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Concentration and susceptibility of dissolved DNA for enzyme degradation in lake water — some methodological remarks

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ABSTRACT: This paper discusses the methodology for determination of dissolved DNA concentration by means of direct DAPI staining of water samples and compares it with the data obtained by the method of dissolved DNA precipitation (in 0.2 µm water filtrates) with the use of cetyltrimethyl-ammonium bromide (CTAB) and DAPI staining. The samples were collected from lakes of varying trophic states. Enzymatically hydrolysable DNA (EH-DNA) was estimated as the difference between the concentration of the DNA in samples without and with DNAse treatment. Concentrations of enzymatically hydrolysable DNA determined by the enzymatic method were 27 to 54% lower than those measured by CTAB-DNA precipitation and DAPI staining. Enzymatically hydrolysable DNA concentrations increased with the trophic state of the lake and correlated positively with algal pigment concentrations and bacterial numbers. The contribution of phosphorus that can be enzymatically liberated from extracellular DNA to the total organic phosphorus concentration in lake water samples varied from 11% (oligo/mesotrophic lake) to 27.6% (hypertrophic lake).

KEY WORDS: Dissolved DNA · DNAse · Phosphorus · Trophic state index · Plankton

INTRODUCTION

Deoxyribonucleic acid (DNA) is a common constituent of the particulate organic matter (POM) in all natural aquatic environments (Minear 1972). In addition, many investigations carried out in marine (Pillai & Ganguly 1972, Bailiff & Karl 1991, Weinbauer et al. 1993, 1995) and freshwater (Paul et al. 1989, 1990, Siuda et al. 1998) ecosystems revealed that DNA may occur also extracellularly in 3 main fractions: (1) a naked free DNA, (2) DNAse resistant naked DNA adsorbed on detrital particles, and (3) protein encapsulated and/or coated DNA forms (e.g. viral DNA) (Maruyama et al. 1993). Although literature data (Minear 1972, Maruyama et al. 1993) and the results of our previous investigations (Siuda et al. 1998) suggested that dissolved DNA (dDNA) may be regarded as one of the most important phosphorus sources for microplankton in water environments, the quantitative aspects of P regeneration from the extracellular DNA pool are still not well known.

The main problem for quantitative analysis of dDNA (measured by chemical methods) decomposition in a natural environment is our poor knowledge of the quantity of the dDNA pool and the susceptibility of various dDNA fractions for degradation by bacterial DNA hydrolysing enzymes. Earlier investigations of Siuda & Güde (1996a) gave some evidence that dDNA concentrations were sometimes overestimated when determined by the CTAB-DAPI method (precipitation by cetyltrimethyl-ammonium bromide [CTAB] and fluorometric detection after 4,6-diamidino-2-phenylindole [DAPI] staining). This was observed occasionally in eutrophic, and often in highly eutrophic lakes. Nonspecific dDNA precipitation by the CTAB technique (Bellamy & Ralph 1968, Karl & Bailiff 1989, Siuda & Güde 1996a) and further partial solubilisation of various fluorescent high-

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molecular weight dDNA contaminants in NaCl solution during dDNA recovery from the dDNA-CTAB precipitate sometimes caused a significant increase of fluorescence of the dDNA extracts during the fluorometric DAPI assay (DeFlaun et al. 1986, Siuda & Güde 1996a). As a result, especially in lakes rich in dissolved organic matter (DOM), the concentration of dDNA-phosphorus, corresponding to ca 8% of DNA (w/w), sometimes reached or even exceeded the total dissolved organic phosphorus (DOP) concentration, measured by the Koroleff (1983) method.

The other source of errors in the measurement of dDNA fluxes in aquatic environments is the influence of particulate organic matter (POM) and of colloidal, high-molecular weight components (HMWC) of DOM. A substantial, but not easily defined, part of the total dDNA may be adsorbed on particles and colloids in lake water, and thus are partially resistant to enzyme degradation (Romanowski et al. 1991, Lorenz & Wackernagel 1994, Ogram et al. 1994). In summary, the great quantitative discrepancies (generally 1 to 2 orders of magnitude) between results obtained by enzymatic (Paul et al. 1989) and nonenzymatic methods (Karl & Bailiff 1989) of extracellular DNA quantification in aquatic environments reflect our ignorance of the real concentrations of free extracellular DNA and their ecological significance in aquatic ecosystems.

