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In situ measurement of algal growth potential in aquatic ecosystems by immobilized algae

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Abstract

Cells of the green alga *Selenastrum capricornutum* were immobilized in alginate beads. The alga was able to grow inside these beads without being grazed by zooplankton. For P-limited immobilized cells, however, a lower μ_m and initial slope of the Monod growth curve μ_m/K_s were found than for free cells.

To study the feasibility of immobilized algae to estimate algal growth potential *in situ* in aquatic ecosystems, a series of experiments were conducted in indoor model ecosystems (microcosms) and in a small stream. The use of immobilized algae allowed a continuous registration of algal growth potential integrated over periods with natural fluctuations in the environment. The method of encapsulation of the algae can, however, still be improved. The alginate matrix is exposed to marked degradation by microorganisms when incubated in polluted streams for a period longer than two weeks. The applicability of other types of matrices should be tested.

Introduction

Immobilized whole cells entrapped in polymeric matrices have, in the past two decades, been studied extensively for biotechnological applications (Scott, 1987). While most efforts have been directed toward bacteria (Kennedy & Cabral, 1983), attention has also been given to eukaryotic cells, including algae (Brouers *et al.*, 1989; Smidsrød & Skjåk-Braek, 1990).

The encapsulation of algal cells in a matrix may offer certain advantages when carrying out algal assays compared to batch or continuous cultures with free cells. Separation of cells and their excreted products is quick and simple, and handling of the beads with test algae is easy. During immobilization algal cells maintain their respiratory and photosynthetic activities, and this method prevents them from being washed out or grazed by herbivores. Immobilized algae can, when stored at low temperature (4 °C) in darkness, grow normally after 3 to more than 12 months after entrapment (Hertzberg & Jensen, 1989).

Practical applications of immobilized algae include until now removal of nutrients (Chevalier & de la Noüe, 1985) and heavy metals (Wilkinson *et al.*, 1990) from wastewater, and also production of secondary metabolites for industrial and pharmaceutical purposes. Further, some laboratory experiments have been conducted to test immobilized algae for use in algal toxicity assays (Bozeman *et al.*, 1989; Van Donk *et al.*, 1992). Entrapment, storage and handling of algae in alginate beads are also useful in stock culture management (Tamponnet *et al.*, 1985; Hertzberg & Jensen, 1989). Recently, laboratory experiments have demonstrated the feasibility of using immobilized algae to estimate bioavailable phosphorus released by zooplankton (Van Donk *et al.*, 1993), since these test algae were not simultaneously exposed to grazing by zooplankton.

Until now most growth potential measurements

have been carried out in the laboratory under standardized conditions with nutrient starved test algae incubated in filtrated water samples (e.g. Skulberg, 1964, 1967; Miller *et al.*, 1978). This method and similar bioassays using the natural phytoplankton population as test organisms (e.g. Løvstad 1984; Van Donk *et al.*, 1988) give only an impression of the nutrient conditions at the moment of sampling. Incubation of test algae in the continuously fluctuating natural environment (light, temperature, nutrients, toxins) is preferable, because it will give an integrated measurement over the whole test period. However, continuous *in situ* measurements of algal growth potential in natural waters is not straightforward because the algal cells will be flushed out in running waters and are removed continuously by other loss processes (sedimentation, grazing, parasitism etc.) in lakes and reservoirs.

The aim of the present study was to test the feasibility of using immobilized algae for monitoring *in situ* algal growth potential in aquatic ecosystems. A series of experiments were carried out in batch cultures, in indoor model ecosystems (microcosms) and in a small stream.

Methods

Immobilized cells (beads)

