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Determination of dissolved deoxyribonucleic acid concentration in lake water

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ABSTRACT: In this paper we discuss the suitability of the cetyltrimethylammonium bromide (CTAB) precipitation method for the determination of dissolved DNA (dDNA) in lake water. Factors affecting dDNA recovery from the precipitate and further DAPI fluorometric assay were also examined. The proposed method was particularly suitable for routine determination of dDNA concentration in low and moderately eutrophicated freshwater environments. The detection limit of the method varied from 1.5 µg dDNA l^{-1} for mesotrophic waters to 2.5 µg dDNA l^{-1} for eutrophic lakes. Preliminary observations on the concentration of dDNA in the surface water of 8 southern German lakes showed that dDNA concentrations were generally related to their trophic status and varied between 2.5 and 46.0 µg l^{-1} in mesotrophic and from 11.5 to 72 µg l^{-1} in eutrophic waters.

KEY WORDS: Dissolved DNA · Lake water · CTAB · DAPI

INTRODUCTION

Deoxyribonucleic acid (DNA) as one of the basic cell components and the fundamental molecule of heredity for all living organisms is widely distributed in all environments. In aquatic ecosystems DNA occurs as a heterogeneous pool, consisting of particulate DNA (pDNA) and extracellular dissolved (dDNA) forms [free naked DNA, viral DNA and uncharacterized, small (<0.2 µm) particle bound DNA]. Although in recent years a variety of sophisticated biochemical techniques have been applied in ecological investigations, highly variable molecular weight distribution and dynamics of various dDNA forms are still poorly described and understood.

Beebee (1991) found 2 distinctive classes of dDNA in various types of aquatic environments: high molecular weight (>20 kbp, kilo base pairs) material derived probably from viruses and much smaller (1 to 500 bp), nonviral dDNA fragments. Similar results were reported by Reisser et al. (1993), who observed both virus-like particles and free, nonviral dDNA (<500 bp) in Lake Plußsee. DeFlaun et al. (1986) found 0.12 to 35.2 kbp dDNA in <0.2 µm seawater filtrate. Additional information about the nature of dDNA was provided by Maruyama et al. (1993). Their results indicated that in eutrophic seawater most of the dDNA in the 0.03 to 0.2 µm size fraction (80%) was in DNase resistant, coated form.

The spatial and temporal distribution of dDNA in both freshwater and marine environments is relatively well characterized. Minear (1972) found from 4 to 30 µg dDNA in oligo- and eutrophic lowland ponds. Similar dDNA concentrations in various oligo- and mesotrophic environments (0.2 to 44 and 0.5 to 25.6 µg l^{-1}, respectively) were documented by the later studies of DeFlaun et al. (1986), Paul et al. (1989), Maruyama et al. (1993) and Weinbauer et al. (1995). Unusually high levels of dDNA (up to 88 µg l^{-1}) were noted by Karl & Bailiff (1989) in a eutrophic Hawaiian pond.

Although a sophisticated, highly sensitive immunochemical-potentiometric method (Kung et al. 1990) allows measurement of DNA quantities even in the picogram range, the most sensitive fluorometric techniques, at least in theory, permit direct determination of DNA concentrations higher than 4 µg l^{-1} (Berdalet &

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Dortch 1991). Since this detection limit is frequently unrealistic in the case of crude DNA solutions (like lake water filtrates) the majority of natural water samples need preliminary dDNA concentration before fluorometric assay.

For large scale isolation of dDNA from natural water filtrates, various precipitation techniques are the most frequently used. These procedures include: precipitation with $BaSO_4$ (Pillai & Ganguly 1970), absolute (100%) ethanol (DeFlaun et al. 1986), CTAB (Breter et al. 1977, Karl & Bailiff 1989, Weinbauer & Peduzzi in press) and polyethylene glycol (Maruyama et al. 1993). Based on these methods even relatively low dDNA concentrations in various aquatic environments could be determined fluorometrically after binding to 3,5-diaminobenzoic acid (Karl & Bailiff 1989), Hoechst 33258 (Mordy & Carlson 1991), ethidium bromide (Abdel-Rahman et al. 1976) and other fluorogenic compounds (Berdalet & Dortch 1991).

The aim of this study was to develop a simple and fast method for routine dDNA determination in freshwater environments useful for ecological investigations. For this purpose we combined the dDNA precipitation technique (by CTAB treatment) and DAPI fluorometric assay.

METHODS

Sample collection. Lake water samples (5 l) were collected into polypropylene bottles from the surface layer (1 m) of Lake Constance and 7 small southern German lakes of various trophic state during the summer stagnation period. All samples were filtered through 0.2 µm Nuclepore polycarbonate filters within a few (2 to 3) hours after sampling and divided into 4 portions of 100 to 1000 ml (in relation to expected dDNA concentration). Three of them were replicates. The fourth, enriched with calf thymus DNA (Serva GmbH, Heidelberg, Germany) to 50–100 µg l^{-1} final concentration, was used for internal standardization of the dDNA assay.

dDNA precipitation. Dissolved DNA was precipitated by slow addition of CTAB-NaCl stock solution to stirred 0.2 µm lake water filtrate (2 ml solution per each 100 ml of sample). Samples supplemented with CTAB were frozen at –28°C and stored for further treatment. CTAB-NaCl stock solution was prepared by dissolving 5 g of CTAB (cetyltrimethyl-ammonium bromide, Sigma Chemical Co., St. Louis, MO, USA) in 1000 ml of 0.5 M NaCl.

Collection and solubilization of DNA-CTA salts. To obtain natural dDNA concentrates, frozen samples (250 to 1000 ml) were thawed at room temperature and insoluble dDNA-CTA salts were collected by centrifugation (15000 rpm, 3400 × *g*, 0.5 h) and solubilized in 10 ml of 1 M NaCl (3 h, 40°C, mixing). Soluble DNA sodium salts were separated from insoluble residue by additional centrifugation (5000 rpm, 3000 × *g*, 15 min). Clear supernatant was used for final dDNA determination.

DNA assay. DNA concentrations in standards and natural dDNA concentrates were determined spectrofluorometrically. Stock solutions (1 mg ml^{-1}) of DAPI (diamidino-phenylindole, Serva GmbH) and DNA from calf thymus (Serva GmbH) were prepared in deionized water and stored at –28°C for not longer than 1 mo. To prepare the standard curve, the DNA stock solution was thawed at room temperature and diluted to the required concentration with 1 M NaCl. DAPI 'working' solution was obtained by dilution of the stock solution in 0.1 M Tris-Na buffer, pH 8.3 (Prasad et al. 1972).

