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Cell-specific detection of phosphorus stress in *Trichodesmium* from the Western North Atlantic

Abstract—The low phosphorus concentrations observed in the western North Atlantic and the western Central Atlantic suggest that phosphorus bioavailability may limit *Trichodesmium* productivity and N_2 fixation. However, the degree to which the concentration and composition of the total phosphorus pool affects actual bioavailability is poorly understood. To better examine how phosphorus bioavailability may constrain primary production and marine N_2 fixation, we have developed a molecular diagnostic tool for quickly detecting the phosphorus status of the diazotroph *Trichodesmium.* This diagnostic method uses a commercially available substrate to fluorescently tag cells expressing the phosphate-regulated enzyme, alkaline phosphatase. Using this diagnostic tool, we were able to distinguish phosphorus-replete from phosphorus-stressed *Trichodesmium* populations along a transect from Grand Bahama Island to Bermuda in November 2000.

The biogeochemical cycles of phosphorus (P) and other biolimiting elements are tightly linked to marine primary production. In particular, the degree to which P may limit marine primary production and concomitant carbon export has been extensively debated. Over geologic time, biological fixation of atmospheric N_2, which has no P counterpart, should eventually force marine systems into a state of P limitation (Redfield 1958). This contradicts the traditional views of many researchers, developed from nutrient addition experiments, that nitrogen (N) or iron (Fe) can limit phytoplankton biomass at a given point in space and time (Ryther and Dunstan 1971; Martin et al. 1994).

In the western North Atlantic, it has been suggested that the Fe supply may control N versus P limitation through modulation of N_2 fixation by the diazotrophic cyanobacterium *Trichodesmium.* This is thought to be due to the relatively high Fe concentrations required by *Trichodesmium* for N_2 fixation (Wu et al. 2000; Berman-Frank et al. 2001). However, recent work in the western Central Atlantic has indicated that reduced rates of N_2 fixation by *Trichodesmium* are not affected by Fe inputs but rather correlate with P limitation (Sanudo-Wilhelmy et al. 2001). Fitting with this observation is the finding that the P concentration is exceedingly low in both areas of the Atlantic relative to other oligotrophic regimes, such as the North Pacific. For example, mean dissolved inorganic phosphate (DIP) concentrations in the western North Atlantic are $<10^{-9}$ mol L^{-1}, whereas in the North Pacific DIP is typically $>10^{-8}$ mol L^{-1} (Wu et al. 2000). Although these chemical concentrations suggest that P is likely to limit productivity in the western North Atlantic, the geochemistry of P is complex, and the degree to which P is bioavailable for phytoplankton growth depends on its speciation. In the surface of oligotrophic waters, organic P (DOP) often comprises a significant proportion of the total P (Jackson and Williams 1985; Orrett and Karl 1987; Karl 1999; Wu et al. 2000). Although DIP is directly available to phytoplankton like cyanobacteria, the degree to which the complex constituents of the DOP pool are bioavailable is poorly understood, especially for different species in diverse field populations (Cembella et al. 1984*a*,*b*). A way to circumvent these interpretive challenges is to directly assay the P status of phytoplankton in a manner that is sensitive to both the DIP and DOP supply.

A common approach to studying P physiology in phytoplankton is to monitor alkaline phosphatase (PhoA) activity (discussed in Cembella et al. 1984*a*). This scavenging enzyme is typically induced to allow cells to acquire inorganic phosphate from different organic P sources in the water column (Kuenzler and Perras 1965). In organisms in which PhoA activity has been studied in detail, it has been shown to respond to intracellular inorganic phosphate pools (e.g., Wanner 1996). Thus the expression and activity of the enzyme is sensitive to both bioavailable DIP and DOP. Herein we have applied a technique for detecting in situ PhoA activity in *Trichodesmium* that uses enzyme-labeled fluorescence (ELF) (González-Gil et al. 1998). With this diagnostic tool we were able to specifically identify P stress in *Trichodesmium* from the western North Atlantic.

Methods—Axenic cultures of *Trichodesmium erythraeum* IMS 101 and *T. thiebautii* were grown as described elsewhere in P-replete medium (8×10^{-6} mol L^{-1}) (Webb et al. 2001). P stress was induced by inoculating *Trichodesmium* colonies into media where P had been omitted. The cultures were harvested when the biomass was reduced compared with that of the P-augmented control, the timing of which depended on inoculum (typically 6–10 d). Field populations and chemical data were collected during a cruise to the western North Atlantic in November of 2000 (*see Table 1 for coordinates*). Colonies were obtained from surface water by towing a 130.0-μm net. Isolated colonies were washed twice in 0.2-μm filter-sterilized seawater and gently filtered onto 5.0-μm polycarbonate filters for storage at -20°C prior to analyses.

To assay for P stress, *Trichodesmium* colonies were isolated from field samples or cultures and transferred to Poly-L Lysine microscopy slides. The colonies were incubated with 70% ethanol for 30 min at 4°C and then rinsed in *Trichodesmium* three-quarters–strength seawater base medium

Table 1. Coordinates, water temperature, and DIP concentration for surface stations along a November 2000 Western North Atlantic transect from the Gulf Stream to offshore.

Station	Coordinates	Temperature (°C)	DIP ($\times 10^{-9}$ mol L^{-1} PO_4^{-3})
1	28°18′N, 79°30′W	29.0	12.1
2	29°32′N, 75°02′W	24.3	7.5
3	29°38′N, 74°47′W	23.7	7.9
4	30°25′N, 71°43′W	24.1	5.0
5	30°32′N, 71°24′W	24.1	7.5

(1.5×10^{-6} M ethylene diaminetetraacetic acid, 5×10^{-8} M Fe (ferric citrate), 10^{-7} M $MnSO_4$, 10^{-8} M $ZnCl_2$, 10^{-8} M $NaMoO_4$, 10^{-10} M $CoCl_2$, 10^{-10} M $NiCl_2$, 10^{-10} M $NaSeO_3$, and 1.5 μg L^{-1} vitamin B_{12}). The slides were subsequently incubated for 1 h with a 1:20 dilution of ELF reagent from the ELF Phosphatase Detection Kit (E-6601; Molecular Probes) and then rinsed once again in media. Cover slips were fixed to the slides with mounting media provided with the ELF Phosphatase Detection Kit. *Trichodesmium* colonies and filaments were visualized with a Zeiss Axioplan2 microscope. The ELF product and phycoerythrin fluorescence were detected with a 4,6-damidino-2-phenylindole (DAPI) long-pass and Rhodamine filter sets, respectively. Digital images were captured with a Zeiss Axiocam and Openlab software. All settings, including exposure and gain, were held constant in order to adequately compare samples.

