±0.08)	0.22(±0.05)	0.42(±0.07)	0.31(±0.05)	0.51(±0.08)
24–2		0.23(±0.05)	0.43(±0.05)	0.26(±0.04)	0.44(±0.04)	0.32(±0.07)	0.48(±0.09)
3–3		0.20(±0.03)	0.42(±0.05)	0.21(±0.02)	0.40(±0.06)	0.26(±0.03)	0.46(±0.07)
10–3		0.08(±0.04)	0.20(±0.03)	0.12(±0.03)	0.23(±0.02)	0.24(±0.06)	0.44(±0.06)
17–3		–	0.12(±0.04)	0.03(±0.02)	0.08(±0.03)	0.15(±0.04)	0.35(±0.08)
24–3		–		0.02(±0.02)	0.04(±0.02)	0.06(±0.02)	0.16(±0.03)
31–3		–		–		0.07(±0.02)	0.08(±0.03)
17–2	*S. hantzschii*	0.20(±0.02)	0.36(±0.07)	0.16(±0.04)	0.38(±0.04)	0.40(±0.07)	0.60(±0.09)
24–2		0.19(±0.04)	0.29(±0.08)	0.15(±0.05)	0.26(±0.05)	0.30(±0.08)	0.52(±0.07)
3–3		0.17(±0.04)	0.28(±0.04)	0.18(±0.05)	0.27(±0.05)	0.35(±0.04)	0.57(±0.05)
10–3		0.02(±0.02)	0.07(±0.02)	0.03(±0.02)	0.08(±0.02)	0.27(±0.03)	0.48(±0.06)
17–3		–	–	–	–	–	–
17–2	*S. astraea*	0.14(±0.03)	0.30(±0.04)	0.16(±0.06)	0.32(±0.06)	0.30(±0.04)	0.52(±0.07)
24–2		0.15(±0.05)	0.32(±0.05)	0.18(±0.04)	0.31(±0.05)	0.29(±0.03)	0.49(±0.05)
3–3		0.12(±0.04)	0.28(±0.06)	0.14(±0.02)	0.25(±0.04)	0.22(±0.03)	0.44(±0.06)
10–3		0.07(±0.02)	0.14(±0.03)	0.06(±0.01)	0.09(±0.02)	0.15(±0.02)	0.36(±0.04)
17–3		–	–	–	–	0.12(±0.03)	0.33(±0.02)

ments such as P, can be characterized by a high uptake capacity (V_{max}) for the limiting nutrient compared to the maximum specific uptake rate (q_{max}) (e.g., Gotham & Rhee, 1981; Zevenboom, 1980; Riegman & Mur, 1984a).

The short-term phosphate uptake has been examined in detail in steady state cultures of *Scenedesmus* sp. (Rhee, 1973; 1974), *Anabaena flos-aquae* and *Microcystis* sp. (Gotham & Rhee, 1981) and *Oscillatoria agardhii* (Riegman & Mur, 1984b). Phosphate uptake in these organisms being a function of both internal and external phosphate concentrations, can be described by an equation resembling non-competitive enzyme inhibition:

$$V = \frac{V_{max}}{\left(1 + \frac{K_s \cdot u}{s}\right)\left(1 + \frac{i}{K_i}\right)}, \qquad (6)$$

where i is the internal total inorganic polyphosphate concentration and K_i is a constant which expresses the degree of inhibition by the internal phosphorus concentration. Rhee (1973) found that V_{max} depends on K_i. A similar negative effect of cellular polyphosphate on phosphate uptake was reported for batch culture studies of *Chlorella* (Jeanjean, 1969; Aitchison & Butt, 1973).

However, in some species this type of feedback control for P-uptake has apparently not been observed. Burmaster & Chrisholm (1979) found that the V_{max} of *Monochrysis lutheri* remains constant with increasing Q. Healey & Hendzel (1975) and Nyholm (1977) found a decrease in V_{max} only when Q approaches Q_{max}.

Our study with *A. formosa* demonstrated a negative linear relationship between V_{max} and Q:

$$V_{max} = 12.1 - 0.08Q. \qquad (7)$$

Using the *A. formosa* reference curve as a guide to define conditions where P is not limiting, the high phosphate uptake capacities of the natural phytoplankton community of Lake Maarsseveen compared to that of the reference curve indicated phosphate limitation throughout the entire study period.

Cell phosphorus content

Rhee (1974) and Harrison *et al.* (1976) found for some phytoplankton species, that under nitrogen or silicon limited conditions, the cell phosphorus contents remained high and generally constant regardless of growth rate. Tilman and Kilham (1976) demonstrated this for *A. formosa* growing under silicon limitation.

The cell phosphorus content of the *A. formosa* population in the lake was 6–12 times lower than the maximum value measured in the laboratory. The values found for natural populations were comparable with the minimum cell phosphorus content measured in the laboratory at 5 °C (9.5×10^{-9} μM P cell^{-1}) (Van Donk, 1983; Van Donk & Kilham, 1989). These low values strongly pointed to a phosphate limitation.

Bioassays

Despite the evidence showing P-limitation in the lake water, as identified by the low cell phosphorus contents and high phosphate uptake capacities over the whole experimental period, the results of the bioassay experiments, somehow, were less supportive. Although the growth of *A. formosa* was consistently higher in 'All' than in 'All-P' and 'LW', a significantly higher growth rate was observed with addition of phosphorus only after March 3. Bioassays, as indicated in our tests, have been considered less sensitive compared to short-term nutrient uptake experiments because the difference between the growth response in the enriched and the control samples was too small to be statistically significant. Furthermore, because the growth responses observed at 5 °C and 10 °C were almost the same, temperature has not been considered to be the factor responsible for the low sensitivity.

Conclusions

According to the phosphate uptake capacity and the cell phosphorus content, we may draw the

conclusion that *A. formosa* was growing under phosphate limitation from the beginning of the experimental period onwards (half February). However, according to the bioassays, *A. formosa* became limited by phosphate only from the beginning of March. At March 10, *A. formosa* was strongly limited by phosphate, while only 15% of the cells were infected by *Z. planktonicum*. Thus it seems likely that phosphate limitation was the primary cause of the end of the spring bloom in 1982 and the fungus infection might have only accelerated the collapse of *A. formosa*.

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