To 2.3 ml of sample (standard or natural dDNA concentrate), 0.1 ml 1 M Tris-Na buffer (pH 8.3) and 0.1 ml (10 µg ml^{-1}) of DAPI dye solution was added. After 10 min incubation in the dark, samples were shaken and their fluorescence was measured with a Perkin-Elmer Spectrofluorometer LS 50 B at 365 nm (excitation) and 445 nm (emission).

All dDNA concentrates and untreated water filtrates were also tested for their autofluorescence (without DAPI) according to DeFlaun et al. (1986). Autofluorescence was treated as a blank and subtracted from the fluorescence of the sample with DAPI. dDNA concentrations were read from the standard curve and corrected finally using the internal standard method (Karl & Bailiff 1989).

RESULTS

We tested various conditions of dDNA precipitation, conversion of dDNA-CTA precipitate into dDNA sodium salts and dDNA-DAPI complex fluorescence measurement. Additionally, potential DNA leakage from cells disrupted during sample filtration was examined. The flow chart of the final recommended procedure for sample treatment during dDNA determination in lake water is presented in Fig. 1.

Sample filtration

Adsorption of dDNA on various types of filters commonly used for separation of particulate organic matter (POM) is shown in Fig. 2. Polycarbonate filters (0.2 µm Nuclepore) adsorbed less than 2.5% of dDNA during filtration of 10 ml lake water filtrate (0.2 µm Nuclepore) enriched with calf thymus DNA to a final

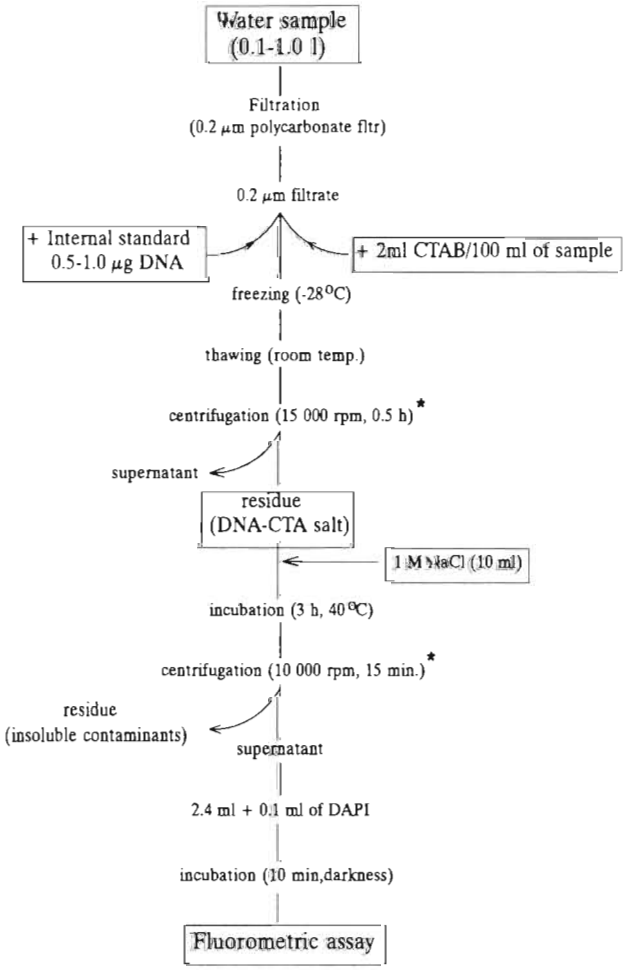

Fig. 1 Final recommended procedure for dissolved DNA determination in lake water. For each sample triplicates and subsample containing internal standard were prepared. *Centrifugation may be replaced by filtration through 0.2 µm polycarbonate membrane filters. Additional explanations in the text

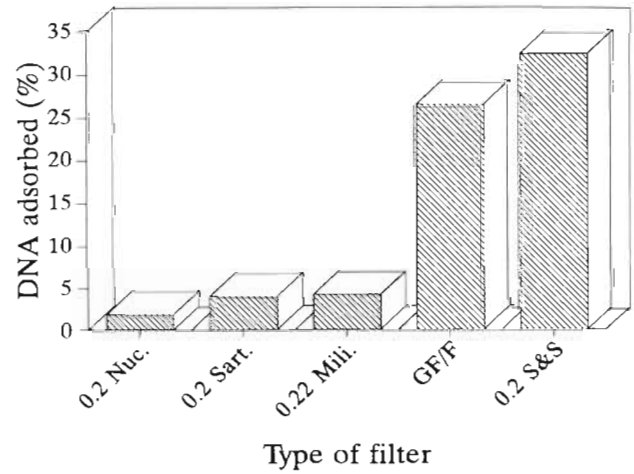

Fig. 2. Adsorption of DNA on various types of filters. Portions (10 ml) of Lake Constance water filtrate (0.2 µm Nuclepore) enriched with calf thymus DNA (1 µg ml^{-1} final conc.) were filtered through: Nuclepore 0.2 µm, polycarbonate (Nuc.); Sartorius 0.2 µm, cellulose acetate (Sart.); Millipore 0.22 µm, cellulose acetate (Milli.); Schleicher and Schuell 0.2 µm, cellulose nitrate (S&S); and Whatman 0.7 µm, glass fiber (GF/F) filters; n = 3, SD ≤ 5