An axenic clone culture of the green alga *Selenastrum capricornutum* NIVA CHL 1, from the Culture Collection at the Norwegian Institute for Water Research (Skulberg & Skulberg, 1990), was selected for the experiments. This clone is probably the most used test alga today (Forsberg &Claesson, 1981; Nygaard *et al.*, 1986). Inoculum cultures were incubated in an inorganic medium Z8 (Skulberg & Skulberg, 1990) diluted to 20% concentration in glass-distilled water. The illumination was provided by coolwhite fluorescent tubes at $4.2 \cdot 10^{19}$ mol photons $m^{-2}\ s^{-1}$ (70 μE $m^{-2}\ s^{-1}$) operating on a 14:10 h LD cycle. To measure algal growth potential, P-limited algal cells were used, because non-limited test algae are able to use intracellularly stored phosphorus to perform high growth rates even under P-limited conditions. To obtain P-limited algae, exponentially growing cells were inoculated into flasks containing a phosphorus-free medium. The cells entered stationary phase after 5 days. The method used for preparing beads of immobilized algal cells was based on the procedure described by Van Donk *et al.* (1992). The test algae were encapsulated in these spherical alginate beads (diam. 3 mm), with Sodium alginate (Sigma No. A-7128) as matrix for the beads. The mean initial cell density was 25,000 ± 1150 (SE) cells/bead.

To determine the actual cell number per bead, each bead was dissolved in 5% sodium hexametaphosphate and the algal cells were counted with a Coulter Multisizer after 15 min.

Growth experiments in batch cultures with free and immobilized cells

Short-term laboratory experiments with batch cultures of immobilized or free cells were used to measure the maximal growth rates. We also studied the relationship between external P concentration and growth rate to compare with field measurements. Short-term batch cultures with free algal cells have been successfully used in previous growth kinetic experiments (Van Donk & Kilham, 1990). When low cell densities are inoculated in large volumes in short-term experiments, the algal cultures approximate steady-state growth conditions because the algae cannot affect the nutrient concentration to any significant degree.

Free cells and beads were inoculated separately into 1-L flasks containing 500 mL of medium Z8 (Skulberg & Skulberg, 1990) diluted to 20% concentration in glass-distilled water with varying concentrations of P (0.1–50 μg L^{-1} P). The initial free cell concentration was ca. 250 cells mL^{-1}. In the experiments with the immobilized cells, eight beads per flask were inoculated at the start of the experiments. The experiments were performed at 10 and 20 °C because incubations in the field studies were performed at approximately these temperatures. Illumination was provided by cool-white fluorescent tubes at $4.2 \cdot 10^{19}$ mol photons $m^{-2}\ s^{-1}$ operating on a 14:10 h LD cycle at 20 °C and 10:14 h LD at 10 °C. The low cell density and large volume of medium 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 of free cells (5 mL suspension) and immobilized cells (2 beads per day) were taken daily. Samples of free cells were preserved with Lugol's solution and counted microscopically. Finally, least-squares regressions of ln (cells mL^{-1}) vs. time (d) were calculated. Because measurements from replicate flasks were close compared to other sources of variation, replicates were pooled. Mean growth rates with SE of the algal cells were calculated by a least squares linear regression analysis of

ln transformed data with more than one value of y per value of x (Sokal & Rolhf, 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).

Tests in indoor microcosms

Eight microcosms were constructed in a climate room (20±1 °C). Each microcosm consisted of a glass aquarium (length 110 cm, width 110 cm, height 70 cm), filled with a 10-cm layer of lake sediment and 50 cm of overlying water. Homogenized sediment from a mesotrophic lake (sandy loam), with approximately 3% organic matter and a nitrogen and phosphorus content of 0.32 and 0.14 mg g^{-1} dry weight, respectively, was used. Evaporated water was replenished by demineralized water twice a week. Philips HPI-T 400W high pressure metal halide lamps were used to provide artificial daylight. The height of the suspended irradiance assembly were adjusted to get an average intensity of about $6 \cdot 10^{19}$ mol photons $m^{-2}\ s^{-1}$ (100 μE $m^{-2}\ s^{-1}$) at the water surface. A daily photoperiod of 14 h was maintained. In the preparatory phase of the experiment, flora and fauna (except fish) characteristic for Dutch drainage ditches, were introduced. During an acclimation period of 3 months a biocoenosis was allowed to develop in the microcosms. In this period all eight systems were interconnected by tubes and the water was circulated by means of a pump.

The eight microcosms were randomly divided into two treatment groups (four replicates each). One group of four microcosms served as controls. The second group was loaded once with 150 μg L^{-1} P and 900 μg L^{-1} N (N/P cosms).

