

Mechanisms underlying the impact of humic acids on DNA quantification by SYBR Green I and consequences for the analysis of soils and aquatic sediments

Hubert Zipper^{1,2}, Christiane Buta¹, Katrin Lämmle^{1,2}, Herwig Brunner¹,
Jürgen Bernhagen^{1,3} and Frank Vitzthum^{1,4,*}

¹Laboratory of Biochemistry, Chair for Interfacial Engineering, University of Stuttgart and Fraunhofer IGB, Nobelstrasse 12, D-70569 Stuttgart, Germany, ²Institute of Technical Biochemistry, Allmandring 31, University of Stuttgart, D-70569 Stuttgart, Germany, ³Department of Biochemistry and Molecular Cell Biology, Institute of Biochemistry, University Hospital RWTH Aachen, Aachen, Germany and ⁴Proteomics Research, Dade Behring Marburg GmbH, D-35001 Marburg, Germany

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ABSTRACT

DNA quantification of soils and sediments is useful for the investigation of microbial communities and for the acquisition of their genomes that are exploited for the production of natural products. However, in such samples DNA quantification is impaired by humic acids (HA). Due to its lack of specificity and sensitivity, UV spectrophotometry cannot be applied. Consequently, fluorimetric assays applying Hoechst (H) 33258 or PicoGreen (PG) are used. Here, we investigated the SYBR Green I (SG) assay, which was also affected by HA, but was found to be 25- and 1.7-fold more sensitive compared to the H 33258 and PG assays, respectively. Spectrophotometric, fluorimetric and quenching studies as well as gel mobility shift assays suggested that the effect of HA on the SG assay was based on an inner filter effect, collisional quenching and binding of SG to HA. As to the latter finding, the standard 6250-fold dilution of the SG reagent was optimised to a 2000-fold dilution. Although the sensitivity of the optimised SG assay was reduced by a factor of 1.3, the interfering effect of HA could be reduced up to 22-fold. A significant reduction of HA interferences by lowering the pH of the assay was not observed. Finally, the performance of the modified SG assay and the corresponding evaluation methods were verified by the determination of DNA recoveries and concentrations of standards and environmental samples in comparison to the PG assay.

INTRODUCTION

The generation of genomic preparations from microbial communities involves the extraction of nucleic acids from environmental samples, the quantification of nucleic acids, especially double-stranded DNA (dsDNA), and the subsequent cloning into culturable organisms in order to establish gene banks. Resultant clones of gene banks may be screened for the production of novel natural products (1–3). According to the ‘great plate count anomaly’, only a minor proportion of microorganisms are culturable (4,5). Thus, the access to the genomes of microbial communities via gene banks is an important key to exploit microbial biodiversity for the discovery of novel enzymes, antibiotics and other medical agents.

An accurate quantification of extracted DNA may not only be important for this approach (6). It can also be necessary for other applications that are important for ecologists, including the elucidation of ecological or biochemical pathways (7), the detection of cryptic microorganisms (8), the monitoring of genotypic diversity (8) and metabolic activities (9), toxicity tests to assess effects of pollutants (10), the estimation of the abundance of microorganisms (11) and the evaluation of biodiversity in general (4). The latter critically depends on the extraction technique applied, as it must provide sufficient amounts of total community DNA (2,8). Consequently, quantification of the extracted DNA is also important for the validation and optimisation of standard extraction protocols and for the development of novel extraction techniques (4,8,12–14).

Components, in particular anionic detergents and chaotropic salts, used for the extraction of DNA may impair DNA quantification (15). DNA quantification of environmental samples is mainly hampered by co-extracted or co-purified humic substances (6). Humic substances consist of a mixture

*To whom correspondence should be addressed at Dade Behring Marburg GmbH, PO Box 1149, D-35001 Marburg, Germany. Tel: +49 6421 39 4473; Fax: +49 6421 39 4940; Email: frank_vitzthum@dadebehring.com

