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Use of immobilized algae for estimating bioavailable phosphorus released by zooplankton

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Abstract. Cells of the green alga *Selenastrum capricornutum* were immobilized in permeable alginate beads to prevent them from being grazed by zooplankton. Algae were able to grow in these beads and were used as a new technique to estimate bioavailable phosphorus (P) released by zooplankton. P-limited algal cells were encapsulated in alginate beads and used to measure P-release by *Daphnia pulex* feeding on P-saturated and P-limited free algal cells. Daphnids grazing on P-saturated cells released 20 times more P available for the immobilized algae than animals grazing on P-limited cells (0.06 versus 0.003 μg P mg^{-1} Daphnia-DW h^{-1}).

Introduction

Nutrient release by zooplankton has been investigated by several workers (e.g. Peters and Lean, 1973; Peters and Rigler, 1973; Ferrante, 1976; Lehman, 1980; Scavia and McFarland, 1982; Olsen and Østgaard, 1985; Den Oude and Gulati, 1988; Hessen and Andersen, 1992). Nutrient regeneration is caused by various processes: excretion, egestion, moulting and food cell damage during feeding of zooplankton (Lehman, 1980; Gardner and Scavia, 1981). During summer stratification, zooplankton release may be the major input of dissolved nutrients for phytoplankton production (e.g. Lehman, 1980). The extent to which released nutrients stimulate phytoplankton growth has been difficult to quantify because zooplankton not only affect phytoplankton growth through nutrient release, but simultaneously also remove phytoplankton by grazing. In many studies, phytoplankton were removed from the experimental vessels when measuring the amount of nutrients released (e.g. Ikeda and Mitchell, 1982). However, since the bulk of nutrients released by zooplankton is produced during the feeding process, its real importance can only be measured with feeding zooplankton (Lampert, 1978; Scavia and Gardner, 1982). Some investigators measured nutrient release by zooplankton in the presence of phytoplankton by correction for algal uptake (Takahasi and Ikeda, 1975) or by adding surplus nutrients to saturate the algal uptake to a constant level (Lehman, 1980). However, these studies give no information on the amount of released nutrients available for algal growth. Fractions of the released phosphorus (P), especially, may be in forms which are not directly available for algal growth (Peters and Lean, 1973; Ferrante, 1976).

To overcome this problem, we present a technique where a fraction of the algal cells in the P-release experiments were encapsulated in a permeable alginate (immobilized algal cells), preventing the algae from being grazed, while growth stimulation by the released nutrients was still possible.

Interest in immobilized cell technology has grown rapidly in the past 15 years (Kennedy and Cabral, 1983). While most efforts have been directed toward bacteria, considerable attention has also been given to eukaryotic animal and plant cells, including algae (Mattiasson, 1983). Applications for immobilized algae up to now included hydrocarbon production for fuels, removal of nitrogen and P from wastewater (Chevalier and De La Noüe, 1985), and ecotoxicology testing (Bozeman *et al.*, 1989; Van Donk *et al.*, 1992).

The purpose of the present study was to test the use of immobilized algal cells for estimating the amount of P available for algal growth regenerated by zooplankton. We used this method to assess P-release from zooplankton feeding on P-limited and P-saturated algae. The release of P by zooplankton depends both on food quantity and quality. Owing to the stable elemental ratio in most zooplankton species (Andersen and Hessen, 1991), the release of P depends not only on the absolute supply of P in the food (Lehman, 1984; Lehman and Naumoski, 1985), but also on the relative content of P (C:P ratio) (Olsen *et al.*, 1986) and the composition of the zooplankton community (Hessen and Andersen, 1992). By extrapolation of regressions on P-release versus C:P ratios in phytoplankton, Olsen *et al.* (1986) estimated zero P-release by daphnids above C:P ratios of 320 in the algal cells.