We have recently improved the ELF labeling procedure described above. Although this procedure was not used during the course of the present study, we have used it for subsequent work. In this improved method, we collect and wash the colonies as above and then place two colonies into 100 μl of sterile seawater combined with 5 μl of the ELF reagent. This sample is allowed to react for 45 min in the dark at room temperature. After this incubation, the colonies are drawn onto a 25-mm 5-μm polycarbonate filter and washed twice with sterile seawater. The filter is then removed and incubated in 1 ml of histology-grade 10% neutral buffered formalin (Surgipath) for 45 min in the dark at room temperature. The resulting suspension of colonies in formalin is then drawn onto a second 25-mm 5-μm polycarbonate filter. Last, the filter is mounted onto glass slides with the mounting media provided in the ELF Phosphatase Detection Kit. This preparation can be imaged as detailed above, and the samples are stable for several months when kept in a damp container at 4°C.

Water samples were analyzed for DIP content. Samples were passed through acid-cleaned 0.4-μm polycarbonate filters and collected in 50 ml polypropylene, screw-cap tubes that were cleaned with trace metal–grade 10% HCl prior to use. All water samples were stored frozen at −20°C prior to analyses. DIP was measured by Susan Becker and Douglas Masten at the Ocean Data Facility of the Scripps Institution of Oceanography using a standard protocol for the Skalar San Plus autoanalyzer with a detection limit of $\sim 3 \times 10^{-9}$ mol L^{-1}.

Results and discussion—Bulk PhoA activity has been shown to be a good indicator of P physiology in *Trichodesmium* cultures and field populations (Stihl et al. 2001). In our study, cell-specific P stress was detected using a modification of the ELF labeling protocols described elsewhere (González-Gil et al. 1998; Dyhrman and Palenik 1999). Parallel cultures of axenic *T. erythraeum* IMS101 were inoculated from the same starter culture into medium with P (P-replete) and without added P (P-stressed). P-stressed colonies can be easily distinguished from P-replete colonies on the basis of the presence of inducible PhoA activity. PhoA-producing cells cleave the ELF reagent, generating an insoluble fluorescent precipitate that tags the P-stressed colonies at the site of the activity (Fig. 1A). Similar results were obtained with *Trichodesmium thiebautii* (data not shown). Despite the fact that previous researchers have detected low-level PhoA activity in P-replete cultures of *Trichodesmium* using a bulk p-NPP–based colorimetric assay (Stihl et al. 2001), we were unable to detect this activity using the ELF method. Although it has been well documented that the signal molecule for the P regulon in bacteria is the available pool of internal inorganic phosphate (P_i) (Wanner 1996), we sought to verify the assumption that high concentrations of water column DOP would also repress the synthesis of the P_i regulated PhoA. Using a protocol similar to the P omission experiment described above, cells were stressed for P (expressing PhoA) and then supplemented with 20.0×10^{-6} mol L^{-1} glucose-6-phosphate. Within approximately three doublings of *Trichodesmium*, the PhoA activity, as assessed by ELF labeling, was undetectable in cultures supplemented with G-6-P (data not shown). PhoA activity responds to both bioavailable DOP and DIP; therefore, ELF labeling of *Trichodesmium* can quickly and directly indicate the physiological need of the cell for P in any oceanic regime.

During a November 2000 cruise in the western North Atlantic (Table 1), clear P stress was demonstrated in *Trichodesmium* samples from four of five stations (Fig. 1C). Station 1 was in the Gulf Stream, as based on higher temperatures and the set of the current, whereas the other stations (2–5) were colder and more oligotrophic (Table 1). *Trichodesmium* colonies isolated from Sta. 1 showed no P stress (lack of ELF labeling), whereas colonies from subsequent stations were clearly P-stressed (ELF-labeled) (Fig. 1C). The morphology and location of the ELF product was similar in cells from the field and culture controls. The shift in *Trichodesmium* P physiology between Sta. 1 and 2 was accompanied by a decrease in water column DIP concentration (Table 1). Although this physiological difference was observed over a relatively small change in DIP concentration ($12.1–7.9 \times 10^{-9}$ mol L^{-1}), these numbers do not represent the *Trichodesmium* P stress threshold, because PhoA activity also responds to DOP, a measure we were unable to obtain in this study. Last, the lack of *Trichodesmium* ELF labeling at Sta. 1 demonstrates the in situ repression of PhoA and indicates that *Trichodesmium*-specific PhoA activity is not constitutively expressed in field populations as an adaptation to low environmental P.

Detailed investigation of the samples from Sta. 1 showed that within the same environment the physiological status of different phytoplankton species could be resolved using the