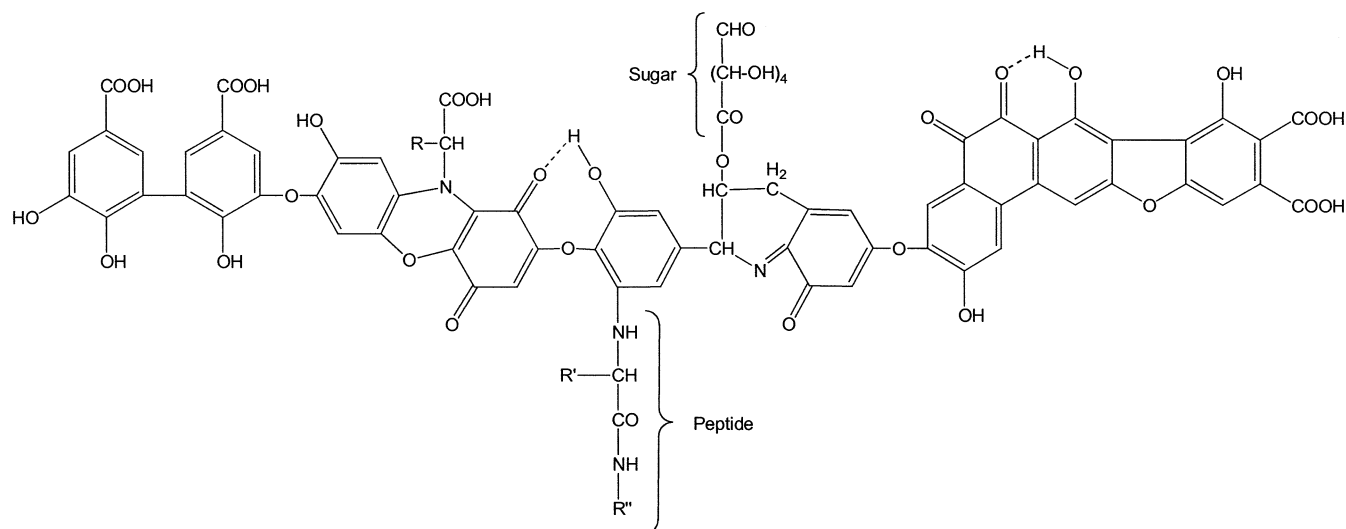


Figure 1. Representative chemical structure of soil HA pointing out key components as proposed by Stevenson (21). Dotted lines show intra-molecular hydrogen bonds. R, R' and R'' indicate different residues.

of plant and animal residues resulting from chemical and/or biological decomposition (16). Characteristic features of humic substances are their structural heterogeneity (17), their ability to bind metal ions by complex formation (18) and their property to interact with a variety of organic compounds (16). Based on their solubility in alkaline and acidic solutions, humic substances may be classified as three fractions: (i) fulvic acids that are soluble in alkali and acid, (ii) humic acids (HA) that are derived from alkaline extracts precipitated by acidification and (iii) humin that cannot be extracted by alkali or acid from soil. Due to these properties, crude DNA extracts from soil or sediment samples are often contaminated by fulvic acids and HA. The amount and composition of humic substances that contaminate DNA preparations may depend on the DNA extraction technique applied (19). Of course, the biology of soils and sediments as well as their fauna and flora also influence the amount and composition of humic substances of preparations (16).

With regard to the chemical heterogeneity of humic substances, it is not possible to describe this class of compounds by unique molecular structures. Statistical calculations indicate that only two identical humic molecules can be expected in a sample of 1 kg humic substances (17). Nevertheless, basic structural moieties and several types of functional groups have been identified as common structural components in unknown humic substance molecules (20). Based on the common structural components, a general model structure was proposed for HA (Fig. 1) (21). This hypothetical macromolecular structure of HA is based on aromatic, quinonic and heterocyclic rings, which are randomly condensed or linked by ether or by aliphatic bridges. Side chains consisting of polysaccharides, peptides and aliphates as well as chemically active functional groups, including carboxylic and carbonyl groups, phenolic and alcoholic hydroxyls, determine the properties of HA.

HA possess high absorption coefficients in the ultraviolet (UV) spectral range, which strongly impairs nucleic acid quantification by UV spectrophotometry and often leads to an

overestimation of DNA concentrations (6,22). Furthermore, the lack of specificity of UV spectrophotometry for DNA quantification with respect to other UV absorbing compounds, including proteins, RNA, nucleotides, some detergents and other potential contaminants, strongly restricts the application of UV spectrophotometry. Even extensive DNA purification may not serve to eliminate this problem as complete removal of HA is difficult to achieve (23).

