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Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA

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ABSTRACT: The fate of the exogenous plasmid DNA, pEGFP, was traced in lake waters collected from the epilimnion and the hypolimnion during a period of marked thermal stratification. Both gel electrophoresis analysis and dot-blot hybridization analysis demonstrated that the seeded pEGFP was completely degraded in epilimnion water within 170 h of incubation, while no pEGFP degradation was observed in hypolimnion water. Removal of microorganisms from the lake waters of the epilimnion and hypolimnion attenuated the degradation of pEGFP but did not stop it. Addition of EDTA to the lake water samples resulted in complete termination of the pEGFP degradation and indicated the involvement of a dissolved enzyme for DNA degradation. A measurable release of phosphate during pEGFP degradation indicated the existence of DNA-mediated phosphate regeneration in epilimnion water. In contrast, no DNA degradation in hypolimnion water suggested the possibility that it is less nutritionally important in the hypolimnion. Transformability of the pEGFP was not altered in hypolimnion water during the incubation, which suggested stability of genetic information that gradually decreased in epilimnion water. Dissolved DNA (dDNA) is commonly observed in aquatic environments and is thought to be an important nutritional source for microorganisms. However, our results suggest that dDNA in the hypolimnion environment may not be so important as a nutritional source for microbes, but might be more important as a genetic pool for further natural transformation.

KEY WORDS: Dissolved DNA · Lake Biwa · Hypolimnion · Phosphate regeneration · DNA stability

INTRODUCTION

It is well known that in many freshwater environments phosphorus is a regulatory element for growth of both phytoplankton and bacterioplankton (Schindler 1977, Horne & Goldman 1994). The reasons why planktonic microorganisms can maintain particular population levels in environments where phosphorus is scarce are not yet clear, but previous studies have suggested that planktonic regeneration of organic matter, caused by viral lysis of bacterial and algal cells, sloppy feeding, excretion and fecal pellets, could sustain the microbial community under limiting nutrient conditions (Wetzel 1983, Hudson et al. 1999). This process of regeneration is mainly caused by the release of dissolved cellular macromolecules, such as DNA, into the water.

Dissolved deoxyribonucleic acid (dDNA, which passes through 0.2 µm pore filters) is commonly observed as micro-particulate matter in all natural aquatic environments. The amount of dDNA has been determined repeatedly and found at concentrations from 0.5 to 88 µg l^{-1} in freshwater environments (Minear 1972, DeFlaun et al. 1986, Karl & Bailiff 1989, Paul et al. 1989, 1991a, Siuda & Güde 1996a, Siuda et al. 1998). Although this <0.2 µm fraction contains viruses, previous studies have demonstrated that virus DNA is a minor contributor, less than 20%, of the total dDNA pool (Paul et al. 1991b, Weinbauer et al. 1993). Jiang &

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Paul (1995) estimated that 50% of the filterable (<0.2 µm) DNA in marine environment was truly soluble DNA while 17 to 30% of the other half could be accounted for by viral particles. Thus, the majority of the dDNA would be composed of either soluble or particle-binding forms. The production of dDNA would be caused by disruption of microbial cells and it seems to be regulated by both physico-chemical and a variety of biological processes such as grazing and viral lysis (Paul et al. 1987, Paul & David 1989, Turk et al. 1992, Reisser et al. 1993, Kawabata et al. 1998). Although the function of dDNA in aquatic environments is still not clear, it is likely that dDNA would be one of the most attractive phosphorus sources for aquatic microorganisms because of its phosphorus-rich atomic ratio (C:N:P ≒ 10:4:1; under 50% GC content) compared to the Redfield ratio found in planktonic cells (C:N:P ≒ 106:16:1; Redfield et al. 1963). In previous studies, Paul et al. (1987, 1989) demonstrated that seeded, radiolabeled extracellular DNA was hydrolyzed and very rapidly incorporated into heterotrophic microorganisms (within 24 h). Jørgensen & Jacobsen (1996) indicated that phosphorus originating from dDNA would account for 46% of the bacterial phosphorus demand in an oligotrophic environment and for 9% in a eutrophic environment. More recently, Siuda & Chróst (2000) demonstrated that 11 to 27.6% of the total phosphorus concentration in German lakes was accounted for by enzymatically liberated phosphorus of dDNA.