The main aims of this paper were to verify the suitability of the CTAB-DAPI technique for determination of biologically available dDNA in aquatic ecosystems and to propose a new method which may help to solve the problem of the quantification of enzymatically hydrolysable extracellular DNA in eutrophic lakes.

MATERIAL AND METHODS

The investigations were carried out in lakes in the Mazurian Lake District (Northern Poland) during the summer stratification period in 1995. The trophic state of the studied lakes (Table 1) varied from mesotrophy (Lake Kuc) to advanced eutrophy (Lake Szymon). Samples (1 l) were collected in polypropylene bottles under non-sterile conditions from the surface layer (1 m) of the pelagic zone of the studied lakes.

Determination of enzymatically hydrolysable DNA (EH-DNA). DNA concentrations were determined spectro-fluorometrically after DAPI (Serva) staining. For each sample 10 replicates (including 5 replicates of the control) were prepared. 0.3 ml of sodium azide solution (final concentration 0.3%) and 0.1 ml DNAse I-$MgCl_2$ mixture (containing 10 mg DNAse, EC. 3.1.21.1, Sigma-Aldrich and 5 mg $MgCl_2$ ml^{-1}) was added to 19 ml portions of prefiltered (100 µm plankton net) lake water. DNA hydrolysis was terminated by adding 0.6 ml of saturated EDTA solution at time 0 (controls), and after 6 to 8 h of incubation at 20°C. For the DNA assay, 4.3 ml portions of replicate were supplemented with 0.1 ml of 1 M Tris-NaCl buffer (Prasad et al. 1972) (final concentration 0.02 M, pH 8.3) and 0.1 ml (10 µg ml^{-1}) of DAPI water solution. After 10 min of staining in the dark, fluorescence was measured with a Shimadzu spectro-fluorimeter RF 1501 at 365 nm (excitation) and 445 nm (emission). The decrease in the fluorescence of the sample after DNAse treatment (in comparison to the control) was calculated as enzymatically hydrolysable extracellular DNA (EH-DNA) concentration from the standard curve. Standard curves were prepared by dilution of calf thymus DNA (Sigma-Aldrich) stock solution (1 mg ml^{-1}) to the required concentration with 0.02 M Tris-NaCl buffer.

Other analyses. For the measurement of DNAse activity triplicate samples of prefiltered (100 µm plankton net) lake water were supplemented with 0.2 ml of calf thymus DNA solution in distilled water to a final concentration of 100 µg l^{-1}. Sodium azide solution (0.3 ml, final concentration 0.3%) was added to prevent bacterial growth. At time 0 (control) and after 12 to 24 h of incubation DNA concentration was determined as described above. The rates of DNAse activity are expressed as the decrease in DNA concentration per litre of the sample per hour. Bacterial secondary production was determined by the [^{3}H]-thymidine method (Chróst et al. 1988). DOP concentrations in the lake water were measured according to Koroleff (1983). Algal pigments (chlorophyll *a* and phaeophytin) were measured spectro-photometrically after extraction with 96% ethanol (Marker et al. 1980). The bacteria were counted directly in an epifluorescence microscope (Zeiss, Germany) after staining (5 min) with DAPI at 2.7 µM final concentration (Güde et al. 1985).