The most commonly used fluorimetric assay for the quantification of DNA extracted from environmental samples employs the dye Hoechst (H) 33258 (10,22) and more recently PicoGreen (PG) (6,24). H 33258 and PG exhibit fair DNA selectivity and quantification is not critically impaired by most contaminants (6,9,25). However, HA significantly affect these assays (6).

Recently, DNA quantification based on the SYBR Green I (SG) method in solution was shown to be highly specific and robust. Such assays exhibit an almost 2-fold higher sensitivity than the PG assay (15,26). We therefore set out to establish an SG assay for the quantification of DNA in soil and sediment samples. In order to do so, influences of HA from Fluka on DNA quantification by SG were compared to the effects observed with the H 33258 and PG dyes. With respect to the influences observed, we investigated possible molecular mechanisms underlying the interfering influence of HA from Fluka on DNA quantification by SG. Based on the conclusions drawn from these mechanistic studies, we went on to devise an altered SG assay format, which was the central goal of the work. To determine the performance of the accordingly altered SG assay and to assess the validity of the conclusions drawn from the mechanistic studies, the altered SG assay was tested further. DNA recoveries of several spiked lysates from soils and sediments were determined by applying the novel SG assay and the standard PG assay to assess their reliability. For the evaluation of the DNA recoveries and concentrations different experimental settings and mathematical approaches were applied. Finally, absolute DNA concentrations of the samples were

determined to obtain further information on the reliability of the devised SG assay.

MATERIALS AND METHODS

Materials

Ethidium bromide (EtBr), H 33258, PG and SG were purchased from Molecular Probes (Leiden, The Netherlands). Highly polymerised calf thymus (ct)DNA and salmon testes (st)DNA were from Sigma-Aldrich (Deisenhofen, Germany). DNA from bacteriophage λ cI857 Sam 7 (λ DNA) was obtained from Roche Diagnostics (Mannheim, Germany). As the amount and composition of HA of a given sample depends on the soils or sediments used and on the extraction technique applied (16,19), we did not choose to take HA we extracted ourselves for the mechanistic studies. Instead, in order to allow for the reproducibility of our experiments, we chose to use HA that could be purchased. Fluka Inc. (Taufkirchen, Germany) provided the HA preparations, which contain terrestrial humic compounds from a brown coal mine in North Germany (lot and filling code 45 729/1 42899). Miscellaneous chemicals and reagents were purchased from Merck KGaA (Darmstadt, Germany), Carl Roth GmbH (Karlsruhe, Germany) and Life Technologies (Karlsruhe, Germany) and were of the highest analytical grade available.

Marine sediments (samples 1 and 2) were a kind gift from F. Brümmer (Biological Institute, University of Stuttgart, Germany). Sediment sampling was carried out in the Adriatic Sea off the Croatian coast in depths of 9 and 9.5 m, respectively, from the upper 10 cm layer of the sediment. Both samples consisted of sandy clay, having pH values of 5.5 and 6.0, respectively. Flatland sediments (samples 3–6) were kindly provided by H.-U. Steeger (Institute for Zoophysiology, Westfälische Wilhelms-University, Germany). These sediments were collected on the mudflat of Spiekeroog, North Germany. They showed a black silt texture. The pH varied from 6.3 to 7.2. M. Schirmer from the Institute for Ecology and Evolutionary Biology, University of Bremen, kindly provided the estuarine sediments (samples 7–10), which were collected from the river Unterweser near Blexen, North Germany. These sediments had a sandy clay loam texture. A pH between 5.5 and 6.5 was measured. Limnic sediments (samples 11–13) and soils (samples 14–17) were from the region Stuttgart-Vaihingen, Germany. The limnic sediment sampling was performed at various locations in the same lake at a depth of ~0.5 m. The texture of the samples consisted of loam mixed with pieces of roots and leaves that were not humified yet (especially for sample 11). The pH varied from 6.0 to 6.5. The soil samples were taken from the surface layer not deeper than 10 cm and revealed pH values between 4.5 and 6.0. Samples 14–16 were soils from various gardens and sample 17 was collected in the forest of Burgstall, near Stuttgart-Vaihingen (Germany).