Most of these studies of DNA turnover have evaluated only the surface water layers. In many lakes, warm surface water, where photosynthesis occurs, is often isolated from underlying waters by a distinct thermocline, which separates the microbial habitats into epilimnion and hypolimnion, which have totally different characteristics. This stratification might create different environments for the fate of dDNA. Microorganisms might hydrolyze and incorporate dDNA as a phosphorus source in the phosphorus scarce epilimnion, but it is not likely that microorganisms will spend extra energy to degrade dDNA in the hypolimnion environment, where phosphorus does not severely limit their growth. If the dDNA remains without degradation, it would occur as a mixture of heterogeneous extracellular DNA from different organisms. Since DNA is a universal genetic material in all organisms, this dDNA remaining would serve as a reservoir of extracellular gene sequences for other microorganisms, via transformation. The finding of naturally competent bacterial strains (Jeffrey et al. 1990, Frischer et al. 1994) suggests the occurrence of dDNA incorporation as a genetic source in aquatic environments. Thus, dDNA might have different characteristics either nutritionally or genetically, in different layers of thermally stratified lake environments.

Our objective in this study was to estimate the possible function of dDNA in thermally distinct lake water environments. For this purpose, we spiked extraneous DNA into lake water samples and traced the fate of this seeded DNA by gel electrophoresis. We also traced the amount of DNA-mediated phosphate and the transformation efficiency of seeded DNA at each time interval. Through these analyses, we estimated the stability of dDNA in thermally distinct lake water environments and suggest a possible function of dDNA in the hypolimnion environment.

MATERIALS AND METHODS

Field sampling site. The sampling of lake water was carried out during the stratification period (6 November 2000) at a pelagic station (35° 9′ N, 135° 57′ E; ~57 m depth) in the mesotrophic north basin of Lake Biwa, Japan. Lake water samples were collected using a Van-Dorn water sampler, from the epilimnion (2.5 m) and the hypolimnion (50 m). The samples obtained were immediately carried to the laboratory and all experimental treatments were conducted within 6 h of sampling.

Analytical measurements. Water temperature and chl *a* were measured at the same time and place with a conductivity/temperature depth profiler (SBE-25, SeaBird Electronics, USA) equipped with a fluorometer (Sea Tech, Inc., USA). Soluble reactive phosphorus (SRP), as phosphate concentration, was determined by an AACSII (Bran + Luebbe Co., USA) continuous flow system using the molybdenum-blue method (Murphy & Riley 1962). Bacteria were counted directly under an epifluorescence microscope (Olympus BH2, Japan) using the di-amidino-phenyl-indole (DAPI) staining method (Porter & Feig 1980).

The concentration of dissolved DNA was determined by CTAB-nucleic acid precipitation (Karl & Bailiff 1989) and the Hoechst-DNA fluorescence method (DeFlaun et al. 1986), as modified by Ishii et al. (1998).

Preparation of extracellular DNA. Plasmid pEGFP DNA (Clontech, USA), 3.4 kilobase pairs (kb) in size, was used as extracellular DNA to trace its fate in lake water. This plasmid was encoding an ampicillin-resistant gene and a jelly fish originated green fluorescent gene as marker. The pEGFP was introduced into and amplified in *Escherichia coli* JM109 (Sambrook et al. 1989). The amplified plasmid was isolated by the Concert™ High Purity Plasmid Maxiprep System (Gibco, USA), eluted in TE (10 mM Tris-Cl and 1 mM EDTA; pH 8.0) and then used for incubation experiments. The concentration of the plasmid was determined by absorption of OD_{260}.