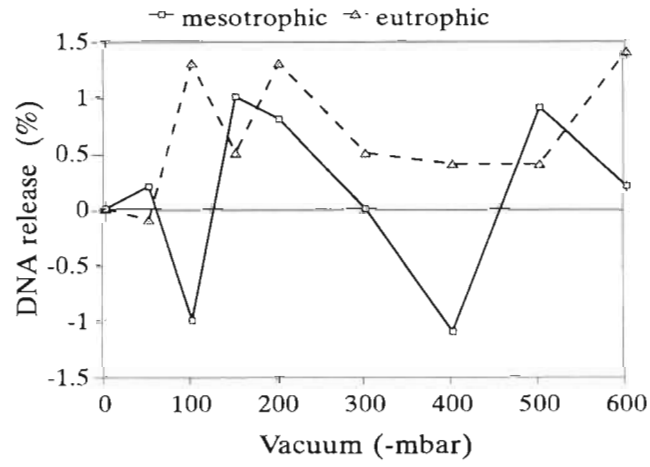

Fig. 3. Effect of filtration pressure on dDNA liberation from the seston in meso- and eutrophic lake water sample (0.5 l) filtered through 0.2 µm Nuclepore membrane filters. Straight line: DNA release (0%) during filtration of the sample under atmospheric pressure (control); n = 3, SD ≤ 1.2

concentration of 1 µg ml^{-1}. Under the same filtration conditions cellulose acetate filters (Sartorius 0.2 µm, Millipore 0.22 µm) removed about 5% of dDNA from the solution. The maximal dDNA loss during filtration was observed when cellulose nitrate (Schleicher and Schuell), membrane filters (32%) or Whatman GF/F glass fiber filters (25%) were used. Moreover, as opposed to polycarbonate filters, adsorption of dDNA to cellulose nitrate and cellulose acetate filters was dependent on dDNA concentration in filtered water until the filter was saturated.

The relationship between filtration pressure and dDNA concentration in filtrate is shown in Fig. 3. We did not observe a distinct increase of dDNA concentration in filtrate of mesotrophic lake water up to −600 mbar filtration pressure (p). However, when water from a eutrophic lake was filtered using p less than −50 mbar, a small increase (0.4 to 1.4%) of dDNA in the liquid phase was observed.

dDNA precipitation

The sensitivity of the dDNA assay by DAPI technique in 0.1 M Tris-NaCl buffer free of fluorescent contaminants allows detection of dDNA concentra-

tions as low as 10 µg l^{-1} (Walser & Güde 1994). However, the DAPI method applied for crude dDNA solutions (i.e. mesotrophic lake water filtrates) permits successful measurement of dDNA concentrations in the range 25 to 30 µg l^{-1}. Therefore, the natural dDNA in most of the eutrophic lake water samples had to be concentrated by the CTAB precipitation technique before fluorescence measurement. We tested the efficiency of dDNA precipitation (from pure dDNA solutions and various 0.2 µm lake water filtrates) and stability of precipitated CTA-DNA complexes during sample storage. The amount of dDNA found in the final concentrate obtained by the proposed method was proportional to the volume of the sample precipitated, in the range 100 to 1000 ml. Theoretically it would allow the concentration of natural dissolved DNA by 100 times and extend the dDNA detection limit up to 0.25 µg l^{-1}

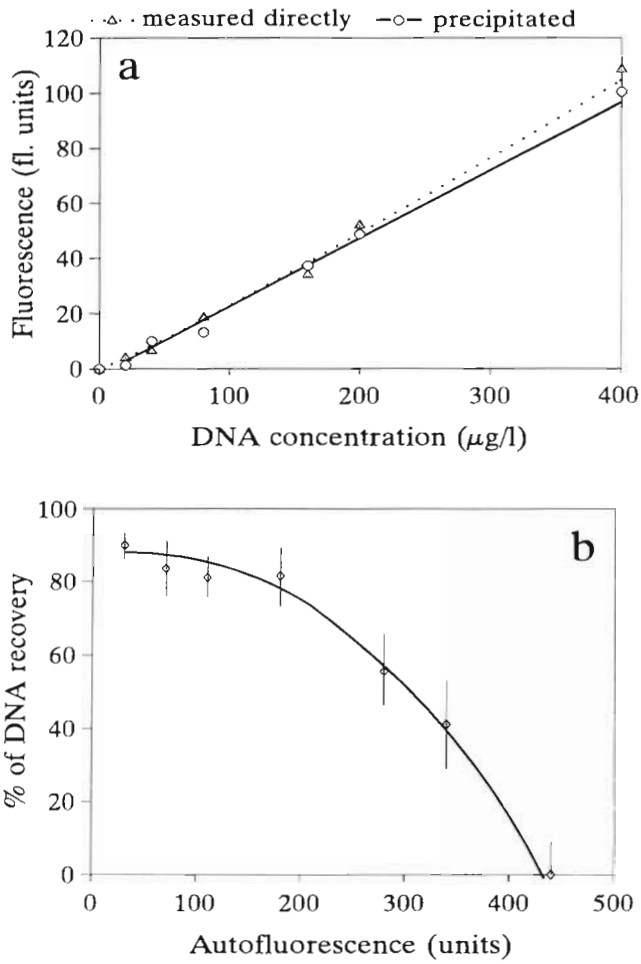

Fig. 4. Recovery of dDNA precipitated with CTAB according to standard procedure. (a) DNA from calf thymus dissolved in distilled water (DNA recovery 95 to 100%). (b) Natural DNA from various 0.2 µm lake water filtrates (DNA recovery was related to fluorogenic contaminant content)

The comparison of standard curves prepared from precipitated and non-treated dDNA standards shows that for contaminant-free dDNA solutions (dDNA stock solution diluted with distilled water) dDNA recovery after precipitation reached 95 to 100% (Fig. 4a). However, dDNA precipitation from lake water by the CTAB method seems not to be as selective as had been observed by Karl & Bailiff (1989) during studies on dDNA concentrations in open ocean habitats. Our methodological experiments revealed that the efficiency of dDNA precipitation and recovery was strongly influenced by the presence of large quantities of undefined, fluorescent organic compounds in filtrates of natural lake water. As can be seen in Fig. 4b these substances decreased dDNA recovery from 85 (in dDNA concentrates with non-DAPI fluorescence lower than 200 fl. units) to almost 0% (in concentrates with background fluorescence higher than 450 fl. units).

Since lake water filtrate may contain dDNA hydrolyzing enzymes (free DNase and DNase liberated from disrupted cells during filtration) the potential possibility of dDNA loss during further steps of dDNA determination had to be tested. We found that precipitated dDNA was well protected against DNase attack during the analysis and sample storage. Although calf thymus DNA added to 0.2 µm lake water filtrates quickly disappeared from the solution (V_{max} varied from 0.367 ± 0.053 SD to 4.588 ± 0.941 µg l^{-1} h^{-1}; Siuda & Güde unpubl.), the concentration of the same DNA in filtrates treated with CTAB remained stable over 72 h (Fig. 5).