Immobilized P-limited algae were incubated in the microcosms two weeks before and one and two weeks after the nutrient loading. The beads were put into a cage consisting of a glass petri-dish (diameter 11.6 cm) with a cover of stainless steel wire (mesh 0.7 mm). The cages were placed 5 cm under the water surface. Samples for algal counts were taken daily (2 beads every day). Maximum incubation time of the beads was seven days.

Depth integrated water samples for nutrient and chlorophyll analysis were taken from each microcosm by means of Perspex tubes (length 40 cm, diam. 7 cm) in at least five localities well distributed over each model ecosystem. In the first week after nutrient loading samples were taken every day and in the following weeks only once a week. The samples were then pooled and ammonium, nitrate/nitrite and soluble molybdate reactive phosphorus (SRP) were analyzed using a Skalar 5100 autoanalyser. Chlorophyll-*a* was measured according to the ethanol extraction method described by Moed & Hallegraeff (1978).

Tests in a small stream

The experiments were carried out at four stations in a small stream, Østernbekken, near Oslo, Norway. Station I was situated in an 'upstream' unpolluted forestry environment. The catchments between stations I and II were dominated by agriculture, whereas between st. II, III and IV resort areas with possibility of sewage leaks were found.

The algal beads were exposed to the running water in the stream by means of flow-through exposure chambers located at the four experimental stations (Fig. 1). A subflow of water from the stream was lead through the chambers via an open rain drain (U-formed crossection, diam. 10 cm). An overflow weir removed surplus water resulting in a constant flow and water level in the exposure chambers, regardless of discharge in the stream. Alginate beads with P-limited *S. capricornutum* were incubated in clear acrylic tubes (diam. 2.5 cm, length 13 cm), closed in both ends with 1 mm mesh gauze. The tubes were placed in the chambers, allowing a low, but steady flow of stream water to pass the beads. Simultaneously, beads were incubated in complete medium in Corning 600 mL acrylic tissue culture flasks also placed in the same chambers (Fig. 1). A constant water level of 1 cm above the tubes and the flasks allowed similar light exposure of the beads in both test systems. Over a period of two weeks (18–30 Sep. 1991) six beads from both the tubes and the flasks were sampled at all stations every second day. The remaining beads were washed in stream water to remove particles from the surface to avoid light reduction to the embedded algae, before continued exposure in the chamber.

Also water samples from the stream (five times at each station) were taken for N and P analysis. Unfortunately, integrated water samples for nutrient analyses were not available to compare nutrient concentrations with growth rates of the test algae in the streams. SRP and dissolved inorganic N were measured on membrane-filtered (0.45 μm) samples. P was analyzed with the molybdate-blue method while NO_3/NO_2 was determined after reduction with cadmium, diazotation