DNA incubation experiments. Triplicate lake water samples (10 ml) were inoculated with plasmid pEGFP DNA solution (final concentration; 4.8 µg DNA ml^{-1}) and incubated at the same water temperature as where they originated (18.0°C for the epilimnion and 7.8°C for the hypolimnion). At each time interval of the incubation, 1.2 ml subsamples were taken and stored at –20°C until analysis (within 2 wk). Each 1.2 ml subsample was subjected to analysis by gel electrophoresis, dot-blot hybridization and transformation experiments, and determination of SRP concentration. To evaluate the contribution of microorganisms and any extracellular enzymes to DNA degradation, the same experiment was also conducted with the filtrate of 0.2 µm pore, polycarbonate filters (Advantec, Japan) with and without the addition of 25 mM EDTA solution.

Gel electrophoresis. Three µl of the subsamples were subjected to gel electrophoresis in a 1.0% agarose gel. Gels were stained with diluted SYBR Green I solution following the manufacturer's protocol (Molecular Probes, USA) and photographed. Fluorescent signals of each DNA band were measured and the relative fluorescent intensity (RFI) of each DNA band was determined, based on the fluorescent intensity of the initial pEGFP band being taken as 100%, using Lumi-Imager F1 system (Roche Molecular Biochemicals, Germany).

Dot-blot hybridization. To quantify the remaining amount of spiked pEGFP plasmid, dilutions of each subsample were blotted on to positively charged nylon membranes (Roche Molecular Biochemicals) using dot-blot manifolds (Bio-Dot, Bio-Rad, USA). The blotted DNA was fixed by UV cross-linking and subject to Southern hybridization. The first 380 bp of the unique green fluorescence gene, *egfp*, were PCR amplified and labeled with dioxigenin (DIG) using a PCR DIG probe synthesis kit (Roche Molecular Biochemicals). The two 17-mer PCR primers for probe construction were (5′-ATGGTGAGCAAGGGCGA-3′) and (5′-GGA CACGCTGAACTTGT-3′), and synthesized by Amersham Pharmacia Biotech (Tokyo, Japan). The DIG-labeled 380 bp fragments were loaded onto 1.5% agarose gels and separated from other DNA fragments in the PCR tube by electrophoresis. The well-separated 380 bp fragment was cut out from the gel, purified using a Prep-A-Gene Purification Kit (Bio-Rad), and used as a probe. Hybridizations were performed overnight at 42°C in hybridization buffer (DIG Easy Hyb; Roche Molecular Biochemicals). Washing was performed under highly stringent conditions: twice for 15 min with 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 42°C. Then twice for 20 min with 0.1 × SSC and 0.1% SDS at 68°C. Quantification of the hybridization signal was performed with a Lumi-imager F1 with Lumi-Analyst 3.0 system (Roche Molecular Biochemicals).

Transformation efficiency. Transformation experiments were performed to evaluate whether the seeded extracellular DNA in the subsamples maintained its genetic information. One µl of each subsample was added to 100 µl of $CaCl_2$-treated competent *Escherichia coli* JM109 (Sambrook et al. 1989), and the bacteria were then selected for resistance to 50 µg ml^{-1} ampicillin in Luria-Bertani (LB) agar medium (Sambrook et al. 1989). Before they were spread onto the agar plate, the transformed competent cells were adequately diluted with LB medium. The plates were incubated for 20 h at 37°C and counts of the colony-forming units (CFU) were obtained. Expression of the green fluorescence gene was also confirmed from green fluorescence color of each colonies. The amount of pEGFP in each subsample was obtained from dot-blot hybridization analysis and the transformation efficiencies (CFU ng^{-1} pEGFP DNA) were determined.

RESULTS

Environmental characteristics of sampling site

The characteristics of the sampling site are shown in Table 1. The thermocline was observed to be between 22 and 26 m on the sampling day, and the samples, from 2.5 and 50 m, taken to be representative of the epilimnion and hypolimnion respectively. The dDNA concentration in the epilimnion was 17 times higher than that in the hypolimnion, but a lower concentration of SRP was observed in the epilimnion. Higher bacterial abundance (2 times) and concentration of chl *a* (8 times) at 2.5 m suggested the possibility of higher biological activity and a scarcity of phosphate in the epilimnion on the sampling day.