Method

Free cell cultures

The green alga *Selenastrum capricornutum* NIVA CHL 1 was selected for the experiments. This species is kept in cultures at the culture collection of the Norwegian Institute for Water Research (Skulberg and Skulberg, 1990). Inoculum phytoplankton cultures were incubated in the inorganic nutrient medium Z8 20% at 20°C (Skulberg and Skulberg, 1990). Illumination was provided by cool-white fluorescent tubes at 70 μE m^{-2} s^{-1} and a 14:10 h light:dark cycle was used.

To obtain P-limited algal cells, exponentially growing cells were inoculated into flasks containing a P-free medium. The cells entered stationary phase after 5 days. A fraction of the P-limited cells was used to produce P-limited immobilized algae (algae–alginate beads).

Immobilized cells (beads)

The method used for preparing beads of immobilized algal cells was based on the procedure described by Van Donk *et al.* (1992). Sodium alginate (Sigma No. A-7128) was dissolved in warm, distilled water to form a 4% w/v solution. The solution was autoclaved for 13 min and cooled to room temperature. Selected volumes of the P-limited algal culture were centrifuged and the algal pellets were transferred into the alginate solution. Aliquots of this mixture were thoroughly stirred, transferred into a 50 ml burette and extruded dropwise into a cool (1–3°C), autoclaved solution of 0.03 M $CaCl_2$. The burette system was carefully kept full of the alginate–algae mixture to ensure a constant flow of ~5 drops s^{-1}

(5 beads s^{-1}, ~16 beads ml^{-1}). The beads, thus produced, had a homogeneous size and a normally distributed number of cells (Abdel-Hamid *et al.*, 1991). They were kept in the cold $CaCl_2$ solution for at least 30 min to allow complete hardening of the alginate, washed several times with distilled water and stored in the dark at 4°C until use. The growth experiments were performed within 2 weeks after the preparation of the beads. To determine the actual cell density in the beads, they were dissolved separately in 5% sodium hexametaphosphate and the algal cells were counted with a Coulter Multisizer after 15 min. The mean initial cell concentration chosen for the beads was 25 000 (±1150) cells $bead^{-1}$.

Growth experiments (immobilized cells and free cells)

Short-term batch culture experiments were used to study the relationship between external P concentrations and the growth rate of immobilized and free cells of *S.capricornutum*. This method was shown to be useful in previous growth kinetic experiments with free algal cells (Van Donk and Kilham, 1990). When low cell densities are incubated in large volumes for relatively short duration, the cultures approximate steady-state conditions because the algae do not significantly affect the nutrient concentration. Free cells and beads were inoculated separately into 1 l flasks containing 500 ml of medium Z8 (Skulberg and Skulberg, 1990) diluted to 20% concentration in glass-distilled water with varying concentrations of P (0.1–50 μg P l^{-1}). The initial free cell concentration was ~250 cells ml^{-1}. Eight beads per flask were inoculated at the start of the experiments. The light and temperature conditions were the same as during the P-release experiments. The low cell density and large volume allowed experiments to proceed for 3 days without the internal concentration becoming measurably reduced. The experiments were carried out in triplicate. Samples for algal counts (free and immobilized cells) were taken daily (two beads every day). Samples of free cells were preserved with Lugol's solution and counted microscopically. Least-squares regressions of ln (cells ml^{-1}) versus time (days) were computed. Mean growth rates with their 95% confidence intervals of the immobilized and free algal cells were calculated by a least-squares linear regression analysis of log-transformed data with more than one value of y per value of x (Sokal and Rohlf, 1969). Growth rate values (μ) and P concentration data (S) were fitted to the Monod relationship ($\mu = (\mu_m S)/(K_s + S)$) by an iterative non-linear regression method (Kilham, 1975).