Methods

Appropriately cultured (27) *Escherichia coli* DH5 α containing the plasmid pACYC 184 (New England Biolabs, Beverly, MA) were used as a pACYC 184 source. The plasmid was purified by applying the Qiaprep Spin Miniprep kit from Qiagen (Hilden, Germany). pACYC 184 was linearised by

*Eco*RI restriction endonuclease digestion in NEBuffer *Eco*RI (New England Biolabs) according to the supplier's protocol and purified by applying the QIAquick PCR purification kit from Qiagen.

DNA solutions were prepared with 10 mM Tris pH 7.5, containing 1 mM EDTA (TE buffer). The concentration of the stock solutions was determined by measuring the absorbance at 260 nm (27). DNA concentrations applied, in particular those of the standard curves, generally included 0.06, 0.14, 0.2, 0.4, 0.6, 0.8 and 1 μ g/ml, unless stated otherwise. ctDNA and stDNA were solubilised by mild sonication. Analysis by agarose gel electrophoresis showed no profuse fragmentation.

HA solutions were obtained from soil samples by incubation in a Winogradsky column as described (28). HA were dissolved in TE buffer pH 7.5, before they were added to the DNA samples. Unless stated otherwise, HA from Fluka were used. The pH value of soils and sediments was determined in a mixture of five parts distilled water and one part soil or sediment (23).

SG reagent was diluted 6250-fold (15), PG reagent 250-fold (15,25) and H 33258 was applied at a concentration of 1 μ g/ml (6). According to the standard protocols, the dilutions and concentrations of the dyes were chosen to yield appropriate dye/base pair ratios that are crucial to obtain maximal linearity and sensitivity of the DNA quantification assays.

All investigations were generally performed at room temperature with the solutions protected from light and incubated for ~10 min before measurements were started.

Spectrophotometric absorption measurements were carried out with a double-beam spectrophotometer Specord 200 from Analytik Jena AG (Jena, Germany) or a Lambda2 spectrophotometer from Perkin Elmer (Überlingen, Germany; now Applied Biosystems) and with a SpectraMax Plus384 plate reader from Molecular Devices (Ismaning, Germany). Interaction studies by differential spectrophotometry were performed as described (29–32) by applying tandem cuvettes from Hellma Inc. (Müllheim, Germany) and microplates in combination with cup-like lids (30) from Ensinger Inc. (Nufringen, Germany) in order to avoid evaporation effects, when the influence of temperature on the quenching process was investigated.

Spectrofluorimetric measurements were performed with a Fluoromax 3 from Jobin Yvon Inc. (HORIBA Groupe, Grasbrunn, Germany), a Spectrafluor Plus from the Tecan Group (Männedorf, Germany) and a Spectramax Gemini XS from Molecular Devices. Unless stated otherwise, excitation of SG and PG was performed at 485 nm and emission registered at 535 nm (15,25). H 33258 was excited at 360 nm and fluorescence emission was detected at 465 nm (6).

Agarose (1% w/v) gel electrophoresis was performed in 20 mM Tris-acetate, 2 mM EDTA, pH 8.2. Imaging was carried out with an IDA Gel Documentation System and the AIDA version 2.0 software from Raytest Isotopengerätebau Inc. (Straubenhardt, Germany). Fluorescence excitation was performed at 312 nm.

Potential effects of the pH on the assay were assessed by applying a buffer that contained 33 mM citric acid, 33 mM *o*-phosphoric acid, 343 mM sodium hydroxide and 57 mM boric acid. Here, the SG reagent was diluted 2000-fold. Aliquots of 10 μ g/ml ctDNA and 30 μ g/ml HA were added. To determine the extent of HA precipitation caused by

protonation of carboxylic groups, HA solutions were incubated for 10 min, centrifuged at 16 060 g for 5 min at 18°C, and the absorption of the supernatants was measured at 260 nm. The fluorescence of the SG/ctDNA complex in the absence and presence of HA was determined. Also, ctDNA hyperchromicity due to pH-dependent denaturation was determined at 260 nm. pH-dependent spectral changes of SG were followed at 495 nm. To compare the different settings, values at pH 1.5 were set as 0%. Sigmoidal curves were fitted and their widths as well as the pH values at 50% of the maximum (pH_{50%}) were determined.