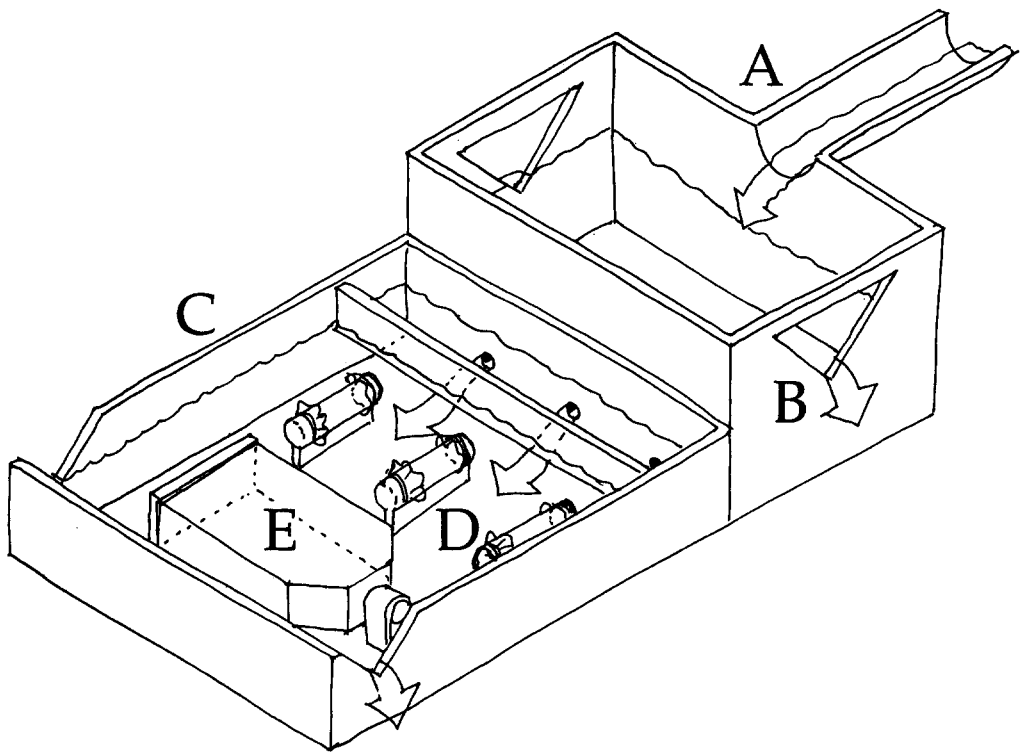

Fig. 1. Flow-through system located at the four experimental stations in Stream Østernbekken consisted of an open rain drain (A) to support a subflow of water from the steam, an overflow weir (B) and a constant water level exposure chamber (C). Alginate beads were incubated in clear acrylic tubes (D), closed in both ends with 1 mm mesh gauze, and in 600 mL acrylic tissue culture flasks with complete medium (E).

and coupling to naphtylethylene-diamine. Ammonium concentrations were negligible during the test period.

Results

Growth experiments (immobilized cells and free cells)

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 1. The maximum growth rate of the immobilized algae and free cells were significantly lower at 10 °C than at 20 °C, 20% and 40% respectively of the values measured at 20 °C. For the P-limited immobilized cells a lower μ_m and initial slope of the Monod growth curve (μ_m/K_s) were found than for the free cells. Further, a lower initial slope was measured at 10 °C than at 20 °C for both types of cultures.

Experiments in indoor microcosms

The mean concentrations of inorganic N and P during the incubation periods of the immobilized algae in the microcosms are given in Table 2. The highest concentrations were measured in the N/P cosms during the first week after the nutrient loading (week 1), however this resulted only in short-term increases in the concentrations of inorganic N and P in the water column (Fig. 2). Especially P decreased rapidly due to absorption to sediment and uptake by macrophytes. One day after nutrient additions, concentrations of inorganic N and P were reduced by more than 50% and after one week the values were close to the detection levels.

Nutrient addition, however, did not result in a significant increase in planktonic algal cells or chlorophyll-*a* concentrations in the cosms; mean chlorophyll-*a* remained below 13 $\mu g\ L^{-1}$. The growth rates of encapsulated *S. capricornutum* cells in the control cosms and the nutrient loaded cosms (N/P cosms) are given in Fig. 3. Two weeks before the addition

Table 1. Calculated Monod constants (mean μ_m and $K_s \pm$SE) of immobilized and free cells of *S. capricornutum* determined under P-limitation at 10 and 20 °C (n=3).

Cell conditions	Temp.(°C)	μ_m (day^{-1})	K_s (μg P L^{-1})	μ_s/K_s
Immobilized	10	0.14±0.03	1.12±0.04	0.13
	20	0.70±0.01	1.00±0.04	0.70
Free	10	0.45±0.05	1.04±0.04	0.43
	20	1.13±0.07	1.22±0.06	0.90

Table 2. Mean concentrations of inorganic N and SRP (μg L^{-1})±SE during the incubation periods of the immobilized algae in the microcosms.

Experimental system	Sampling week					
	−2		1		2	
	inorg. N	SRP	inorg. N	SRP	inorg. N	SRP
Control	30±17	7.9±4.9	22±5	3.7±1.5	7±5	<1±
N/P cosm	5±10	2.8±1.0	277±12	20.3±2.4	17±5	1.7±1.1

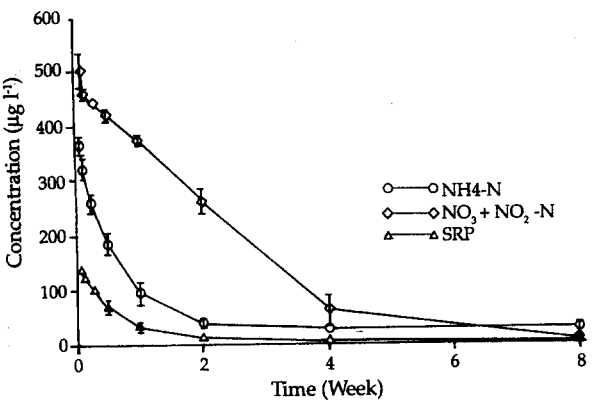

Fig. 2. Development of mean concentrations of inorganic nitrogen and phosphorus (SRP = soluble reactive phosphorus) in the four microcosms (N/P cosms) after the application of 150 μg L^{-1} P and 900 μg L^{-1} N (Bars indicate SE).