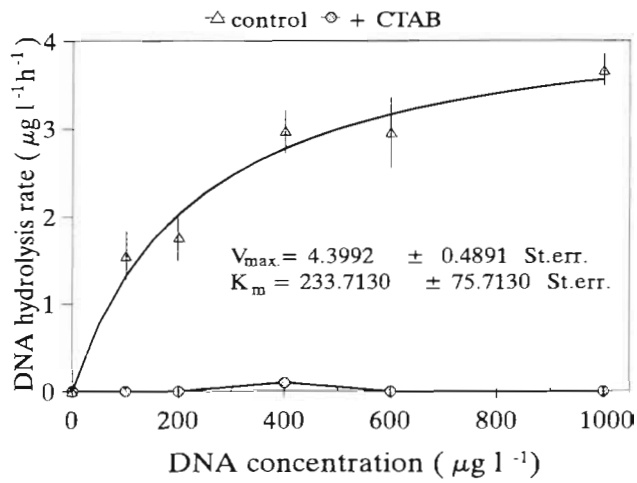

Fig. 5. Inhibition of calf thymus DNA hydrolysis by CTAB, in natural lake water sample. Portions (20 ml) of 0.2 µm lake water filtrate were supplemented with DNA and incubated for 24 h at 2°C. To maintain sterility NaN_2 was added (to 0.3% final conc.). DNA hydrolysis rate was expressed as DNA decrease in the sample per time unit

Solubilization of DNA-CTA precipitate

For fluorometric determination by the DAPI method, DNA had to be liberated from insoluble DNA-CTA salts and transferred into the liquid phase. For this purpose various concentrations of Tris-Na buffer and NaCl solutions as possible solubilizers were tested. Although the solubility of the DNA-CTA complex in both types of solvents was similar (Siuda unpubl. data), due to higher background fluorescence of concentrated (1 M) Tris-Na buffer we decided to use an NaCl solution for further analyses.

DNA recovery from the precipitate increased with NaCl concentration, temperature and time of resolubilization. Good efficiency of DNA-CTA conversion into DNA sodium salts (almost 100% of DNA recovery) can be achieved in 2 M NaCl after 4.0 h of incubation at room temperature (20°C) or alternatively after 1.5 to 2.0 h of incubation in 1 M NaCl at 40°C (Fig. 6a, b). A further increase of the temperature of the process could have shortened this step of the DNA determination procedure but this is not recommended; for example, we found that incubation of the DNA solution (100 µg l^{-1} in 1 M NaCl) for 1 h at 80°C caused partial DNA hydrolysis and approximately 50% of DNA-DAPI fluorescence loss. Finally, we decided to solubilize the DNA-CTA precipitate routinely in 1 M NaCl for 3 h at 40°C. These conditions sufficiently accelerated the formation of dDNA sodium salts, prevented possible DNA hydrolysis and resulted in highest (>90%) dDNA recovery.

Collection of DNA-CTA precipitate and soluble dDNA sodium salts

Suitability of filtration and centrifugation techniques for collection of DNA-CTA precipitate and separation of dissolved DNA sodium salts from insoluble contaminants was tested on natural Lake Constance water samples. During filtration of dDNA concentrates resuspended in 1 M NaCl (the last step of the procedure) through 0.2 µm Nuclepore membrane filters, usually 84 to 93% of DNA was recovered in the filtrate. This was about 10% more than after centrifugation (78 to 85% of dDNA recovery) and about 60% more than after filtration of the concentrates through GF/F or cellulose nitrate filters (23 to 30% recovery) which strongly adsorbed dDNA sodium salts from the solution. Although we have found that filtration through polycarbonate filters is the more useful technique for dDNA concentration than centrifugation, for routine work we decided to use centrifugation, which was faster and made it possible to process several samples simultaneously.

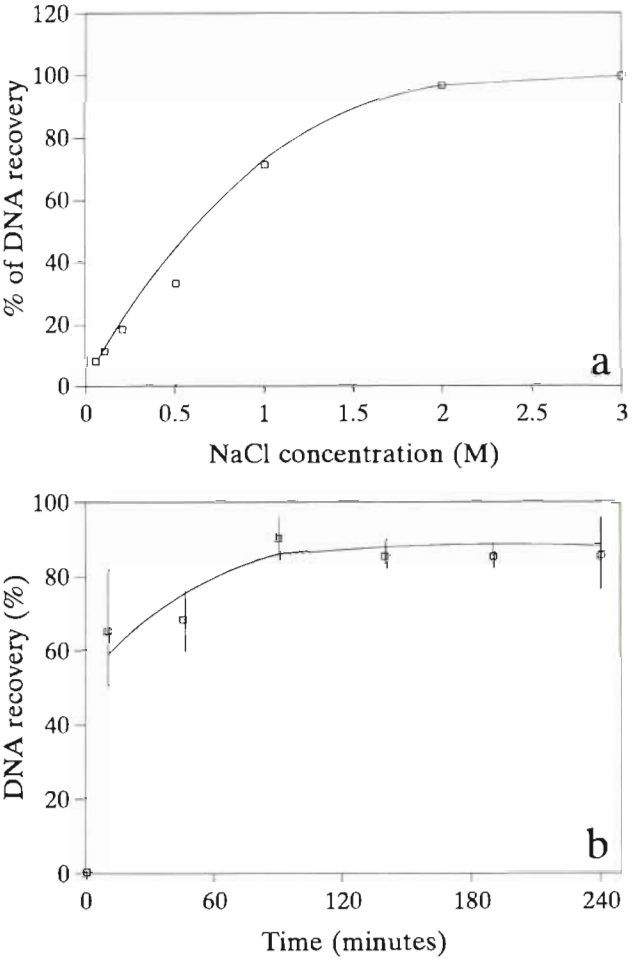

Fig. 6. DNA recovery during DNA-CTA salt solubilization as a function of: (a) concentration of the solubilizer (NaCl solution), precipitates were incubated for 3 h at 40°C, n = 3, SD ≤ 6; (b) time of solubilization (in 1 M NaCl at 40°C)

Measurement of dDNA-DAPI fluorescence

At this point of our methodological investigations we focused mainly on the enhancement of dDNA-DAPI fluorescence by solubilizers of the DNA-CTAB complex and on interference from undefined compounds coprecipitated with dDNA during the CTAB precipitation procedure. We found that the detection of dDNA-DAPI complex dissolved in Tris-Na buffer resulted in a fluorescence yield almost linearly dependent on buffer concentration (Fig. 7a), but poorly related (linear regression slope, $a = 0.0938, \pm 0.0065$) to the amount of dDNA in the solution (Fig. 7b). dDNA-DAPI fluorescence in NaCl was relatively stable in the 0.5 to 2.0 M NaCl concentration range (Fig. 7a) and well related to dDNA concentration in the solution (Fig. 7b, $a = 0.2361, \pm 0.01227$).