Tracing the seeded pEGFP by gel electrophoresis

The stability of the pEGFP DNA inoculated into the lake water samples was traced by gel electrophoresis. Fig. 1A shows the gel picture of DNA in the epilimnion

Table 1. Characteristics of the sampling site. WT: water temperature; PO_4-P: soluble reactive phosphorus; BA: bacterial abundance

Depth (m)	WT (°C)	PO_4-P (µg l^{-1})	dDNA (µg l^{-1})	BA (10^6 cells ml^{-1})	Chl *a* (µg l^{-1})
2.5	18.0	0.7	23.8 ± 4.3	2.6	2.1
50	7.8	1.2	1.4 ± 0.2	1.2	0.26

sample with different treatments. In the intact lake water, gradual degradation of the added DNA was observed after 24 h of incubation and no DNA bands were found after 170 h of incubation. The degradation of pEGFP was also observed in the filtered lake water sample although it occurred much more slowly than in the intact lake water. No apparent pEGFP degradation was observed in the filtered water supplemented with 25 mM EDTA, which indicated the involvement of some enzymatic reaction during the DNA degradation process.

The gel picture of DNA in the hypolimnion samples (Fig. 1B) showed a different pattern of bands from that in the epilimnion. There was no apparent pEGFP degradation in any of the hypolimnion subsamples, although some conformational band-shifting was observed in both the intact and filtered sub-samples after more than 3 h of incubation (Fig. 1D). No conformational changes were observed in the filtered water sample supplemented with EDTA.

Quantification of pEGFP and SRP during incubation periods

We conducted the dot-blot hybridization analysis in order to quantify the pEGFP remaining during incubation (Fig. 2A,B). We also determined the SRP concentration which would be released by DNA degradation (Fig. 2C,D). In the intact epilimnion water samples, the