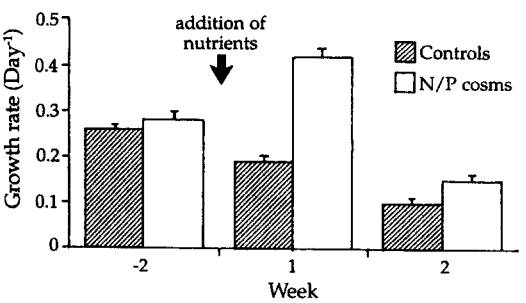

Fig. 3. Growth rates of *S. capricornutum* (mean + SE) encapsulated in alginate beads and incubated in the eight microcosms (four control cosms and four N/P cosms) before (week −2) and after the addition of nutrients (weeks 1 and 2). The arrow indicate the moment of nutrient loading.

of nutrients (week −2) rates were very similar in all cosms. During the first week after loading with N and P the rates in the nutrient loaded cosms were significantly higher (p<0.05) than in the controls, but after two weeks growth rates decreased and did not differ from the controls.

Before nutrient addition the immobilized algae demonstrated a growth rate of ca. 40% of μ_m as measured in the batch cultures of immobilized cells (Table 1). The growth rate decreased in the control cosms to 27% of μ_m in week 1 and 14% of μ_m in week 2. Only in the first week after nutrient addition in the N/P cosms, growth of the algae in the beads increased to 60% of μ_m.

Tests in small streams

At the upstream station I the mean P concentration of five samples was low, total P 2.6±0.4 μg L^{-1}. The mean total N concentration was 446±33 μg L^{-1} during

the 14 days sampling period. At the downstream stations the water quality was markedly affected by nutrient pollution giving concentrations of total P>29 μg L^{-1} and total N>1490 μg L^{-1} (Table 3). The dissolved fractions of P and N at stations II, III and IV all exceeded expected growth limiting values for the test algae.

All growth rates were calculated from day three onwards, due to a lag phase in growth response of the test algae. The growth rates of the immobilized algae incubated in complete medium were similar ($p<0.05$) (Fig. 4). The growth rates of the algae incubated in stream water were significantly lower ($p<0.05$) than in complete medium at stations I, II and III, but the rate was not significantly lower at station IV. The lowest growth potential was measured at station I (41% of the complete medium). At stations II, III and IV the growth rates were 67%, 57% and 62% of the rates in complete medium, respectively. Because light and temperature conditions were the same for the test algae incubated in complete medium and in stream water, the growth rate of the algae in stream water must be limited or inhibited by other factors. For station I phosphorus limitation is the most likely factor. According to the batch culture experiments at 10 °C in the laboratory (Table 1), immobilized *Selenastrum* may reach a growth rate of 0.049 d^{-1} at 0.6 μg SRP L^{-1}. This is only slightly higher than the rate measured in the stream at station I (0.038 d^{-1} at 8.5 °C). This may be due to the differences in temperature and light conditions between the batch cultures and the stream (μ_m is 0.14 d^{-1} at 10 °C in batch culture, Table 1: μ measured in complete medium is 0.088 d^{-1} at 8.5 °C in the stream, Fig. 4) or to slightly lower mean P-concentrations in the stream water during the incubation period than showing by the intermittent water samples. At the other stations the measured P- and N-concentrations were higher (Table 3), hence, other factors must have been responsible for the slightly lower growth potential in the stream than in the complete medium.