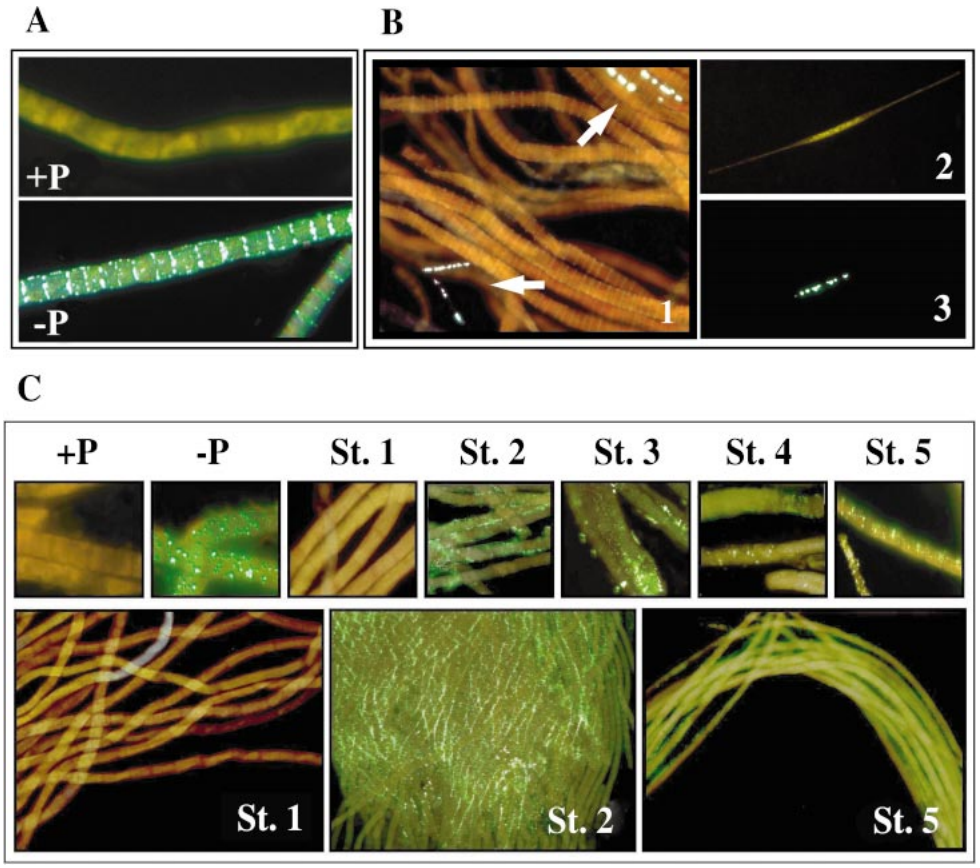

Fig. 1. (A) ELF-assayed phosphorus-replete (+P) and phosphorus-stressed (−P) *T. erythraeum* controls imaged with a DAPI long-pass filter set. Phycoerythrin autofluorescence is visible in the +P cells, whereas the green fluorescent product is PhoA-cleaved ELF. (B) Analyses of the specificity of the ELF reaction. (1) P-replete *Trichodesmium* samples (not ELF-labeled) from Sta. 1 associated with *Plectonema* sp. (arrows) that do have PhoA activity (ELF-labeled). (2) Phycoerythrin fluorescence in *Plectonema* from Sta. 1 observed by use of the Rhodamine filter set. (3) The same cell when viewed with a DAPI long-pass filter set to detect the fluorescent ELF product. (C) Culture controls and field populations of *Trichodesmium* from the western North Atlantic assayed for PhoA activity with ELF. Thumbnail images (top panel) of ELF-assayed trichomes are from +P and −P culture controls as well as stations (1–5) along a November 2000 cruise track. P-stressed cells are evident due to the PhoA-generated green fluorescent ELF precipitate. Phycoerythrin autofluorescence is also visible. In the bottom panel, larger images of representative colonies are shown for Sta. 1 (no ELF labeling), 2 (ELF-labeled), and 5 (ELF-labeled), respectively.

ELF method. Although we hand-picked and carefully washed each *Trichodesmium* colony, we were unable to completely eliminate contaminating organisms. At Sta. 1, the *Trichodesmium* trichomes were not P-stressed (not labeled), yet clear labeling of several contaminating *Plectonema* sp. could be seen (Fig. 1B). This further demonstrates the utility of the ELF labeling protocol and emphasizes that cyanobacteria from different genera can respond very differently to the same external P environment. In this instance, it is clear that measurements of PhoA activity on the *Trichodesmium* colony in bulk, using common colorimetric (Stihl et al. 2001) or fluorometric (Perry 1972) techniques, could be misleading. Herein the high PhoA activity in *Plectonema* would be detected and erroneously attributed to *Trichodesmium*.

Like other molecular methods that probe in situ phytoplankton physiology (González-Gil et al. 1998; Scanlan and Wilson 1999; Dyhrman and Palenik 2001), our technique was developed to adequately diagnose nutrient limitation or stress in field populations by directly assessing a cell's physiological condition. Our diagnostic tool avoids many of the complications of nutrient addition experiments (i.e., grazing) and cellular quotas (i.e., contaminating organisms and detritus). Moreover, it can be used to directly assess the bioavailability of P to different *Trichodesmium* species. Such molecular diagnostics of physiological condition can provide near-real-time information about the physiological status of different species and thus represent a powerful new approach to studying nutrient limitation in complex marine systems (reviewed in Scanlan and Wilson 1999). This ELF labeling detection of *Trichodesmium* PhoA activity is specific to P stress, easy to implement in shipboard laboratories, and uses commercially available reagents. Furthermore, it allows increased resolution over the common bulk PhoA measurements that are often performed to assess P stress. We describe above two proce-

dures for ELF labeling *Trichodesmium.* We would recommend the latter procedure; it uses a formalin fixation step that best maintains the *Trichodesmium* morphology without losing the ELF signal. *Trichodesmium* samples labeled in this manner are stable for many months. The limitation of the assay is that it is qualitative. Samples are categorized as stressed or not on the basis of their fluorescence, without regard to relative intensity. At this juncture, ELF labeling is indicative of the P stress experienced by P-starved *Trichodesmium*; further work with culture controls is needed to determine to what extent ELF labeling indicates P limitation of CO_2 and N_2 fixation.

Using ELF labeling, we have precisely demonstrated that *Trichodesmium* field samples from the oligotrophic western North Atlantic are indeed stressed for P relative to samples obtained from the Gulf Stream, where there are higher DIP concentrations. To our knowledge, this work represents the first implementation of a cell-specific diagnostic to assess nutrient stress in *Trichodesmium*, an organism that is critically important to marine production and biogeochemical cycling (Capone et al. 1997; Falkowski 1997). This diagnostic tool can monitor the impact of P bioavailability on the physiological condition of *Trichodesmium* in diverse field environments where there may be considerable spatial and temporal variability in P supply. For example, chemical and biological observations from the Pacific (Falkowski et al. 1998; Letelier and Karl 1998; Karl 1999) suggest that P bioavailability in this region may influence *Trichodesmium* abundances and N_2 fixation rates. The systematic application of this molecular diagnostic, in concert with rigorous chemical analyses, could provide an invaluable link between chemical cycling and *Trichodesmium* physiology, in this and more poorly studied systems.

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Effects of bioturbation on recruitment of algal cells from the ‘‘seed bank’’ of lake sediments

Abstract—Effects of different bioturbators on recruitment of several nuisance algae, *Anabaena* spp. (Cyanophyta), *Microcystis* spp. (Cyanophyta), and *Gonyostomum semen* (Raphidophyta), from sediment to water were studied in a long-term laboratory experiment. Natural sediment, where macrofauna larger than 1 mm had been removed, was added to 18 aquaria. To each of six aquaria, individuals of *Asellus aquaticus* (Isopoda) or *Chironomus plumosus* (Arthropoda) larvae were added, and six aquaria were left as bioturbation-free controls. Recruitment of *Anabaena, Microcystis,* and *G. semen* from the sediment was detected using inverted traps that were sampled once a week during 8 weeks. The activities of the isopod *A. aquaticus* increased recruitment rates of all algal groups investigated, whereas chironomids had a less pronounced effect. With respect to *Anabaena,* increased recruitment rate was expressed as a promotion of growth in the pelagic habitat. To our knowledge, these results are the first to demonstrate that bioturbating invertebrates affect the recruitment of phytoplankton resting stages. Moreover, our results suggest that recruitment rate might be more pronounced in littoral areas, which are often dominated by *A. aquaticus,* rather than in profundal areas of a lake, generally dominated by chironomids. Hence, with respect to algal dynamics, the strength of the coupling between the benthic and pelagic zones might vary both spatially and temporally, depending on composition of the benthic invertebrate community and the ontogenetic development of the individuals within it.