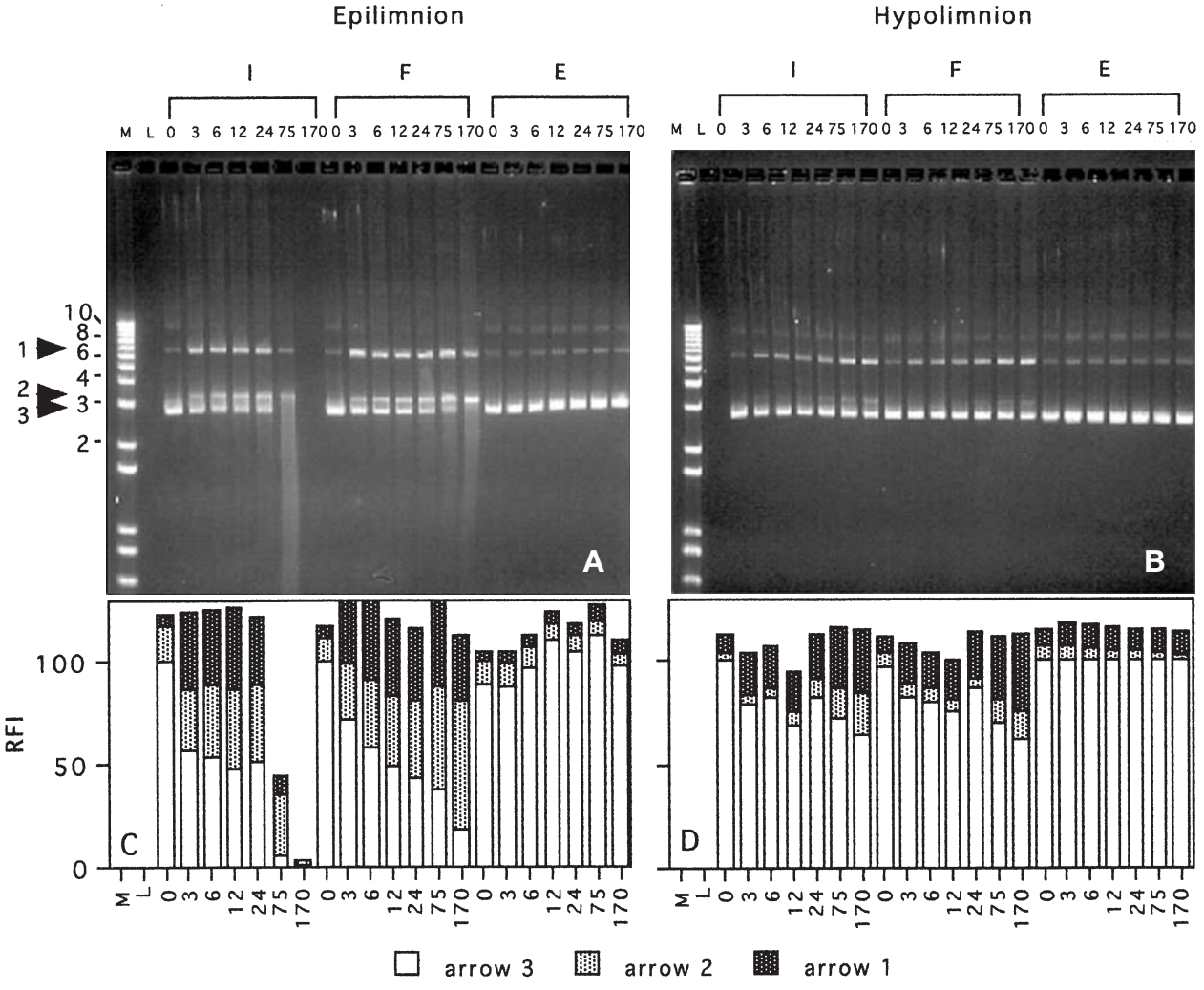

Fig. 1. Agarose gel electrophoresis of seeded extracellular DNA from incubation experiments and relative fluorescence intensity (RFI) of DNA bands (Arrows 1, 2 and 3) in the epilimnion lake water sample (A,C); in the hypolimnion lake water sample (B,D). Lane M is the DNA standard (1 kb plus DNA ladder™, Gibco) and the molecular weights of the bands are shown in kb pairs. Numbers of each lane indicate the incubation periods of the subsamples. Group I, DNA in intact lake water; Group F, DNA in filtered lake water; Group E, DNA in filtered lake water containing 25 mM EDTA; Lane L contained 15 µl of intact lake water and no bands were detected

amount of pEGFP gradually decreased in proportion to the incubation period (Fig. 2A). After 75 h, the amount of pEGFP had decreased to less than half of the initial amount inoculated and no hybridization signals were detected after 170 h of incubation. In the filtered epilimnion water samples, obvious pEGFP degradation was observed only after 170 h of incubation. Although there were some conformational changes after 3 h in the filtered lake water (Fig. 1A,B), dot-blot hybridization analysis showed the persistence of the inoculated