Table 1. Basic morphological and limnological parameters of the studied lakes. Lakes are arranged according to increasing trophic status. Chl *a*: chlorophyll *a*; P_T: total phosphorus; N_T: total nitrogen, TSI: trophic state index (Carlson 1977) calculated from mean annual chl *a* ($TSI_{chl\ a}$) and PT (TSI_{P_T}) values

Lake	Area (ha)	Depth Max. (m)	Depth Mean (m)	Chl *a*	P_T (µg l^{-1})	N_T	$TSI_{chl\ a}$	TSI_{P_T}
Kuc	99	28.0	8.0	1.7	25.0	440.0	51	35
Ryńskie	676	51.0	13.8	26.5	122.0	1650.0	73	63
Mikołajskie	498	25.9	11.2	35.1	63.0	960.0	64	65
Szymon	154	2.9	1.1	101.1	214.4	2120.0	77	82

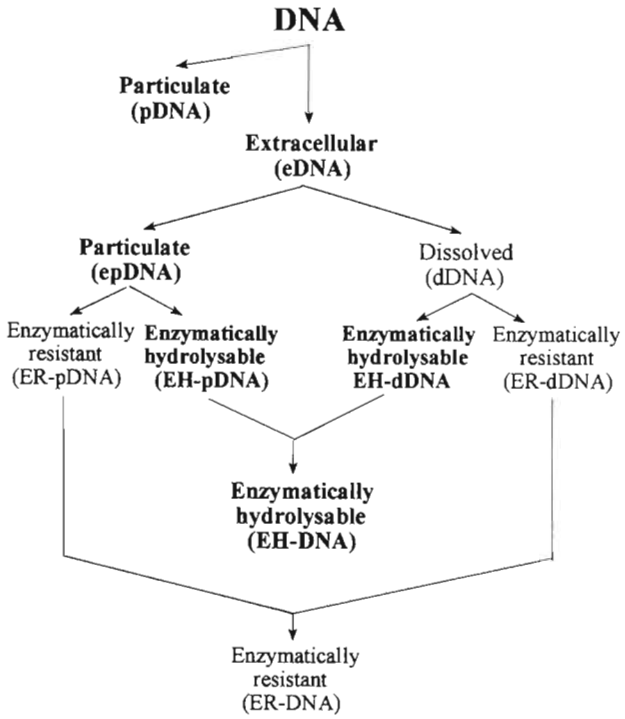

Fig. 1. Various DNA pools in lake water. Abbreviations used in the text are in bold

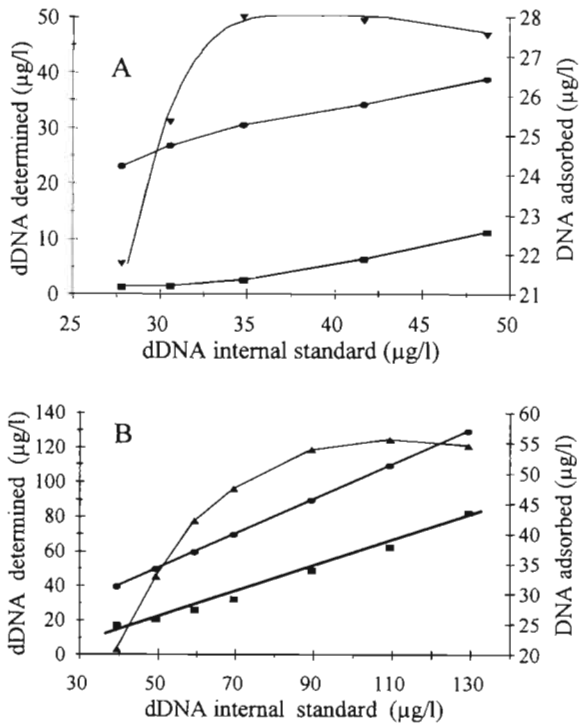

Fig. 2. Calf thymus DNA standard curves obtained with epilimnetic (1 m) water from: (A) Lake Constance, 3 October 1993; (B) Lake Mikołajskie, 26 June 1995. (■) DNA was added to 20 ml of unfiltered lake water and then standards were filtered through 0.2 µm Nuclepore. (●) Standards were prepared by DNA addition to lake water filtrate (0.2 µm Nuclepore) and filtered again (▲) DNA adsorbed by seston particles

RESULTS AND DISCUSSION

Extracellular DNA, dDNA and EH-DNA in aquatic environments — some methodological remarks