Through eutrophication and acidification, humans have negatively altered aquatic community composition; one such effect is an increase in prevalence of nuisance algae. High nutrient loads from anthropogenic activities to lakes and coastal waters have increased the stock of algae and caused noxious and toxic blooms of *Anabaena* spp. and *Microcystis* spp. (Cyanophyta). Acidification and its secondary effects have led to the spread, and sometimes complete dominance, of *Gonyostomum semen* (Raphidophyta) (Cronberg et al. 1988; Lepistö et al. 1994; Hansson 1996). High concentrations of *G. semen* produce itching and allergic reactions, resulting in restrictions on recreation and swimming in lakes.

Both *Anabaena* and *G. semen* can form morphologically distinguishable resting stages, whereas *Microcystis* can produce colonies that are temporarily inactive in the sediment. A resting strategy is adopted by many organisms (e.g., as a way to avoid times of harsh environmental conditions, Fryxell 1983; Lampert 1995; Boero et al. 1996; Marcus and Boero 1998). Because resting stages can remain viable for a long time—from years to centuries—in the sediment (Stockner and Lund 1970; Livingstone and Jaworski 1980; Hairston et al. 1995), they can constitute a ‘‘seed bank’’ for later outbreaks. In lakes, these seed banks accumulate mostly in the deeper portions, where sedimentation rate is high (Head et al. 1998; Baker 1999). Recruitment from resting stages occurs in response to a stimulus, such as altered temperature (Fryxell 1983; Huber 1985; Rengefors et al 1998), light conditions (Fryxell 1983; Huber 1985; Hansson 1993; van Dok and Hart 1997), nutrient concentrations (Fryxell 1983; Huber 1985; van Dok and Hart 1997), or oxygen concentrations (Hansson 1993). Several observations have been made where the germination of resting stages has provided the first propagules for development of new plankton populations (Anderson and Wall 1978; Preston et al. 1980; Baker 1999; Head et al. 1999) and where recruited resting stages have served as major contributors to growing plankton populations (Barbiero and Welch 1992; Forsell and Pettersson 1995; Perakis et al. 1996). Hence, recruitment from resting stages might be an important process for algal population dynamics.

Although resting stages can have a lower density than sand and clay particles (Anderson et al. 1982), they are often found deep in the sediment. Stockner and Lund (1970) observed that the depth at which viable resting stages could be found was partially correlated with the biomass of burrowing invertebrates, which implies that invertebrate bioturbating activities could affect the vertical distribution of resting stages. Several studies have confirmed that material, including resting stages, can be transported both upward and downward as a consequence of burrowing and feeding activities (Jónasson 1972; Marcus and Schmidt-Gengenbach 1986; Fu-

kuhara 1987; Kearns et al. 1996). The directionality can vary, depending on the invertebrate involved; for example, tubificids (Tubificidae, Oligochaeta) feed head down and excrete at the sediment–water interface, whereas chironomids (Chironomidae, Arthropoda) feed and excrete in the opposite direction (Jónasson 1972; McCall and Tevesz 1982). Another effect of bioturbation is oxygenation of the sediment (Granéli 1979; Fukuhara 1987; Hansen et al. 1998), which enhances mineralization processes, by which nutrients (mainly phosphate and ammonia) are released to the overlying waters (Fukuhara and Sakamoto 1987; Hansen et al. 1998).

An outcome from the above-mentioned findings is that benthic invertebrates can generate suitable or obstructive conditions for the recruitment of resting stages. Benthic invertebrates can affect algal recruitment rates positively by moving the resting stages to the surface, and thereby exposing them to germination cues in the water, or by increasing the nutrient concentrations in the water and thereby improving the environmental conditions for subsequent rapid proliferation. On the other hand, benthic invertebrates might have a negative effect on the number of algal recruits by destroying them through digestion or by moving them to deeper layers through defecation or burrowing activities. We investigated whether bioturbation affects the recruitment rate of certain algae by performing a long-term laboratory study using sediment that has an abundant supply of resting stages of *Anabaena, Microcystis,* and *G. semen.* We chose *Chironomus plumosus* (Arthropoda) and *Asellus aquaticus* (Isopoda) as representatives of common bioturbating invertebrates in profundal and littoral sediments (Jónasson 1972; McCall and Tevesz 1982), respectively. Perhaps more important, they affect the sediment mechanically in completely different ways: *C. plumosus* digs and filters vertically (Jónasson 1972; McCall and Tevesz 1982), whereas *A. aquaticus* migrates mainly horizontally. Thus, their effect on resting stages could be hypothesized to differ considerably.

Materials and methods—Water and sediment were collected from Lake Dagstorpssjön (55°52′N, 13°32′E) in southern Sweden in the beginning of April. This lake is known to have high abundances of algal resting stages of *Anabaena, Microcystis,* and *Gonyostomum semen* (Hansson 2000). Sediment was collected with an Ekman grab at a water depth of 3 m. Excessive water was removed and the remaining sediment was kept in a bucket with a lid to avoid contact with air and light. From the same site, 200 L of lake surface water was collected. Individuals of *A. aquaticus* were collected in the nearby Lake Krankesjön (55°42′N, 13°28′E) by shaking *Chara* sp. above a sieve. *Chironomus plumosus* larvae were gathered from the nearby Lake Krageholmssjön (55°29′N, 13°45′E) and from a pond close to Lund by taking sediment with an Ekman grab and sieving the sediment (mesh size 1 mm).

All sediment was sieved (mesh size 1 mm) in the lab under low light (1 μmol m^{-2} s^{-1}) and at low temperature (10°C) to remove the macrofauna. Approximately 5 liters of sieved sediment (grain size $<$ 1 mm) was put into each of 18 aquaria (315 mm long $\times$ 200 mm wide $\times$ 200 mm high) to a depth of 50 mm. Approximately 8 liters of filtered in situ water (filter size 10 μm) mixed with copper-free tap water was added to fill the aquaria. The aquaria were left to allow sediment particles to settle in low light and at increasing air temperature (from 10 to 16°C) for 11 d, after which the light was intensified to 100 μmol m^{-2} s^{-1} at a 16 : 8 h light : dark cycle, and the water was oxygenated with compressed air.