P-release experiments

The zooplankter *Daphnia pulex* used in these experiments was taken from a laboratory monoculture, which has been growing on *S.capricornutum* for >2 years. The experiments were carried out in 1 l Erlenmeyer flasks containing 500 ml culture medium (Z8 20%) with a low P concentration (0.5 μg P l^{-1}). Before inoculation, *S.capricornutum* free cells were washed by centrifuging in the low-P medium. The P-saturated and P-limited algal cells were inoculated in different flasks (P-saturated in Experiments I and III, and P-limited in Experiments II and IV). At the start of the experiment, the C, N and P contents

of the algal cells were analysed. C and N contents were measured on a Carlo-Erba CHN 1106 elemental analyser, and P content on the total samples after peroxo-disulphate digestion. The initial free algal cell concentration was ~5 × 10^4 cells ml^{-1}. To each flask were also added six alginate beads with immobilized P-limited algae. At the start of experiments, 30 individuals of *D.pulex* of similar length [mean length 2 mm, mean weight 44 μg dry weight (DW)] were brought into flasks of Experiments I and II. Before incubation, the animals were brought in medium without algae to remove surface contamination and to allow them to empty their guts. Free cells and beads incubated together without zooplankton served as controls for P-excretion by the algae (Experiments III and IV). The release experiments lasted 3 days and were carried out in triplicate. Flasks were shaken manually four times a day. Procedures for sampling, counting and calculating growth rates were the same as described for the growth experiments.

The amount of P available for algal growth was estimated by calculating from the Monod equation, as determined in the batch growth experiments, the P concentration corresponding to the measured specific growth rate.

Results

The Monod constants (μ_m and K_s) of P-limited immobilized cells and free cells, determined in the batch growth experiments, are given in Table I. For the P-limited immobilized cells a lower initial slope of the Monod growth curve (μ_m/K_s = 0.70) was found than for the free cells (μ_m/K_s = 0.93). This difference in initial slope of the Monod growth curve is probably caused by resistance to diffusion of P into the beads, as this must diffuse through the alginate pores in order to reach the algal cells. Consequently, a P-concentration gradient is established within the pores, resulting in concentration decreasing with increasing distance (in depth) from the surface of the bead (Kennedy and Cabral, 1983).

The course of the numbers of immobilized and free cells of *S.capricornutum* in the P-release experiments (I–IV) is given in Figures 1 and 2, respectively, and the growth rates in Table II. The immobilized algae incubated together with P-saturated free cells and *D.pulex* in Experiment I experienced the highest growth rate (0.62 ± 0.02 day^{-1}). The growth rate of the immobilized cells measured in the control without *Daphnia* (Experiment III) was significantly lower (0.18 ± 0.06 day^{-1}). Also, the growth rate of the immobilized algae incubated together with P-limited free cells and *D.pulex* increased compared with the control (0.29 ± 0.09 day^{-1} in Experiment II versus 0.17 ± 0.08 day^{-1} in Experiment

Table I. The calculated Monod constants (μ_m and K_s) of immobilized and free cells of *S.capricornutum* determined under P-limitation. The 95% confidence intervals are given in parentheses

	μ_m (day^{-1})	K_s (μg P l^{-1})
Immobilized cells	0.70 (±0.02)	1.00 (±0.04)
Free cells	1.13 (±0.14)	1.22 (±0.12)

IV). The growth rates of the immobilized cells in controls III and IV did not differ significantly from the growth rate measured at 0.5 μg P l^{-1} in the batch growth experiments (μ was 0.5 day^{-1}), indicating that excretion of P from the free algal cells was insignificant. Owing to intracellular P storage, P-saturated free cells incubated without *Daphnia* showed a higher growth rate than the P-limited cells (0.52 ± 0.3 day^{-1} in III versus 0.32 ± 0.13 day^{-1} in IV).

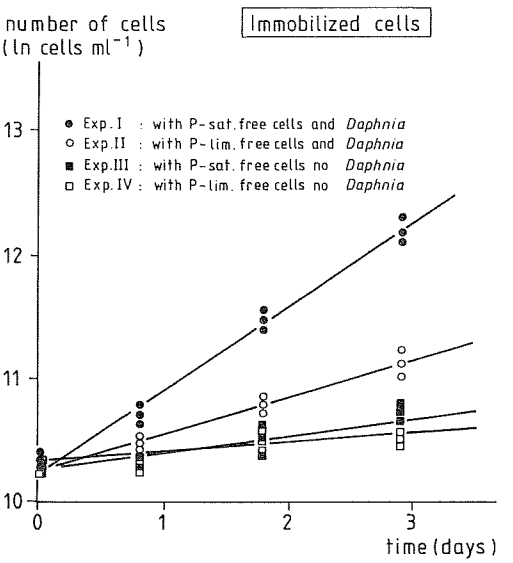

Fig. 1. The mean numbers of immobilized cells of *S.capricornutum* in the P-release experiments (I–IV) performed in triplicate (see the legend to Table II).