The autofluorescence (fluorescence of DAPI-free sample) of mesotrophic and of some eutrophic, non-

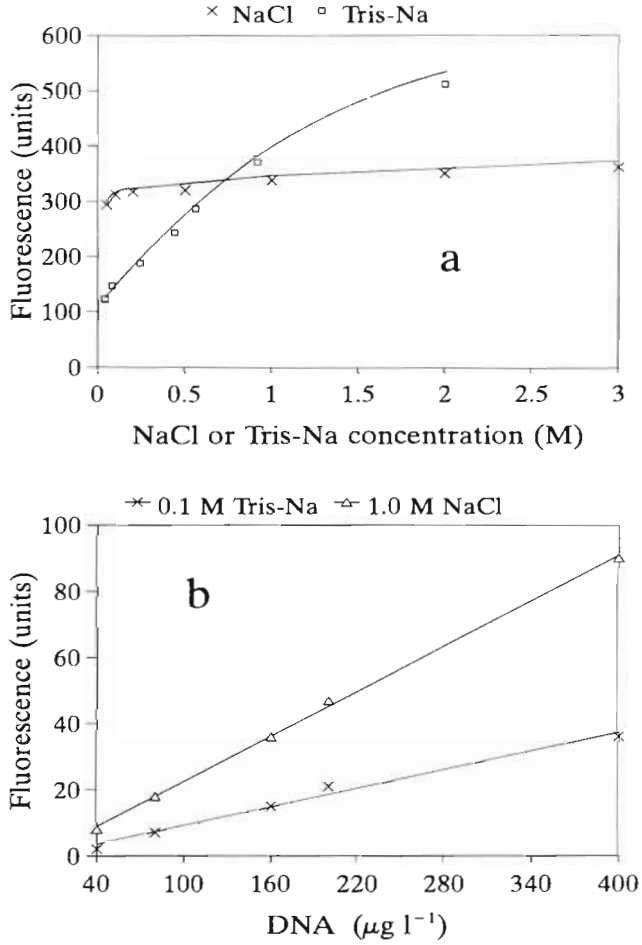

Fig. 7 (a) Fluorescence of DNA-DAPI (200 µg l^{-1} calf thymus DNA) complex in various NaCl and Tris-Na solutions, n = 3, SD ≤ 5. (b) DNA standard curves prepared on 1 M NaCl and 0.1 M Tris-Na buffer, n = 3, SD ≤ 7

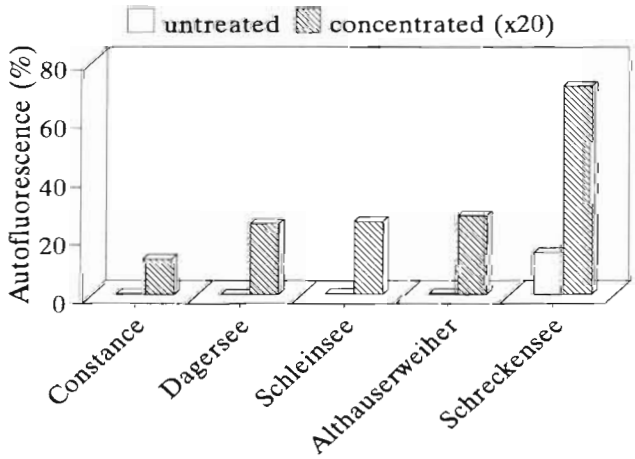

Fig. 8. Autofluorescence (non-DAPI fluorescence) of unknown fluorogenic contaminations in untreated 0.2 µm lake water filtrates and natural dDNA concentrates (in 1 M NaCl) obtained by the CTAB technique

concentrated samples did not exceed 1% of their total fluorescence (DAPI-dDNA + non-DAPI fluorescence) and in general could be negligible. However, we had to consider this for natural dDNA concentrates, where not only dDNA but also other fluorescent contaminants (precipitated by CTAB and solubilized by 1 M NaCl) could have been concentrated. These resulted in the sample autofluorescence increase to 15–65% (Fig. 8). The lowest level of fluorogenic contaminants was found in nonconcentrated (<0.3%) and concentrated (<24%) samples from mesotrophic lakes (Lake Dagersee and Lake Constance) whereas in Lake Schreckensee, highly eutrophicated and rich in organic substances, the highest autofluorescence of nonconcentrated as well as concentrated samples (14.1 and 71.3%, respectively) was observed.

Concentrations of dDNA in lake water

Most of the measurements of dDNA concentrations were carried out in mesotrophic (Lake Constance) and eutrophic (Lake Schleinsee) waters. Additionally some small Bavarian lakes in various trophic states were examined (Table 1).

dDNA concentration in the surface layer of these lakes was generally related to their trophic status. The lowest concentrations were found in the pelagial of mesotrophic Lake Constance (up to 15 µg l^{-1}); medium and highest dDNA contents were observed in samples from Lake Constance taken from sites close to the littoral zone (15 to 22 µg l^{-1}) and in eutrophic lakes (20 to 72 µg l^{-1}). Sometimes, relatively high concentrations of free deoxyribonucleic acid were detected in mesotrophic lakes (Lake Illmensee and Lake Dagersee: 44.9 and 46.0 µg l^{-1}, respectively) and relatively low (20.8 µg l^{-1}, Lake Althauserweiher) dDNA content in hypertrophic lakes was found. Moreover, we could not detect any free dDNA in samples taken from the dystrophic, brown water Lake Buchsee (data not shown).

The concentration of dDNA in the surface layer of Lake Constance during summer stagnation was examined more extensively. Relatively low amounts of dDNA were observed in the beginning of June during the clear water phase (below 5 µg l^{-1}). In July and in the first days of August the dDNA content in lake water was relatively stable (10 to 15 µg l^{-1}) and reached its maximum during first phytoplankton blooms.