The experimental design included *A. aquaticus, C. plumosus,* and controls (no invertebrates), each in six replicates. To six aquaria, we added 20 *A. aquaticus* of different sizes, and to six other aquaria, we added 20 *C. plumosus* larvae (animal abundances corresponded to 300 individuals m^{-2}, which is a typical natural density). During the entire experiment, when dead animals were observed on the sediment surface, they were carefully removed with a pair of tweezers and replaced with new organisms without disturbing the sediment. Likewise, hatched animals were replaced with new ones when skins from larvae were detected in the aquaria. However, no replacements of *A. aquaticus* were made after 4 weeks because of natural reproduction; no replacement of chironomids could be made during the last week because of a lack of spare organisms. To evaluate whether the *C. plumosus* larvae were alive, the holes that appeared newly made in the sediment were counted.

Before starting the experiment, an integrated sample of the water column was taken from the surface to the bottom of the tanks by pooling four samples obtained with a tube sampler (130 mm long $\times$ 30 mm diameter). Some of the water was preserved with Lugol's solution for analysis of the phytoplankton contents, and some was filtered through a Whatman GF/C filter and put into acid-rinsed bottles for analyses of dissolved nitrogen and phosphorus (NH_4^+, NO_3^- and PO_4^{3-}). The remaining water was put into acid-rinsed bottles for analyses of total nitrogen (TN) and phosphorus (TP).

Once a week, in order to collect recruited algae, an inverted trap was put into each aquarium, where it was left hanging approximately 10 mm from the sediment surface for 48 h. Different sampling locations were used every sampling date. The traps consist of a glass container (40 ml volume) with a funnel (55 mm diameter) through the lid (modified from Hansson et al. 1994). Before lowering the traps into the aquaria, they were filled with copper-free tap water to avoid contamination with phytoplankton from the water column. The surface temperatures of the aquaria were measured with a thermometer ($\pm$0.1°C) when the traps were in the water. After 48 h, the traps were removed and the samples were preserved with Lugol's solution. Phytoplankton samples were also taken by means of pooling three random tube samples (as described above) from surface to bottom of the aquaria. After each sampling, the aquaria were carefully topped up with copper-free tap water without disturbing the sediment. At the end of the experiment, we also took an integrated water sample, with the sample technique described above, for analyses of TN, TP, and dissolved nitrogen and phosphorus (NH_4^+, NO_3^-, PO_4^{3-}) and for turbidity and pH measurements. Turbidity was analyzed on a turbidometer (Hach 1860), and pH was determined using a pH electrode (Philips PW9460). Oxygen concentrations and saturation values were also determined with an oxygen electrode (Oxy Guard Handy Gamma). The depth ($\pm$0.5 mm) of

the darker organic sediment layer was measured by placing a ruler on the outside of the transparent aquarium walls.

After 8 weeks, the experiment was terminated and the aquaria were emptied of water and the sediment was sieved (mesh size 1 mm) to evaluate the final number of active animals in each aquarium. Dry weights of the animals were determined using a microbalance after drying at 70°C for 24 h.

Organisms from the traps and from the phytoplankton samples were counted using an inverted microscope. A minimum of 100 organisms or 20 fields were counted at ×100 magnification. *Anabaena, Microcystis,* and *G. semen* were counted for all dates, as were the major zooplankton species—cladocerans, copepods, and *Asplanchna* sp. (Rotatoria).

Total nutrients were analyzed using a Technicon Auto Analyzer, whereas dissolved nutrients were analyzed by flow injection analysis (FIA).

All statistics were made using SPSS 10 software with $\alpha = 0.05$. Treatment effects were tested using analysis of variance (one-way and repeated-measures) after checking for normality or equal variances. Where necessary, data were log- or square root–transformed. If the normality and equal variance assumptions were still not fulfilled, treatment effects were analyzed with Kruskal-Wallis one-way analysis of variance. Post hoc analyses were done using Bonferroni or Mann–Whitney *U*-tests. Correlation analysis between recruitment and abundance of *Anabaena* was made by Pearson's correlation test after checking for normality.

Results and discussion—In the *A. aquaticus* treatment, recruitment to the traps and abundance in the water of *Anabaena, Microcystis,* and *G. semen* increased in the beginning of the experiment and then decreased, whereas zooplankton recruitment and abundance increased continuously (Fig. 1). On the other hand, recruitment to the traps and abundance in the water of *Anabaena* in the *C. plumosus* treatment and in the control were almost absent, whereas some recruitment and abundance could be detected for both *Microcystis* and *G. semen* in these treatments (Fig. 1). As in the *A. aquaticus* treatment, there was a continuous increase of zooplankton recruitment to the traps and abundance in the water in both the *C. plumosus* treatment and in the control (Fig. 1). Both recruitment from the sediment to the traps and abundance in the water of *Anabaena, Microcystis,* and *G. semen* were significantly increased by bioturbation, with the most pronounced effect in the *A. aquaticus* treatment (Table 1; Fig. 1). Zooplankton recruitment to the traps was also significantly increased in the *A. aquaticus* treatment (Table 1), evidenced mainly as a small, but patchy, increase in the number of *Asplanchna* individuals. Cladocerans and copepods made up the largest part of the zooplankton fraction, though, and neither of these groups showed any increase in either recruitment or abundance due to bioturbation (not significant, repeated-measures ANOVA). Total zooplankton abundance in the water did not show any treatment influence either (Table 1; Fig. 1).

Abundances of *Anabaena, Microcystis, G. semen,* and total zooplankton in the water generally followed their recruitment patterns, especially with respect to *Anabaena* in the *A. aquaticus* treatment (Fig. 1, 2). Abundance of *Anabaena* was positively correlated with the recruitment of *Anabaena* after a time lag of 1 d ($r = 0.832$; $t = 10.2$; $p < 0.001$; $n = 48$). However, *G. semen* and *Microcystis* were detected in the water column at the pretreatment sampling (Fig. 1). This can be explained by their buoyant capacities: *G. semen* has a flagellum and *Microcystis* has gas vacuoles. These devices counteract sedimentation and were probably activated when the water was added and the sediment left to settle.

At the end of the experiment, concentrations of nitrogen and TP in the water were significantly higher in the *A. aquaticus* treatment (Table 2). Overall, nutrient concentrations decreased from the start and throughout the experiment, but to a lesser extent in the *A. aquaticus* treatment, and ammonium concentrations increased from 71.3 to 90.5 μg L^{-1} in the *A. aquaticus* treatment.

We observed, at the end of the experiment, that particles from the sediment surface had been mechanically transported by bioturbation from *A. aquaticus.* This contributed to a significant increase in turbidity and depth of the organic layer (Table 3).

Compared with the control, oxygen concentrations at the end of the experiment were lower in both the *A. aquaticus* and the *C. plumosus* treatments, a difference that could not be detected midway through the experiment (Table 3).