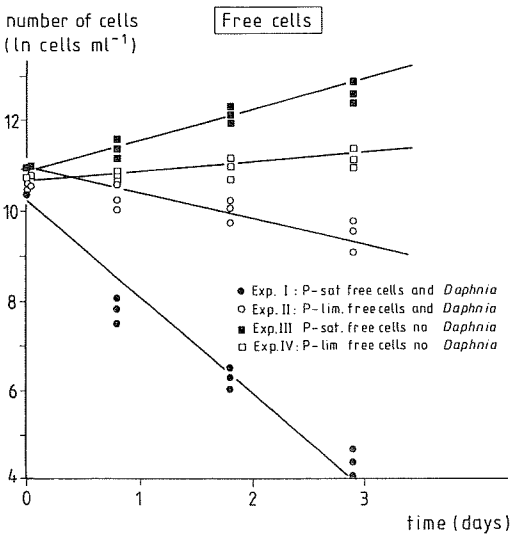

Fig. 2. The mean numbers of free cells of *S.capricornutum* in the P-release experiments (I–IV) performed in triplicate (see the legend to Table II).

Table II. Growth rates (μ day^{-1}) of the immobilized and free cells of *S.capricornutum* as determine in the P-release experiments (I–IV)

	Free cells (μ day^{-1})	Immobilized cells (μ day^{-1})	P available for alga (μg P l^{-1})
I	−2.10 (±0.07)	0.62 (±0.02)	7.75 (6.00–10.60)
II	−0.62 (±0.04)	0.29 (±0.09)	0.71 (0.40–1.18)
III	0.52 (±0.30)	0.18 (±0.06)	0.35 (0.21–0.52)
IV	0.32 (±0.13)	0.17 (±0.08)	0.32 (0.15–0.49)

Phosphorus (μg P l^{-1}) available for growth of the immobilized *S.capricornutum* in the P-releas experiments was calculated according to the Monod equation (see Table I) (the 95% confidenc intervals are given in parentheses). Experiment I, P-saturated free cells plus *D.pulex*; Experiment II P-limited free cells plus *D.pulex*; Experiment III, P-saturated free cells, no zooplankton Experiment IV, P-limited free cells, no zooplankton. P-limited immobilized algae were present in al four combinations.

Table III. The C:N:P ratio and the absolute amount of P in the P-saturated and P-limited cells o *S.capricornutum* used in the P-release Experiments I and II (see the legend to Table II). Also P ingestion rates of *D.pulex* during the three experimental days and release rates of P available fo algal growth after the first 2 days of incubation (the 95% confidence intervals are given i parentheses)

C:N:P (atomic ratio algae)	μg P (cell^{-1})	Total P ingested (μg P mg DW^{-1} h^{-1})			P available (μg P mg DW^{-1} h^{-1}) over days 1 and 2
		Day 1	Day 2	Day 3	
I 159:14:1	19×10^{-8}	0.24	0.30	0	0.06 (0.04–0.08)
II 1032:46:1	3×10^{-8}	0.02	0.01	0.006	0.003 (0.0006–0.006)

The amount of P available for growth of the immobilized *S.capricornutum* i the P-release experiments was calculated from the Monod equation (Tables and II). Subtracting the amount of P available in the controls gave the amount o P regenerated by *Daphnia* available for algal growth.