A critical review of the literature on various DNA forms existing in aquatic ecosystems leads to the conclusion that the most commonly used differentiation of the total DNA pool into living or nonliving particulate DNA (pDNA) and dDNA fractions is comfortable indeed from the analytical point of view, but does not reflect the ecological role and significance of extracellular DNA (eDNA) in the environment. For instance our earlier investigations and some literature data (Weinbauer & Peduzzi 1996) provided evidence that the term 'dDNA' reserved for the fraction of eDNA that passes through a 0.2 µm filter and may be precipitated by CTAB is inaccurate and erroneous when used for the description of eDNA decomposition in aquatic ecosystems. Apart from free naked enzymatically hydrolysable DNA (EH-dDNA), CTAB could also precipitate viral DNA (Weinbauer & Peduzzi 1996), other DNA-containing particles <0.2 µm (Maruyama et al. 1993) and perhaps some non-DNA contaminants (Siuda & Güde 1996a). It should be pointed out that the contribution of viral DNA to the total dDNA pool may be substantial. It may vary from 0.01 to 9.42 µg l^{-1}, i.e. from 0.1 to 96.1% of the dDNA concentration in the environment (Beebee 1991, Weinbauer et al. 1995). As an important conclusion arising from these observations one can say that the eDNA pool in lake water is structurally and physiologically non-homogeneous (Fig. 1). Our old method for determination of dDNA concentrations (Siuda & Güde 1996a) might have underestimated the total eDNA concentrations but overestimated the really dissolved DNA pool, especially in hypertrophic and some eutrophic environments.

The analysis of DNA-DAPI standard curves prepared with filtered (0.2 µm Nuclepore) lake water before and after calf thymus DNA supplementation (Fig. 2) showed that a large portion of calf thymus DNA was immediately removed from the liquid phase. Similarly, the sigmoidal shape of DNAse kinetic curves obtained for Lake Mikołajskie (Fig. 3) and other eutrophic lakes strongly suggested that an essential part of highly polymerized DNA from calf thymus added to the natural (unfiltered) lake water could have been adsorbed on the filter or seston particles, which, to some extent, protects it from hydrolysis by nucleases. Since adsorption of calf thymus DNA by polycarbonate (0.2 µm Nuclepore) filters could

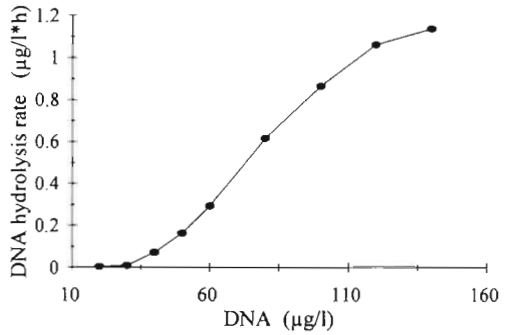

Fig. 3. Kinetics of calf thymus DNA hydrolysis by DNAse in epilimnetic (1 m depth) water from Lake Mikołajskie (26 June 1995). Various amounts of calf thymus DNA were added to triplicates of unfiltered lake water. The amounts of hydrolysed DNA were calculated from the differences between the DNA concentration at time 0 and after 24 h of incubation in 0.2 µm filtrates of these subsamples. The standard curve for recalculation of the fluorescence into DNA concentration was prepared by dilution of DNA stock solution (1 mg ml^{-1}) to required concentrations with 0.02 M Tris-NaCl buffer (pH 8.3)

Table 2. Comparison of mean values of dDNA and EH-DNA concentration in lakes of various trophic states. dDNA: dissolved DNA determined by the CTAB-DAPI method (Siuda et al. 1998), EH-DNA: enzymatically hydrolysable DNA determined by the 'enzymatic method'. *Number of studied lakes, **number of determinations. Range of variability is given within parentheses

Type of lake	dDNA (µg l^{-1})	EH-DNA (µg l^{-1})
Mesotrophic	16.7	7.7
	(2.3–27.3)	(3.2–14.2)
	*5	*1
	**25	**4
Eutrophic	37.6	21.8
	(17.2–57.0)	(13.4–32.3)
	*12	*2
	**22	**7
Hypertrophic	50.0	36.6
	(35.4–88.3)	(21.9–79.6)
	*4	*1
	**12	**4

be negligible (it does not exceed 5%, Siuda & Güde 1996a), one can suppose that originally dissolved DNA was adsorbed on seston and perhaps also on large colloidal particles suspended in lake water and became, in fact, extracellular particulate DNA (epDNA). Generally, in mesotrophic environments less dDNA was adsorbed than (Fig. 2A) in the seston-rich hypertrophic lakes (Fig. 2B). For instance, we found that up to 55 µg of calf thymus DNA added to unfiltered water sample taken from Lake Mikołajskie can be immobilized by the seston and could not be detected in 0.2 µm filtrate by the CTAB-DAPI method.