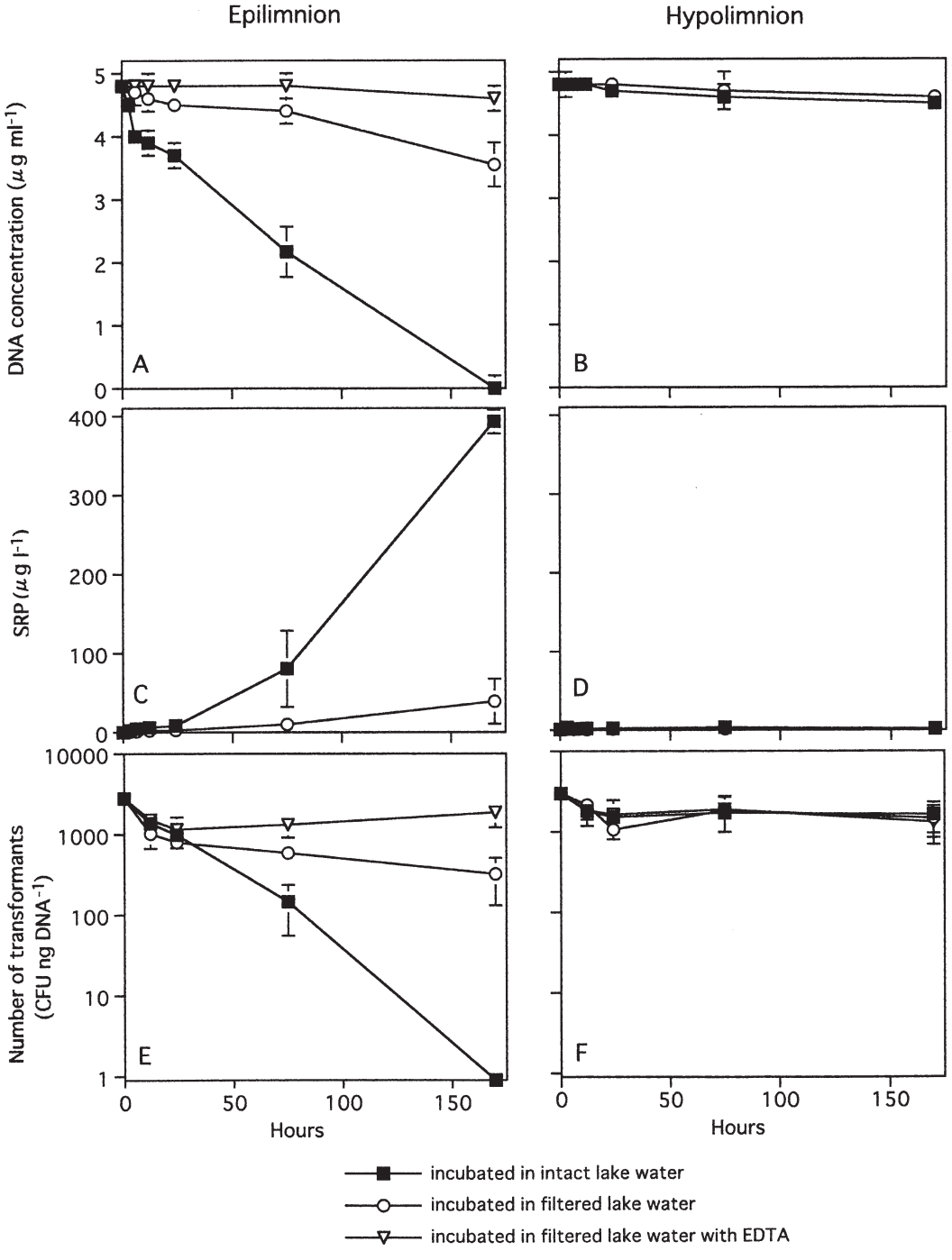

Fig. 2. Parameters measured during the incubation experiments with epilimnion water (A,C,E) and hypolimnion water (B,D,F). DNA concentration obtained from dot-blot hybridization (A,B); SRP (C,D); Transformation efficiency (E,F). Error bars indicate the standard deviations from the means of triplicate experiments

pEGFP sequences for up to 75 h of incubation. No pEGFP degradation was detected in the EDTA-treated filtered epilimnion water nor in any of the hypolimnion water samples (Fig. 2B).

To elucidate whether the degradation of DNA resulted in phosphorus regeneration, especially inorganic phosphorus, we measured the amount of SRP at each incubation interval (Fig. 2C,D). SRP increase related to the period of incubation in the intact epilimnion water and reached 393 µg l^{-1} after 170 h. Increased SRP was also observed after 170 h in the filtered epilimnion water. These data indicated the possibility of DNA-mediated phosphate regeneration in the epilimnion environment. In contrast, there was no phosphate production in the hypolimnion water samples and phosphate regeneration would be less possible under hypolimnetic conditions.