DISCUSSION

This report describes the factors influencing the assay of dissolved deoxyribonucleic acids in lake water based on the enhancement of fluorescence seen when

Table 1 Concentration of dissolved DNA in surface waters (1 m) of 7 south German lakes. Each value represents a mean of triplicate determinations, standard deviation ≤ 9%

Lake (trophic status)	Date	dDNA (µg l^{-1})
Constance (mesotrophic)	10 Oct 1991	18.8
	17 Nov 1991	9.0
	20 Nov 1991	22.0
	8 Jun 1991	4.6
	15 Jun 1991	2.5
	24 Jun 1991	14.8
	1 Jul 1991	9.0
	6 Jul 1991	12.2
	14 Jul 1991	5.3
	24 Jul 1991	11.5
	28 Jul 1991	12.0
	3 Aug 1991	7.7
	11 Aug 1991	21.3
	17 Aug 1991	18.6
Dagersee (mesotrophic)	21 Oct 1991	5.9
	22 Jun 1992	46.0
Illmensee (mesotrophic)	22 Oct 1991	32.4
	22 Jun 1992	44.9
Schleinsee (eutrophic)	3 Dec 1991	46.7
	29 Jun 1992	36.2
	30 Jul 1992	49.0
	1 Sep 1993	11.5
Schreckensee (eutrophic)	22 Jun 1992	72.0
Althauserweiher (highly eutrophic)	21 Oct 1991	20.8
	22 Jun 1992	50.1
Lengenweiler (highly eutrophic)	21 Oct 1991	41.7

the fluorogenic dye DAPI binds to DNA. The determination of DNA by the DAPI technique in tissue and cell homogenates was described in detail by Kapuściński & Skoczylas (1977) and Brunk et al. (1979). The suitability of this technique for ecophysiological applications was confirmed by earlier studies made in our laboratory (Krückemeier 1987, Walser & Güde 1994). However, to adapt the method to the determination of the relatively low concentrations of dDNA commonly present in lake water it was necessary to develop a simple method of dDNA concentration. For this purpose we chose the method of dDNA precipitation by cetyltrimethylammonium bromide (CTAB).

In this paper the term 'dissolved DNA' is operationally defined as DNA which passes through a 0.2 µm membrane filter, can be removed from the filtrate by CTAB precipitation and is able to create a fluorescent complex with DAPI dye.

Filtered (0.2 µm) natural water may contain various kinds of dDNA: both double and single stranded free naked dDNA molecules, free dDNA adsorbed by colloids or other small (<0.2 µm) detrital particles, protein encapsulated, viral DNA (Bergh et al. 1989, Beebee 1991, Maruyama et al. 1993), and cell membrane coated dDNA, i.e. ultramicrobacteria (Tabor et al. 1981, Velimirov 1994) and phospholipid membranous vesicles (Nagata & Kirchman 1992). Although CTAB precipitates both double (<121 bp long) and single stranded (>200 nucleotides) DNA forms (Sclafani & Wechsler 1981, Cockerill 1988), using DAPI technique for DNA detection restricts the measurable DNA pool exclusively to double stranded DNA fragments >121 bp long. Nevertheless, because up to now it has not been clear what part of the viral DNA (also detectable by DAPI method) can be precipitated with CTAB, more recent investigations (Weinbauer et al. 1993) revealed that the contribution of viral DNA to the DNA-CTA precipitate may be substantial. Viral DNA may account for 0.7 to 88.3% of the total dDNA pool in the Adriatic Sea.

The proposed technique has 4 critical steps: separation of dDNA from the total nucleic acids pool existing in lake water, precipitation as a CTA salt, conversion of insoluble precipitate into soluble dDNA sodium salts and detection of dDNA-DAPI complex in the dDNA concentrate obtained.

Sample filtration

Although considerable release of dDNA from living microorganisms during filtration of both eutrophic and mesotrophic lake water, under pressure up to −500 mbar, was not observed (Fig. 4), we suggest that a pressure of −50 mbar should not be exceeded in order to prevent eventual dDNA release from disrupted cells. Similar conditions of sample filtration were recommended by DeFlaun et al. (1986) who found that vacuum up to 150 mbar did not cause DNA release from the particulate to the liquid phase. The lack of distinct 'filtration effect' during dDNA separation can probably be explained by the fact that prokaryotic cells which contain naked DNA are relatively resistant to degradation. On the other hand DNA released rapidly from damaged eukaryotic microorganisms is usually enclosed inside the nucleus and thus additionally protected from fast solubilization by the nuclear membrane. Since nuclei are retained by a 0.2 µm filter the increase in dDNA concentration of eukaryotic origin in the filtrate was not observed.

In our opinion, the opposite phenomenon is more important. We found (Siuda & Güde unpubl.) that part of the potentially measurable dDNA can be lost during sample filtration. dDNA has a strong tendency to be adsorbed on some types of filters and on seston particles suspended in natural waters (Romanowski et al.

1991). Ogram et al. (1994) observed a similar phenomenon in soils.

We realized that highly polymerized calf thymus DNA was probably not the best standard for testing various filters for adsorbtion of natural dDNA, but we used it instead of natural dDNA extract to avoid additional problems with interpretation of results that might be caused by fluorogenic contaminants coprecipitated with dDNA during extraction. Assuming that shorter dDNA fragments adsorb to the detrital particles to a greater extent than longer particles, which was proved for most kinds of soils (Ogram et al. 1994), one could conclude that filters strongly adsorbing calf thymus DNA should probably also, even more strongly, adsorb less polymerized natural dDNA.

Cellulose nitrate (S&S) and glass fiber filters (Whatman GF/F) were found to be of limited usefulness for dDNA determination. They retained 25 to 30% of calf thymus DNA dissolved in natural water filtrate (Fig. 1). The amount of dDNA adsorbed on those types of filters is a function of both dDNA concentration in filtered water and the active surface of the filter. For each step of quantitative dDNA analysis we recommend using polycarbonate filters (Nuclepore) retaining only negligible amounts of DNA dissolved in the sample, in concentrations close to the range of natural concentrations (Fig. 2).