The disappearance rate of the P-limited free cells due to grazing i Experiment II was less than that of the P-saturated free cells in Experiment (−0.62 ± 0.02 day^{-1} in II versus −2.1 ± 0.07 day^{-1} in I, see Table II and Figur 2). This can be explained by the fact that P-limited *S.capricornutum* were largel intact after passing once through the gut of *Daphnia*, while P-saturated cell were efficiently assimilated (Van Donk and Hessen, 1993). After 2 days o incubation with *Daphnia*, >95% of the free cells were ingested in Experiment and >75% in Experiment II (Figures 1 and 2).

The ingestion rates of P were calculated and compared with the release rate of P available for algal growth calculated over the first 2 days of incubation. Fo the immobilized cells incubated with *Daphnia* grazing on P-saturated algal cells 20 times more P was made available than for algae incubated with *Daphnia* grazing on P-limited cells (0.06 μg P mg^{-1} Daphnia-DW h^{-1} in I versus 0.003 μg P mg^{-1} Daphnia-DW h^{-1} in II). Averaged over two days, zooplankton released to the immobilized algae ~22% of ingested P when feeding on P-saturated algae in Experiment I and ~10% when feeding on P-limited algae in Experiment II (Table III).

Discussion

The stimulated growth of the immobilized P-limited algae when incubated with free algal cells and *Daphnia* demonstrated the extent to which P released by zooplankton became available again for algal growth. Owing to the stable elemental ratio in most zooplankton species, the release of P from zooplankton may be described as a simple relationship between C:P ratios of the consumed food and that of the grazer (Hessen and Andersen, 1992). The model presented by these authors predicts release of P (R_p) per unit of ingested C (I_c) as: $R_p/I_c = (1 - K_1^*)Q_f$ for $Q_f > Q_z$ (Q_f = C:P weight ratio of food, Q_z = C:P weight ratio of zooplankton). K_1^* is the dimensionless growth efficiency of zooplankton (gross assimilated C minus respiratory loss). Based on this model, it was assumed that *Daphnia* grazing on P-limited cells would release less P than those grazing on P-saturated cells. P-release from *Daphnia* in Experiments I and II would, according to this model and the measured ingestion rates, be 0.05 and 0.006 μg P mg^{-1} Daphnia-DW h^{-1}, respectively, assuming a growth efficiency of *Daphnia* of 60% ($K_1^* = 0.6$) for both P-saturated and P-limited algae. This is in accordance with the rates based on the observed growth of immobilized algae in beads (cf. Table III). Release rates would, however, be sensitive to K_1^* and, for the P-limited algae, K_1^* would probably decrease (cf. Van Donk and Hessen, 1993), meaning that calculated R_p would be a minimum estimate. The release model of Olsen *et al.* (1986) predicts zero release above C:P atomic ratios of 320, while the model of Hessen and Andersen (1992) predicts zero P-release only when the food is totally deprived of P. In our grazing Experiments I and II, the sestonic C:P atomic ratios were 159 and 1032, respectively. The measurable release of P even from the P-limited algae (C:P = 1032) in these experiments, gives support to the view of a declining, but continuous, release even at very high sestonic C:P ratios.

A comparison of P release rates measured by different workers, summarized by Den Oude and Gulati (1988), showed more than an order of magnitude difference (0.03–1.5 μg P mg DW^{-1} h^{-1}). This span in measured release rates may by attributed to methodological problems, but also food quality (C:P ratios) and digestibility of the food particles. Some of these studies also report very low P release rates when zooplankton were fed P-limited algae (e.g. Lehman, 1980; Olsen and Østgaard, 1985). These rates give no direct information on the amount of released P available for algal growth, however, and this aspect may be investigated using immobilized, non-grazable algae. It has been shown in the present study that immobilized algae can be used successfully in laboratory experiments for the measurement of available P for algal growth. Encapsulation of the algal cells in alginate gave some resistance to diffusion of P within the bead, but comparison with growth of immobilized cells under standardized conditions allowed quantification of the available P. Further applications of immobilized algae for measurement of algal growth rates and available nutrients for algal growth under field conditions are being developed (B.A.Faafeng, personal communication).

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