Despite the fact that some data concerning the protection of dDNA from DNA hydrolysing enzymes by glass and mineral surfaces are known, we still lack information on the susceptibility of DNA bound to organic particles to DNAse (Lorenz & Wackernagel 1994). The mechanisms of these processes are also not fully understood. Two possible solutions have been proposed to explain the protection of epDNA against enzymatic degradation: DNAse has only limited access to the adsorbed DNA molecules (Lorenz & Wackernagel 1987, 1992, Romanowski et al. 1991) and/or the nuclease itself adsorbs to the seston and may thus be inhibited or inactivated similarly as several other adsorbed enzymes (Sarkar et al. 1989).

dDNA concentrations (20 to 88 µg l^{-1}) reported for the 0.2 µm filtrates of samples from eutrophic and hypertrophic lakes (Minear 1972, Karl & Bailiff 1989, Siuda et al. 1998) are commonly higher than the lower limit of DNA-DAPI assay (20 µg dDNA l^{-1}, Siuda & Güde 1996a). Moreover, eDNA adsorbed on the seston particles (epDNA) may also, similar to dDNA, bind DAPI particles and increase the total fluorescence yield. Therefore, one can assume that, at least in theory, in the majority of lake water samples it should be possible to determine the dDNA concentrations without preliminary filtration of samples and dDNA precipitation from the filtrates. A knowledge of eDNA instead of dDNA concentrations in lake water would be probably the same or even more profitable for the description of DNA decomposition processes in aquatic environments. Additionally, during the eDNA assay some errors caused by preliminary filtration of samples and precipitation of dDNA from the filtrates by CTAB or other precipitating agents could be eliminated.

In practice, however, direct measurement of absolute eDNA concentration in unfiltered lake water is not possible. Firstly, DAPI binds not only to eDNA but also to DNA of living cells (Porter & Feig 1980) and to other organic compounds present in natural lake water (Sherr et al. 1993), which results in strong fluorescence that usually masks the fluorescence of the eDNA-DAPI complex (Siuda & Güde 1996a). Secondly, fluorometric DNA assays in non-homogenous solutions are significantly affected by a variety of physical phenomena, i.e. light absorption, fluorescence quenching and light scattering (Undenfriend 1964). However, considering some limitations (mainly regarding low sensitivity and variations of the fluorescence among the repetitions), it still seems possible to measure the concentration of EH-DNA in lake water, expressed as the difference between fluorescence of the DNA-DAPI complex in the same sample before and after DNAse treatment. The above-mentioned effects of nonspecific fluorescence and errors caused by other physical phenomena

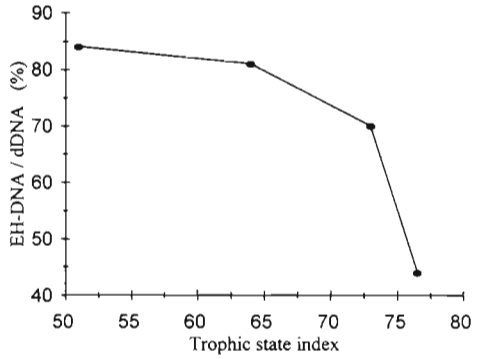

Fig. 4. EH-DNA/dDNA ratio as a function of the trophic state index of the lakes studied. Mean values of EH-DNA and dDNA were calculated from 4 determinations for each lake. The trophic state index was calculated from chl *a* data collected during summer periods 1991 to 1994