Further results of our experiments (Siuda & Güde unpubl.) showed that natural seston could also effectively eliminate dDNA from the solution. Considerable amounts of dDNA from calf thymus added to unfiltered water samples taken from lakes of various trophic states disappeared immediately from the liquid phase and could not be found in 0.2 µm sample filtrates. Although the amount of seston in mesotrophic lake water was often small and did not cause distinct adsorption effect, in highly eutrophicated lake water samples even 15 to 30 µg of calf thymus DNA could be removed by the seston from liquid phase. dDNA adsorption phenomena occurring in lake water had 2 important ecological implications. Firstly, we could measure only a part of the total amount of extracellular DNA in 0.2 µm sample filtrate, and secondly, adsorption and complexation phenomena may seriously alter dDNA transformations in aquatic environments. This last assumption needs more intensive study in the future.

During our investigations we also tried to quantify the possible loss of dDNA from the filtrate caused by deoxyribonuclease action during sample filtration and DNA-CTA precipitate storage. The results of a set of experiments not reported here (Siuda & Güde unpubl.) showed that dDNA hydrolysis rate (v) in surface waters of meso- or eutrophic lakes calculated for unfiltered sample and ambient dDNA concentrations varied from 0.019 to 0.169 µg l^{-1} h^{-1}. These values were comparable with the results of Turk et al. (1992) obtained by a radioisotope method in sea water (v = 0.4 µg l^{-1} h^{-1}). Therefore, dDNA loss during 1 to 2 h of preparation of the sample is probably insignificant and does not substantially affect the measurable dDNA pool. Additional protection against dDNA enzymatic degradation was achieved by gentle filtration ($p > -50$ mbar) of the sample that prevented DNase liberation from filtration-sensitive microplankton cells to some extent. Finally, CTAB addition to the filtrate also efficiently inhibited nuclease action (Fig. 5).

dDNA precipitation

CTAB is a cationic detergent that is used in molecular biology for isolation of nucleic acid and nucleoproteins by precipitating them from dilute sodium chloride solutions (Bellamy & Ralph 1968). It creates insoluble CTA-nucleic acid salts that can be easily separated from the solution by filtration or centrifugation techniques. Freezing of CTAB supplemented samples (Sibatini 1970) allows for easier separation of the DNA-CTA complexes from the liquid phase and prevents micelle or gel formation, which sometimes occurs if excess CTAB is added rapidly (Bellamy & Ralph 1968). NaCl addition (10 nmol l^{-1}) restricts the potential possibility of precipitation with CTAB of various acidic polysaccharides (Bellamy & Ralph 1968, Karl & Bailiff 1989).

The CTAB method was introduced by Breter et al. (1977) for isolation of dDNA from aquatic environments and developed by Karl & Bailiff (1989). In spite of some inconveniences (see below) CTAB precipitation still has some important advantages over the other techniques of dDNA concentration from natural water filtrates.

(1) Creation of DNA-CTA salts inhibits nuclease action (Fig. 5) and permits preliminary fractionation of dDNA based on molecular weight and conformation (Honig et al. 1973).

(2) Use of CTAB as a dDNA precipitating factor also allows purification of dDNA from low molecular weight dissolved organic compounds and considerably reduces the amount of fluorescently active contaminants in the dDNA concentrate (Karl & Bailiff 1989).

(3) It also seems to be simpler and less time consuming than other methods of dDNA concentration (i.e. methods reported by Pillai & Ganguly 1970, Minear 1972, DeFlaun et al. 1986).

However, we do not recommend this procedure in environments rich in high molecular weight organic compounds (HMWC). Our investigations revealed that CTAB did not precipitate dDNA in filtrates of lake

water as selectively as was reported by Karl & Bailiff (1989) for HMWC-poor oceanic waters. In some of our samples undefined dissolved organic material that coprecipitated with dDNA significantly affected conversion of DNA-CTA precipitate into DNA sodium salts (Fig. 4b) and by increasing of background fluorescence and quenching influenced the dDNA-DAPI fluorescence yield (Fig. 8). These phenomena as well as strong dDNA adsorption on detrital particles probably caused underestimation of dDNA concentration in highly eutrophicated Lake Althauserweiher in October 1991 and were responsible for difficulties with dDNA detection in polyhumic Lake Buchsee.

In optimal conditions (standard curves prepared with 1 M NaCl or deionized water) the DAPI method permits determination of 20 to 25 µg dDNA l^{-1}. This means that the difference between 'blank' and sample fluorescence for those dDNA concentrations was statistically significant. The recovery of dDNA from concentrates obtained from natural samples by CTAB treatment was, in most cases, about 80 to 90%. Therefore the lowest dDNA concentrations that could be reliably determined in those concentrates were 25 to 31 µg DNA l^{-1}. In some analyzed mesotrophic lake water samples dDNA was concentrated 100× and dDNA concentration as low as 0.25 to 0.31 µg l^{-1} could still be successfully measured. However, in practice we do not recommend concentration of dDNA by more than 10 to 50× (for eutrophic and mesotrophic lake water, respectively). These concentration conditions do not cause excessive accumulation of undefined dDNA contaminants in the concentrate and permit the determination of 0.12 to 0.15 µg dDNA l^{-1}, which seems to be sufficient for the majority of freshwater samples.

We determined dDNA in 8 south German lakes and found a relatively high dDNA concentration in their surface water (Table 1), similar to that reported for other freshwater environments (Minear 1972, DeFlaun et al. 1986, Paul et al. 1989, 1990, Reisser et al. 1993). These results may suggest that dissolved deoxyribonucleic acid represents a quantitatively significant reservoir of potentially available P for planktonic microorganisms. Cautious calculations showed that dDNA phosphorus may constitute 30 to 60% of the total dissolved organic P pool in the lakes studied. Therefore, enzymatic liberation of P from dDNA by DNase (Paul et al. 1989) and subsequently by 5'-nucleotidase action (Ammerman & Azam 1991, Siuda & Güde 1994) could probably be one of the most effective pathways of P regeneration in freshwater ecosystems.

Finally, we conclude that the techniques reported in this paper were designed to provide a rapid, simple and economical means of recovering and quantifying dDNA from aquatic environments. We do not recommend this method for precise and detailed biochemical dDNA studies. However, its acceptable accuracy, combined with cost effectiveness, the possibility of dDNA-CTA precipitate storage and analysis of a large number of samples in minimal processing time, make it useful, in ecological studies, for routine dDNA determination in all mesotrophic and in the majority of eutrophic environments.