There was a small but significant difference in pH values at the end of the experiment, ranging from 6.8 in the *C. plumosus* treatment to 7.3 in the control. However, they were not considered to be of relevance because daily oscillations of this magnitude are common in natural systems. There was a minor increase in temperature from 15.0°C at the start to 16.6°C at the end, although no difference was detected between treatments during the whole period (Table 3).

The dry weight of benthic macrofauna in the different treatments changed during the experiment (Table 4). *A. aquaticus* had increased from 0.1 to 2.9 g m^{-2} dry weight, whereas the numbers of chironomids had decreased by almost 50% because of hatching during the last week of experiment (Table 4). In all aquaria, some new organisms of macrofauna (oligochaetes and small chironomids) were detected at the end of the experiment, probably as a result of hatching from eggs in the sediment, although their numbers and dry weights were low (*see Control,* Table 4).

Anthropogenic influences often disturb environments and alter species compositions. For example, blooms of nuisance algae are common today in both lakes and coastal waters, forcing many governments and local authorities to struggle to reduce them. Because many nuisance algae produce resting stages, knowledge of how to manage these may be an important tool for managing algal nuisance blooms in general. Resting stages are the source of subsequent algal recruits, and the process of recruitment might be affected by invertebrate bioturbation. Invertebrate digging and grazing can result in the vertical and horizontal redistribution of resting stages; resting stages can be resuspended to the sediment surface and thus reexposed to oxygen and light. Also, bioturbation can promote mineralization, thereby increasing exposure of resting stages to factors, such as oxygen and nutrients, that induce their germination and recruitment (Huber 1985; Hansson 1993; van Dok and Hart 1997). Our results

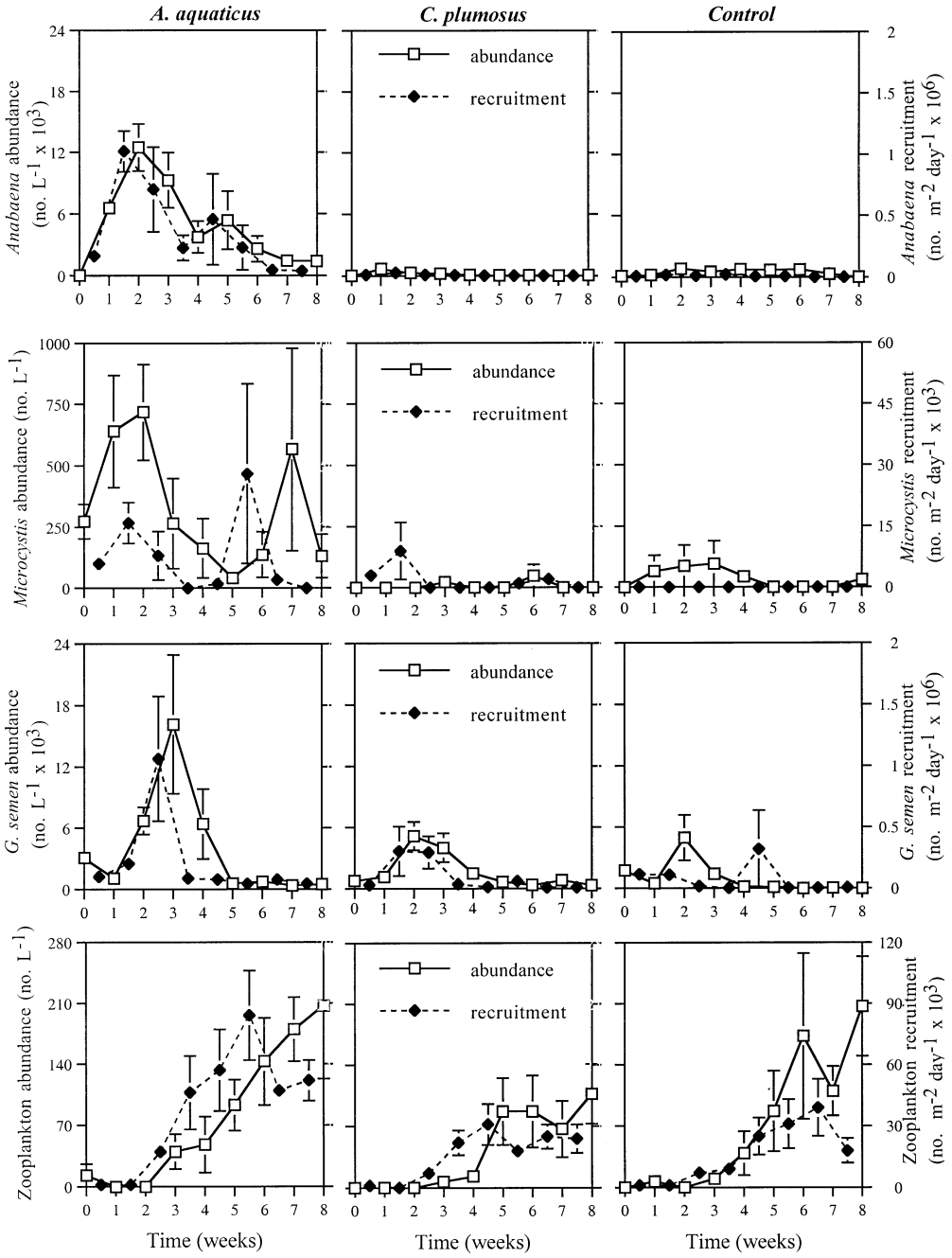

Fig. 1. Abundance (individuals L^{-1}) and recruitment (individuals m^{-2} d^{-1}) of *Anabaena*, *Microcystis*, *G. semen*, and major zooplankton groups (cladocerans, copepods, and *Asplanchna*) during 8 weeks of sampling, including one pretreatment sampling (week 0), in different treatments (*Asellus aquaticus*, *Chironomus plumosus*, and Control). Note that the scales are different for different organisms.

show that bioturbation has a positive effect on recruitment rate, and this was especially pronounced in the *A. aquaticus* treatment (Table 1; Fig. 1). The difference in magnitude of recruitment rates between *C. plumosus* and *A. aquaticus* is probably caused by the very different behavior of the two invertebrates: *A. aquaticus* moves actively around in the sediment, whereas *C. plumosus* remains mainly within its tube, filtering water or collecting nearby surface particles. Thus, in our experiment, *A. aquaticus* had a major effect by mechanically disturbing the whole sediment surface, whereas *C. plumosus* produced a patchier bioturbation.