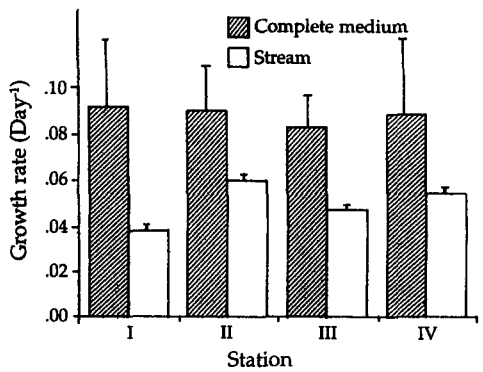

Fig. 4. Growth rates of the immobilized alga *S. capricornutum* (mean + SE) in Østernbekken at stations I–IV during 20–30 Sep. 1991 (in complete medium and in stream water).

Discussion

Fluctuations in nutrient loading were measured in all three test systems (batch cultures, microcosms, stream) by changes in growth rate of the immobilized algae. In the indoor microcosms addition of N and P only resulted in short-term increases in the concentrations of inorganic N and P in the water column. Application of immobilized algae in these systems made detection of intermittent nutrient loading possible, while chlorophyll-*a* concentrations in the cosms did not change. Probably grazing by zooplankton on the free algal cells in the cosms was responsible for the constant low chlorophyll concentration. Such pulses of nutrients to aquatic ecosystems may be realistic after heavy rainfall due to sewerage overflow and washout from arable land or due to daily changes from domestic sources. Encapsulation of algae seems suitable for the elimination of grazing (Van Donk *et al.*, 1993) and make measurement of the gross growth potential of the algae possible. Incubation of immobilized algae in the stream allowed detection of different nutrient levels at upstream and downstream locations.

Dialysis techniques with test algae have been used (e.g. Jensen *et al.*, 1972; Laake, 1978) to monitor *in situ* nutrient loading to aquatic ecosystems. Both dialysis bags and immobilized algae give an integrated measurement over the whole test period, in contrast to the widely used point measurements of algal growth potential tests (AGP-tests). The use of dialysis techniques to measure phytoplankton growth potential *in situ* may, however, be hampered by diffusion limited growth, sedimentation of algae in the dialysis bags and growth of algae and bacteria on the membranes. Advantages of immobilized algae compared to dialysis bags are: (1) easy handling of the beads, (2) good statistical treatment, (3) no sedimentation of the algae, (4) no growth of algae on membranes, (5) immobilized algae can be stored at low temperature in darkness for more than 12 months after entrapment. Both immobilized algae and dialysis bags, however, have the disadvantage of restricted nutrient diffusion (Jensen *et al.*, 1972; Van Donk *et al.*, 1993). Nutrients must diffuse through the

Table 3. Mean concentrations of nutrients ($\mu g\ L^{-1}$)±SE and mean temperature (°C)±SE measured between 20–30 Sep. 1991 at the four different stations in Østernbekken.

Station	Parameter				
	tP	SRP	tN	NO_3-N	Temp.
I	2.6±0.4	0.6±0.2	446±33	300±25	8.5±0.5
II	36.0±0.5	27.2±1.0	1552±277	1206±227	9.0±0.4
III	29.4±2.7	17.0±4.0	1490±209	1247±187	9.5±0.5
IV	32.2±4.4	21.0±1.0	1496±207	1065±308	9.7±0.5

alginate pores or the dialysis membrane to reach the algal cells. The difference in initial slope of the Monod growth curve of the free cells and the immobilized cells was probably due to physical restrictions for algal multiplication inside the alginate matrix (Hertzberg & Jensen, 1989). If algae are allowed to grow to high densities in the beads, resistance to diffusion of P into the beads is also possible as the diffusion rate through the alginate pores may restrict nutrition of the inner algal cells in the beads (Kennedy & Cabral, 1983). As the beads used had a small diameter (3 mm) and included only a low concentration of algal cells, diffusion limited growth of the algal cells in the beads may be minor.

The method of encapsulation of the algae can still be developed further and improved. Different species of algae with different physiological characteristics may be encapsulated in tests beads for evaluation of environmental conditions. Also biotesting in the marine environment may be feasible. Our experiments demonstrated that the alginate matrix is exposed to degradation by microorganisms when incubated in polluted streams for a period longer than two weeks. Application of other types of alginates may result in a higher stability and permeability of the matrix (Martinsen *et al.*, 1989).

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