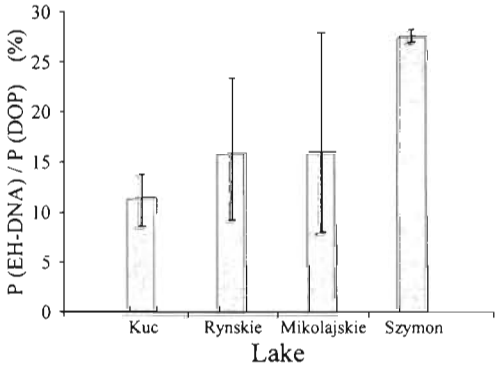

Fig. 5. The mean contribution of EH-dDNA phosphorus to the total dissolved organic phosphorus pool in the lakes studied. Variability ranges of 4 determinations are given

may, in our opinion, be excluded by subtraction of the DNAse activity free blank from the DNAse-supplemented sample. Five replicates instead of commonly used triplicates may reduce standard deviations of the results to acceptable levels (SD < 5%).

The comparison of dDNA concentration data collected during our previous studies (Siuda et al. 1998) with EH-DNA concentrations obtained during this investigation (Table 2) showed that EH-DNA concentrations estimated by the enzymatic method were generally (27 to 54%) lower than concentrations of dDNA measured by the CTAB-DAPI technique. The difference between the results obtained by both methods seems to depend on the trophic state of the lake (Fig. 4), i.e. the more eutrophied the lake, the more eDNA was enzymatically resistant. However, it should be noted that even the pool of DNAse-resistant eDNA adsorbed on detritus ('particulate DNA' according to the old definition) may probably serve as an enzymatically hydrolysable dissolved DNA (EH-dDNA) source. The adsorption of dDNA on detrital particles, similar to the adsorption of other organic molecules, can be seemingly partially reversible. Therefore, desorption of epDNA may additionally increase the concentration of EH-dDNA in lake water. Consequently, the pool of eDNA potentially available for the bacteria in lake water must, in fact, even exceed the EH-DNA pool detected by the proposed enzymatic method.

EH-DNA in lake water — concentration, origin and ecological importance

Differentiation of EH-DNA (assayed by the enzymatic approach) from the total dDNA pool (determined chemically) might be especially important for description of the ecological role of free deoxyribonucleic acid as a regenerated P source for aquatic microorganisms. Although EH-DNA concentration was generally lower than dDNA concentrations (Table 2), it still amounts to on average from 7.7 to 79.6 µg l^{-1} in mesotrophic and hypertrophic lakes, respectively. The mean contribution of EH-DNA phosphorus to the total DOP varied from 11% in the mesotrophic Lake Kuc to 27.6% in the hypertrophic Lake Szymon (Fig. 5). The single values changed from 8.8 to 28.1% except for Lake Szymon, where the content of EH-DNA phosphorus in DOP was relatively stable (26.9 to 28.1%).

Table 3. Linear correlations between EH-DNA concentrations and selected ecological parameters in the surface (1 m depth) of the studied lakes. Correlations and linear regressions (± standard error) were calculated from individual pairs of data collected during the summer stagnation period (17 May to 10 August 1995). Sl: slope, Cst: constant

Parameter	Secchi depth	Chl *a* + phaeophytin	Bacterial number	DNAse activity
EH-DNA	r = 0.57 n = 12, p < 0.05 Sl = −5.8434 ± 2.6420 Cst = 38.1053 ± 18.5434	r = 0.91 n = 11, p < 0.01 Sl = 0.4952 ± 0.0761 Cst = 6.9305 ± 9.8935	r = 0.79 n = 11, p < 0.01 Sl = 4.0918 ± 1.0720 Cst = −13.1251 ± 14.6019	r = 0.80 n = 15, p < 0.01 Sl = 18.1844 ± 3.6778 Cst = 14.2266 ± 13.8716
DNAse activity			r = 0.80 n = 11, p < 0.01 Sl. = 0.2138 ± 0.0528 Cst = −1.2890 ± 0.7186	