Transformation efficiency

To evaluate whether the seeded DNA maintained its genetic information in the lake waters, we conducted the transformation experiment using competent *Escherichia coli* bacteria. Because the amounts of DNA were different among the subsamples, transformation efficiencies were adjusted using the DNA concentration quantified from dot-blot hybridization analysis. Fig. 2 shows the periodical transformation efficiencies in the samples from the epilimnion (Fig. 2E) and hypolimnion (Fig. 2F). Transformation efficiency dropped markedly when the DNA was incubated in intact epilimnion water. A decrease of transformation efficiency was also observed with the DNA incubated for 170 h in filtered epilimnion water and its efficiency was 10 times lower than the initial efficiency. Incubation in hypolimnion water samples did not alter the transformation efficiency throughout the incubation (Fig. 2F). Although the amount of DNA used in this experiment was much higher than the dDNA concentration in natural environments, our results suggest that DNA could maintain its genetic information for several weeks under conditions found in the hypolimnion but lost its information in epilimnion water within a week.

DISCUSSION

In this study, we inoculated samples with exogenous plasmid DNA and traced its longevity in thermally distinct lake waters. Seeded DNA was degraded completely in intact epilimnion water within 170 h of incubation, which suggested dDNA degradation in the epilimnion environment (Fig. 1A). The consequent increase in SRP concentration in intact epilimnion water reached 393 µg l^{-1} after 170 h incubation, which corresponded to 65.5% of the initial amount of phosphorus in the DNA. This suggests that DNA-mediated phosphorus mineralization could occur in the epilimnion environment. This phosphorus mineralization would be necessary for the growth of microorganisms, which are frequently limited by inorganic phosphorus (Toolan et al. 1991, Coveney & Wetzel 1992). Several researchers have reported that phosphorus is an important growth-limiting factor for microorganisms, especially for phytoplankton, in the epilimnion of Lake Biwa, during stratification periods (Tezuka 1984, 1985, Frenette et al. 1996, Urabe et al. 1999). During our sampling period, a concentration of 23.8 µg l^{-1} dDNA was detected in the epilimnion. It contained 2.26 µg l^{-1} phosphorus, which was 4 times higher than the measured SRP concentration in the water (Table 1). In previous studies, we had also found 5 to 30 µg l^{-1} dDNA during stratification (Kawabata et al. unpubl. data) corresponding to 0.47 to 2.85 µg l^{-1} of phosphorus. This quantity of phosphorus was 1 to 3 times higher than the concentration of dissolved inorganic phosphorus in the lake water. Thus, in the epilimnion of our sampling site, dDNA could be an important source of phosphate for microorganisms.

Sequential enzymatic reactions would be involved in this process of DNA hydrolysis and mineralization. In both intact and filtered lake water samples, the additional DNA bands were observed at around 3.4 and 6.5 kb, respectively, after a 3 h incubation period (Fig. 1A; Arrows 1 and 2). We speculate that these bands indicated conformational change of the supplemented pEGFP DNA. From the comparison of its fluorescence intensity on each of the DNA bands, the added super-coiled plasmid (Arrow 3) had conformationally shifted to either open-circular form (Arrow 1) or linear form (Arrow 2) and then smeared (degraded) (Fig. 1C). However, DNA hydrolysis was only apparent after 75 h incubation and indicated that this would be a rate-limiting step for DNA-mediated DNA mineralization. In a previous study, Siuda & Güde (1996b) also suggested that DNA hydrolysis is a rate-limiting step for utilization of DNA as a phosphorus source, and our data support their suggestion. Ammerman & Azam (1985) have shown that the bacterial 5′-nucleotidase plays a significant role in the supply of phosphorus to planktonic algae. Production of phosphate in the pEGFP-seeded epilimnion water in our study indicated the existence of a nucleotidase in a dissolved form and agreed with their finding. In addition to the importance of nucleotidase, our data also showed the involvement of some nuclease activity for DNA-mediated phosphate regeneration.