Resting stages accumulate in the sediment of mainly the profundal zone (Head et al. 1998; Baker 1999). However, recruitment rate is often higher in the littoral zone of lakes (Hansson et al. 1994; Hansson 1995; Head et al. 1999). For example, Hansson et al. (1994) observed that a major part

Table 1. Bioturbation effects on biota (repeated measures ANOVA; $n = 48$) (Kruskal–Wallis one-way analysis; $n = 144$) together with post hoc analyses (Bonferroni and Mann–Whitney U-test). rec, recruitment; ab, abundance; A, *A. aquaticus*; Ch, *C. plumosus*; C, Control; NS, not significant.

Organism	Treatment		Post hoc			
Anabaena rec	$F_{2,15} = 24.4$;	$p < 0.001$	A–Ch:	$p < 0.001$;	A–C:	$p < 0.001$
Anabaena ab	$F_{2,15} = 29.1$;	$p < 0.001$	A–Ch:	$p < 0.001$;	A–C:	$p < 0.001$
Microcystic rec	$H = 24.7$;	$p < 0.001$	A–Ch: Ch–C:	$p = 0.008$; $p = 0.008$	A–C;	$p < 0.001$;
Microcystic ab	$H = 31.6$;	$p = 0.001$	A–Ch:	$p = 0.001$;	A–C:	$p = 0.001$
G. semen rec	$F_{2,15} = 15.5$;	$p < 0.001$	A–Ch:	$p < 0.001$;	C–Ch:	$p = 0.007$
G. semen ab	$F_{2,15} = 15.2$;	$p < 0.001$	A–Ch:	$p < 0.001$;	Ch–C:	$p = 0.005$
Total zooplankton rec	$F_{2,15} = 9.5$;	$p = 0.002$	A–Ch:	$p = 0.005$;	A–C:	$p = 0.006$
Total zooplankton ab	NS		NS			

of the recruitment of *Microcystis* occurred from the littoral zone. Furthermore, Hansson (1995) observed that the recruitment rate of *Anabaena* was 10 times higher from the littoral zone compared to the profundal zone. Such differences can be explained by the physical differences in the two habitats. In the profundal zone, darkness and low temperature might limit the recruitment rate, whereas in the littoral zone, good light penetration capacity, higher temperature, and high wind resuspension might promote recruitment. Our results here suggest that recruitment rate might also be affected by the species composition of the benthic macrofauna. The macrofauna community differs considerably between the littoral and profundal zones, with higher species diversity in the littoral zone (Jónasson 1972). *C. plumosus* is generally abundant in the profundal zone, where they dig extensively through the sediment, whereas *A. aquaticus* is often abundant in the littoral zone, living near the sediment surface and associating with macrophytes (Jónasson 1972; McCall and Tevesz 1982).

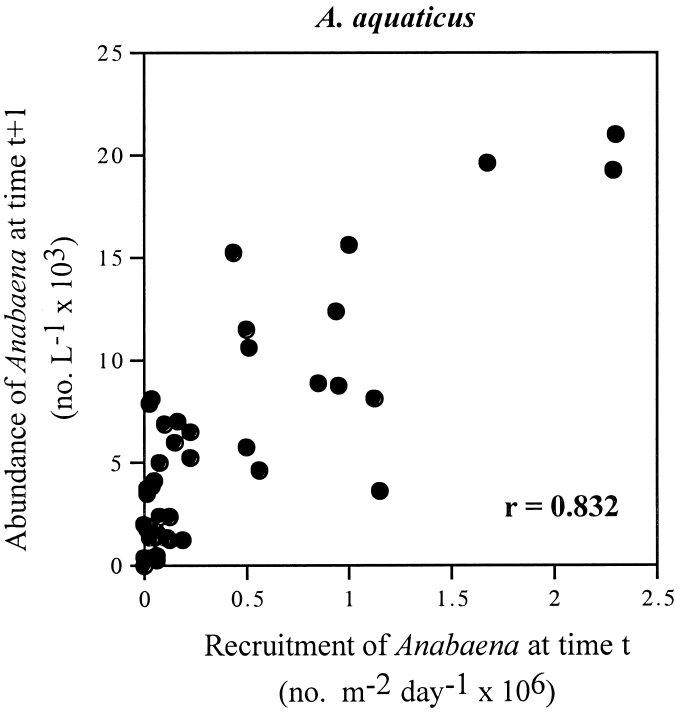

Fig. 2. Abundance of *Anabaena* (individuals $L^{-1} \times 10^3$) at time $t + 1$ d as a function of recruitment of *Anabaena* (individuals m^{-2} $d^{-1} \times 10^6$) at time t ($r = 0.832$).

The mechanisms that seem to be of major importance in promoting recruitment are transport of particles and mineralization. In the *A. aquaticus* treatment, a high rate of particle transport was evidenced by the deep organic layer and high turbidity (Table 3). The deep organic layer was a direct effect of the migration of the invertebrates, and this mechanical disturbance probably aided transport of particles from the sediment surface to the water column, thus increasing turbidity. By the same process, resting stages were probably translocated to the sediment–water interface or to the water column from deeper sediment layers, which made them come into contact with the right germination stimuli (e.g., oxygen, light, or nutrients). In contrast, because the *C. plumosus* treatment displayed neither a deepened organic layer nor increased turbidity, particle transport was probably of minor importance.

Mineralization processes could also have been important in promoting recruitment in the deeper layers of the sediment by the addition of oxygen and by the subsequent liberation of nutrients, both known as recruitment-promoting stimuli (e.g., Hansson 1993; van Dok and Hart 1997). However, nutrient concentrations were lower at the end of the experiment in all treatments, with the exception of an increase in ammonium in the *A. aquaticus* treatment. The lower nutrient concentrations could have been due to chemical binding to the sediment surface, sedimentation of nutrient-rich particles (including algae), or assimilation by the macrofauna. Nevertheless, in the *A. aquaticus* treatment at the end of the experiment, total amounts of nitrogen and phosphorus, as well as dissolved nitrogen, were higher than in the other treatments (Table 2). Thus, mineralization processes could have been important for increasing the recruitment rate. The higher concentrations for these nutrient parameters could have been due to excretion, spill-over from feeding activities by *A. aquaticus,* or simply the mechanical disturbance of the sediment surface that either increased the number of suspended particles in the water or released the nutrients from the interstitial water. Increased concentrations of nutrients in the water have also been observed in nature as a result of

Table 2. Bioturbation effects on nutrient concentrations at the end of the experiment (one-way ANOVA; $n = 6$) together with means ($\mu g\ L^{-1}$). Standard errors are in parentheses. Post hoc analyses (Bonferroni) are given for significant effects. NS, not significant; A, *A. aquaticus*; Ch, *C. plumosus*; C, control.