Table 4. Concentrations of enzymatically hydrolysable DNA (EH-DNA) in the surface layer (1 m depth) of Lake Mikołajskie 26 July 1995. EH-DNA: enzymatically hydrolysable dissolved DNA (EH-dDNA) + enzymatically hydrolysable DNA adsorbed on seston particles (EH-pDNA); SD: standard deviation of 5 replicates

	Unfiltered sample			Filtrate (0.2 µm Nuclepore)			EH-pDNA
	Control (fluorescence units)	+ DNAse	EH-DNA (µg l^{-1})	Control (fluorescence units)	+ DNAse	EH-dDNA (µg l^{-1})	(µg l^{-1})
Mean (±SD)	163.6 (±2.9)	142.8 (±1.9)	41.5 (±7.0)	133.7 (±1.5)	117.9 (±2.3)	31.6 (±4.7)	9.9

EH-DNA concentrations were, similar to dDNA, positively correlated with TSI (trophic state index), Secchi depth and algal pigments but, as opposed to dDNA concentrations, also well correlated with bacterial numbers (Table 3). These results confirm the role of phytoplankton as an important EH-DNA source in lakes (Siuda et al. 1998). They suggest also that part of the EH-DNA may be of bacterial origin. However, because bacterial secondary production (that compensated bacterial mortality and probably caused increased EH-DNA concentrations in the environment) was not significantly correlated with EH-DNA ($r = 0.41$, $n = 12$), we assume that bacteria were not a significant EH-DNA source. For the apparent positive relationship between EH-DNA concentrations and bacterial numbers one can find other explanations. Bacterial numbers, similar to EH-DNA concentrations in lake water, were dependent on algal pigments concentrations that can be considered as a measure of algal biomass ($r = 0.92$, $n = 11$, $p < 0.001$, slope = 0.0963 ± 0.0138, constant = 5.8745 ± 1.7930). Algae probably not only stimulated bacterial growth by supporting the bacteria with easily utilizable organic carbon compounds (Siuda et al. 1991) but also were an important source (grazing, sloppy feeding, autolysis and phage lysis) of EH-DNA liberated into the environment (Bailiff & Karl 1991).

In spite of the dDNA turnover times (Tt) observed in eutrophied waters that were relatively short (<24 h, Paul et al. 1987, 1989, Siuda & Güde 1996b), concentrations of dDNA reported for these environments were commonly high (up to 88 µg l^{-1}, Karl & Bailiff 1989). However, the accumulation of EH-DNA in eutrophic and hypertrophic lakes observed during the present studies (Table 2) could be, at least partially, explained. Firstly, EH-DNA concentrations were about 30 to 50% smaller than dDNA concentrations reported for analogous types of environment. Moreover, a large part of the EH-DNA pool may be adsorbed on detrital particles (Table 4) and is probably relatively slowly hydrolysable by DNAse. Nevertheless the numbers of the bacteria able to produce DNAses as well as DNAse activity are usually high in these types of environments (Maeda & Taga 1974). This can be, at least partially, confirmed by DNAse overproduction by aquatic bacteria observed during our investigations as a positive, statistically significant correlation between EH-DNA concentration and DNAse activity of single bacterial cells (specific DNAse activity). A similar phenomenon was observed in the case of our studies on aminopeptidase activity in seston-rich environments (Siuda & Chróst unpubl. data). Finally, it also seems possible that dDNA turnover times in freshwater environments reported in the literature might be underestimated because they were commonly calculated just for water samples enriched with *stricte* dissolved DNA easily degradable by DNAses.

In summary, the results of our observations on concentrations, origin, physical properties and availability of eDNA for aquatic bacteria allow us to conclude that applying pure chemical methods for the estimation of DNA susceptible to microbial degradation in freshwater environments cannot be recommended. We propose a quantification of this kind of DNA based on enzymatic hydrolysis of eDNA. Our observations suggest that the EH-DNA pool (and perhaps also extremely labile extracellular RNA) is the most important organic phosphorus source for microplankton in lakes. Enzymatic decomposition of relatively large pool of EH-DNA (supplemented constantly by desorption from ER-dDNA complexes) in highly eutrophic lakes may prevent those environments for most of the time from becoming phosphorus limited.

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