In contrast to the obvious pEGFP degradation in the water from the epilimnion, gel electrophoresis analysis

and dot-blot hybridization analysis showed the stability of DNA in the hypolimnion water, although some conformational changes to the seeded pEGFP (Figs 1B & 2B) were recognized. The lack of phosphate production in the hypolimnion water sample suggested that dDNA would not be utilized as a phosphorus source. We can suggest 2 possible interpretations of this hypolimnion data. First, in the hypolimnion, microorganisms were not limited in their growth by phosphorus and, therefore, did not produce the enzymes necessary for utilization of DNA. Second, microorganisms also produced the nucleotidase and phosphatase, but the low temperature limited enzymatic activity in the hypolimnion. We did not have a clear answer to whether enzyme availability or cold temperature regulated the DNA degradation, but our results showed that DNA regeneration does not seem to be sustaining the growth of microbial communities in the hypolimnion, and dDNA is persisting without degradation.

Because DNA is a universal genetic macromolecule for all organisms, the stability of the DNA seeded into the hypolimnion water suggested that dDNA in the hypolimnion could be a source of natural transformation. An earlier finding of 0.12 to 35.2 kb of DNA in aquatic environments (DeFlaun et al. 1987) indicated that dDNA might have sufficient length to carry certain genetic information. It was with this in mind that we conducted the transformation experiments in order to evaluate whether the seeded pEGFP could maintain the longevity of its genetic information. Fig. 2F showed that in the hypolimnion the seeded pEGFP did indeed maintain its genetic information through 170 h of the experimental period, in a transformable form. In epilimnion water, the seeded pEGFP lost its transformability in proportion to the incubation time (Fig. 2E). In intact epilimnion water, the dot-blot hybridization analysis showed that 55% of the pEGFP degraded during 75 h incubation (Fig. 2A) while its transformation efficiency dropped to 5.2% of the initial level (Fig. 2E). The probability of the transfer of plasmid DNA by transformation is highly dependent on the availability of DNA and the existence of bacteria which can take up exogenous DNA by transformation (Lorenz & Wackernagel 1994). Although we did not know of the existence of such bacteria at our sampling site, our results indicated that DNA could retain its transformability without any alteration in the hypolimnetic environment for at least 170 h. Thus, genetic information on dDNA in the hypolimnion would have considerable longevity and might function as a pool of genetic material instead of any nutritional function.

The stability of the DNA molecule was also interesting with regard to the release of genetically modified organisms, and their recombinant DNA, into aquatic environments. Previously, Alvarez et al. (1996) have shown that seeded 10 µg ml^{-1} of plasmid DNA was degraded within a few hours in warm (23 to 25°C) marine and river water, and they suggested that concerns about the uptake of novel genes by resident bacteria may be overstated. DeFlaun & Paul (1989) also reported complete degradation within 36 h of 15 ng ml^{-1} of plasmid DNA added to warm (25°C) estuarine water. They suggested that while transformation in the marine environment might occur to DNA in protected microenvironments, such as the surface of sediment grains, it was less possible in a water body. However, the present studies have shown that while seeded plasmid pEGFP DNA (4.8 µg ml^{-1}) was gradually degraded in epilimnion water (18°C) the seeded pEGFP maintained its initial transformability for at least 170 h in hypolimnion water (7.8°C). This suggests that released recombinant DNA might survive as an extracellular DNA pool in the hypolimnion environment. We have also recently demonstrated the possible survival, through microbial interactions, of genetically modified *Escherichia coli* bacteria which might be unintentionally released from a laboratory (Matsui et al. 2001). The large contribution of autochthonous planktonic microbial processes to the production of dDNA (Paul et al. 1991a) suggested the release of recombinant DNA sequences to the aquatic environment from surviving microbes. Thus, the present study also suggests that the recombinant DNA in genetically modified microorganisms, either intentionally or unintentionally released, would remain in the hypolimnion of thermally stratified aquatic environments.

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