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LITERATURE CITED

Abdel-Rahman A, El-Hamalawi J, Thompson S, Barker GR (1976) The fluorometric determination of nucleic acids in pea seeds by use of ethidium bromide complexes. Analyt Biochem 67:384–391

Ammerman JW, Azam F (1991) Bacterial 5'-nucleotidase activity in estuarine and coastal marine waters: role in phosphorus regeneration. Limnol Oceanogr 36:1437–1447

Beebee T (1991) Analysis, purification and quantification of extracellular DNA from aquatic environments. Freshwater Biol 25:525–532

Bellamy AR, Ralph RK (1968) Recovery and purification of nucleic acids by means of cetyltrimethylammonium bromide. In: Grossman L, Moldave K (eds) Methods in enzymology. Academic Press, New York, p 156–160

Berdalet E, Dortch Q (1991) New double-staining technique for RNA and DNA measurement in marine phytoplankton. Mar Ecol Prog Ser 73:295–305

Bergh O, Børsheim KY, Bratbak G, Heldal M (1989) High abundance of viruses found in aquatic environments. Nature 340:467–468

Breter HJ, Kurelec B, Müller WEG, Zahn RK (1977) Thymidine content of seawater as a measure of biosynthetic potential. Mar Biol 40:1–8

Brunk CF, Jones KC, James TW (1979) Assay for nannogram quantities of DNA in cellular homogenates. Analyt Biochem 92:497–500

Cockerill PN (1988) Two methods that faciliate autoradiography of small ^{32}P-labelled DNA fragments following electrophoresis in agarose gels. Analyt Biochem 168:451–454

DeFlaun MF, Paul JH, Davis D (1986) Simplified method for dissolved DNA determination in aquatic environments. Appl Environ Microbiol 52:654–659

Honig W, Zachn RK, Heintz W (1973) A method for characterization and fractionation of deoxyribonucleic acid by continuous turbidimetry of cetyltrimethylammonium-DNA precipitates. Analyt Biochem 55:34–50

Kapuściński J, Skoczylas B (1977) Simple and rapid fluorometric method for DNA microassay. Analyt Biochem 83: 252–257

Karl DM, Bailiff MD (1989) The measurement and distribution of dissolved nucleic acids in aquatic environments. Limnol Oceanogr 34:543–558

Krückemeier G (1987) Kombinierte fluorometrische DNA- und RNA-Bestimmung mit DAPI und Ethidiumbromid nach quantitativer Nukleinsäureextraktion aus *Scenedes-*

mus und *Fragilaria*-Zellen. Diploma thesis, University of Essen - Gesamthochschule

Kung VT, Panfili PR, Sheldon EL, King RS, Naganis PA, Gomez B Jr, Ross DA, Briggs J, Zuk RF (1990) Picogram quantitation of total DNA using DNA-binding proteins in silicon sensor-based system. Analyt Biochem 187:220–227

Maruyama A, Oda M, Higashihara T (1993) Abundance of virus-sized non-DNase-digestible DNA (coated DNA) in eutrophic seawater. Appl Environ Microbiol 59:712–716

Minear RA (1972) Characterization of naturally occurring dissolved organophosphorus compounds. Environ Sci Technol 6:431–437

Mordy CW, Carlson DJ (1991) An evaluation of fluorescence techniques for measuring DNA and RNA in marine microorganisms. Mar Ecol Prog Ser 73:283–293

Nagata T, Kirchman DL (1992) Release of macromolecular organic complexes by heterotrophic marine flagellates. Mar Ecol Prog Ser 83:233–240

Ogram AV, Mathot ML, Harsh JB, Boyle J, Pettigrew CA Jr (1994) Effects of DNA polymer length on its adsorption to soils. Appl Environ Microbiol 60:393–396

Paul JH, Jeffrey WH, Cannon JP (1990) Production of dissolved DNA, RNA, and protein by microbial populations in a Florida reservoir. Appl Environ Microbiol 56:2957–2962

Paul JH, Jeffrey WH, David AW, DeFlaun MF, Cezares LH (1989) Turnover of extracellular DNA in eutrophic and oligotrophic freshwater environments of southwest Florida. Appl Environ Microbiol 55:1823–1828

Pillai TNV, Ganguly AK (1970) Nucleic acid in the dissolved constituents of sea water. J Mar Biol Ass India 14:384–390

Prasad A, Du Mouchelle E, Koniuch D, Oberleas D (1972) A simple fluorometric method for determination of RNA and DNA in tissues. J Lab Clin Med 80:598–602

Reisser W, Grein S, Krambeck Ch (1993) Extracellular DNA in aquatic ecosystems may in part be due to phycovirus activity. Hydrobiologia 252:199–201

Romanowski G, Lorenz MG, Wackernagel W (1991) Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. Appl Environ Microbiol 57: 1057–1061

Sclafani RA, Wechsler JA (1981) High-yield purification of small, single stranded DNA Analyt Biochem 115: 197–202

Sibatini A (1970) Precipitation and counting of minute quantities of labelled nucleic acids as cetyltrimethylammonium salt. Analyt Biochem 33:279–285

Siuda W, Güde H (1994) A comparative study on 5'-nucleotidase (5'-nase) and alkaline phosphatase (APA) activities in two lakes. Arch Hydrobiol 131:211–229

Tabor PS, Ohwada K, Colwell RR (1981) Filterable marine bacteria found in the deep sea: distribution, taxonomy, and response to starvation. Microb Ecol 7:67–83

Turk V, Rehnstam A, Lundberg E, Hagström A (1992) Release of bacterial DNA by marine nannoflagellates, an intermediate step in phosphorus regeneration. Appl Environ Microbiol 58:3744–3750

Velimirov B (1994) Carbon fluxes in the microbial loop: comments. Microb Ecol 28:205–207

Walser R, Güde H (1994) DNA and RNA contents of bacteria-sized particles from Lake Constance. Arch Hydrobiol 131: 257–276

Weinbauer MG, Fuks D, Peduzzi P (1993) Distribution of viruses and dissolved DNA along a coastal trophic gradient in the Northern Adriatic Sea. Appl Environ Microbiol 59:4074–4082

Weinbauer MG, Fuks D, Puskaric S, Peduzzi P (1995) Diel, seasonal, and depth-related variability of viruses and dissolved DNA in the northern Adriatic Sea. Microb Ecol 30: 25–41

Weinbauer MG, Peduzzi P (in press) Comments on the determination of nucleic acids in natural waters by the CTAB-DABA-orcinol method. Sci Total Environ

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