Nutrient species	Treatment	Post hoc
Ammonium	$F_{2,15} = 22.0$; $p < 0.001$	A–Ch: $p < 0.001$;
	$\text{Mean}_A = 91(15)$	A–C: $p < 0.001$
	$\text{Mean}_{Ch} = 17(3)$	
	$\text{Mean}_C = 21(1)$	
Nitrate	$F_{2,15} = 32.9$; $p < 0.001$	A–Ch: $p < 0.001$;
	$\text{Mean}_A = 68(17)$	A–C: $p < 0.001$
	$\text{Mean}_{Ch} = 4(0.5)$	
	$\text{Mean}_C = 5(0.5)$	
Phosphate	NS	NS
	$\text{Mean}_A = 4(1.0)$	
	$\text{Mean}_{Ch} = 1(0.5)$	
	$\text{Mean}_C = 2(1)$	
Total nitrogen	$F_{2,15} = 41.7$; $p < 0.001$	A–Ch: $p < 0.001$;
	$\text{Mean}_A = 1335(67)$	A–C: $p < 0.001$
	$\text{Mean}_{Ch} = 650(26)$	
	$\text{Mean}_C = 833(62)$	
Total phosphorus	$F_{2,15} = 9.9$; $p < 0.002$	A–Ch: $p < 0.015$;
	$\text{Mean}_A = 25(3)$	A–C: $p < 0.002$
	$\text{Mean}_{Ch} = 16(1)$	
	$\text{Mean}_C = 13(1)$	

Table 3. Bioturbation effects on abiotic parameters (one-way ANOVA; $n = 6$) at the end of the experiment (temperature is analyzed for all 8 weeks with repeated measures ANOVA; $n = 48$) together with mean values (standard errors in parentheses). Post hoc analyses (Bonferroni) are given for significant effects. NS, not significant; A, *A. aquaticus*; Ch, *C. plumosus*; C, control.

Parameter	Treatment	Post hoc
Turbidity (JTU)	$F_{2,15} = 27.2$; $p < 0.001$	A–Ch: $p < 0.001$;
	$\text{Mean}_A = 7.6(1.5)$	A–C: $p < 0.001$
	$\text{Mean}_{Ch} = 1.9(0.3)$	
	$\text{Mean}_C = 1.7(0.3)$	
Organic layer (mm)	$F_{2,15} = 22.8$; $p < 0.001$	A–Ch: $p < 0.001$;
	$\text{Mean}_A = 9.5(0.6)$	A–C: $p < 0.001$
	$\text{Mean}_{Ch} = 4.7(0.3)$	
	$\text{Mean}_C = 5.5(0.6)$	
pH	$F_{2,15} = 35.0$; $p < 0.001$	A–Ch: $p = 0.001$;
	$\text{Mean}_A = 7.1(0.05)$	A–C: $p = 0.010$
	$\text{Mean}_{Ch} = 6.8(0.05)$	Ch–C: $p < 0.001$
	$\text{Mean}_C = 7.3(0.05)$	
Oxygen concentration ($mg\ L^{-1}$)	$F_{2,15} = 5.7$; $p < 0.015$	A–Ch: $p < 0.016$
	$\text{Mean}_A = 8.7(0.2)$	
	$\text{Mean}_{Ch} = 8.9(0.2)$	
	$\text{Mean}_C = 9.5(0.1)$	
Temperature (°C)	F = NS	NS
	$\text{Mean}_A = 16.1(0.1)$	
	$\text{Mean}_{Ch} = 16.3(0.1)$	
	$\text{Mean}_C = 16.0(0.1)$	

Table 4. Mean dry weight (g m^{-2} ± SE) of benthic fauna for different treatments at the start and end of the experiment.

Time/ Treatment	*A. aquaticus*	*C plumosus*	Control
Start	0.1 (0.0)	1.3 (0.0)	0.0 (0.0)
End	2.9 (0.1)	0.6 (0.1)	0.1 (0.05)

the activities of benthic-feeding fish. For example, Breukelaar et al. (1994) found that when benthivorous fish fed on the sediment, nutrient concentrations increased through fecal production and resuspension of particles from the sediment. In contrast to *A. aquaticus*, the *C. plumosus* treatment showed no detectable elevation in nutrient concentrations. Thus, their contribution to mineralization processes was probably minor in this study and therefore had a limited effect on recruitment rate.

The abundance of *Anabaena*, *Microcystis*, *G. semen*, and zooplankton in the water followed their recruitment patterns (Figs. 1. 2). Dependence between recruitment from the sediment and abundance in the water has been observed in natural systems as well (Hansson et al. 1994; Baker 1999; Head et al. 1999), suggesting that benthic–pelagic coupling might be important with respect to algal dynamics.

In general, recruitment of algae was higher in the beginning of the experiment than in the end (Fig. 1), which might be due to a reduction in the number of resting stages at the sediment surface because of previous recruitment. However, in the *A. aquaticus* treatment, there was a second peak in recruitment rate before it leveled off. Perhaps the pool of resting stages was refilled from deeper layers as a result of increased bioturbation (Table 4); when this pool had recruited, the number of potential resting stages was low and the recruitment rate decreased again. Another more likely reason for the decline in numbers of recruiting algae is that the grazing pressure from the eventually increasing numbers of zooplankton was high (Fig. 1). Grazing on blue-green algae are normally not substantial, but some zooplankton groups could be effective grazers (de Bernardi and Giussani 1990; DeMott and Moxter 1991), and Davidowicz et al. (1988) have observed that filamentous forms can get cut into pieces and made accessible to further grazing. Not only direct grazing, but also active inhibition of algal recruitment by zooplankton might have been a mechanism behind the reduced recruitment (Hansson 1993, 1996, 2000; Rengefors et al. 1998). For example, Hansson (1996, 2000) noticed that recruitment of *G. semen* was negatively affected by the presence of zooplankton and that even the chemical cues from the grazers made the species remain as resting stages and thereby avoid recruitment.

Because many nuisance algae, including the three taxa studied here, rely on recruitment from the sediment for their presence in the water, it is important to gain insight into the mechanisms regulating recruitment, in order to overcome the problems of deleterious blooms. As we have demonstrated in this study, bioturbation by invertebrates could be important in creating conditions favorable for recruitment of algae. We have also discovered that the magnitude of promoting activities might be different because of species composition. Earlier studies on zooplankton have revealed that their resting stages might be directly affected by bioturbation from invertebrates and that the effect can be either positive or negative depending on the species composition of benthos (Marcus and Schmidt-Gengenbach 1986; Kearns et al. 1996). To our knowledge, this is the first study to investigate the importance of benthic macrofauna on the recruitment of algae, and it should lead to further studies to reveal their significance in nature.

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