Experiments to validate the performance of the modified SG assay were carried out with solution that contained 0.5 mg/ml HA from Fluka HA and different amounts of ctDNA. Also, different environmental samples were analysed. To determine the dry weight of the samples, they were dried to constant weight at ~70°C as described (19,33). The drying took ~3 days. If not stated otherwise DNA concentrations refer to the dry weight of a sample. These environmental samples were lysed as described (34) by applying a mixer mill MM 200 from Retsch GmbH (Haan, Germany). The preparation of 1–2 g of soil and sediment according to the protocol of More *et al.* (34) yielded ~1 ml of crude sample extract. As the DNA and HA contents of the crude extracts were unknown, dilutions series in the range between 10- and 250-fold, in particular 20- and 40-fold, were prepared in TE buffer before fluorescence measurements were performed with these samples. The following two protocols were used for the quantification of DNA in crude soil and sediment extracts.

Experimental setting for fluorescence determination of single dilutions in triplicate or a dilution series. Aliquots of 50 µl of the diluted samples were transferred to 96-well microplates and 200 µl of 1600-fold diluted SG reagent was added. After an incubation time of ~10 min, fluorescence intensities were measured. For the calibration curves, 50 µl of ctDNA standards with concentrations of 0, 0.01, 0.02, 0.1 and 0.2 µg/ml were used. The highest dilution of samples showing fluorescence intensities within the calibration curve was taken for further experiments or quantification. If necessary, two different dilutions of a sample were applied. The data generated by this setting were evaluated by a method that was based on the fluorescence determination of a single dilution, e.g. at one point, performed in triplicate (35). Another evaluation method was based on the determination of the fluorescence values of a dilution series, e.g. by slope (35). In both cases, fluorescence values were converted into DNA concentrations according to the fluorescence intensities of ctDNA calibration curves.

Experimental setting for the standard addition technique. Aliquots of 50 µl of appropriately diluted samples were mixed with 50 µl of each ctDNA standard (see above). Then 150 µl of 1200-fold diluted SG reagent was added. This setting is referred to as the standard addition calibration curve. ctDNA calibration curves for the standard addition technique were prepared by adding 50 µl aliquots of TE buffer instead of diluted sample. The standard addition technique was used to estimate the extent of quenching on the SG/DNA complex by the sample matrix, e.g. HA. Linear regression by least squares fit of ctDNA calibration curves and standard addition

calibration curves led to different slopes and axis intercepts. Evaluation method I was based on the determination of DNA concentration considering the axis intercepts (36). In contrast, evaluation method II made use of the ratios of the slopes of the ctDNA calibration curves and those that additionally contained sample matrix (37). Evaluation method III combined methods II and I by correcting the slopes of the standard addition curves according to Maji *et al.* (37) and subsequently considering the axis intercepts to determine the DNA concentration of the samples (36).

To evaluate the performance of the assays applied and to assess the validity of the conclusions drawn from the mechanistic studies, ctDNA recoveries in the presence of co-extracted matrix components were determined. For the determination of analytes in complex matrices the recoveries are a common quality check for the accuracy of the quantification (37). In order to do so, a known amount of ctDNA was added to an aliquot, e.g. 0.4 ml, of a crude extract. Of note, the concentration of the ctDNA solution used for spiking was determined by the SG assay (15). To prevent excessive dilution of the matrix components, the added volume of ctDNA was kept below 5% of the volume of the aliquots. The amount of ctDNA added was in the range of the DNA concentration of the aliquot determined before. After spiking, the DNA concentrations of the sample aliquots were determined as described (see above). The difference between the DNA concentration of the original and the spiked sample aliquots denotes the recoveries.

In order to validate the DNA quantification assay, we investigated replicates of the same environmental extract of each sediment and soil sample, because DNA quantification after replicated extraction procedures would include variations due to the extraction procedure. However, we solely wanted to address the reproducibility of the DNA quantification assay. The same experimental settings and crude extracts were used when PG was applied.

RESULTS

Absorption spectra

Quantification of DNA in environmental soil samples by UV spectrophotometry is strongly impaired by the presence of HA, because of their spectral properties. Generally, the spectral properties of HA from different environmental sources are similar and not characteristic in the visible and UV range (16). Consequently, it may be argued that the ratio of certain absorbances in the visible or UV range could be used to determine the impact of humic material on DNA quantification and thereby the correct DNA concentration may be deduced. However, we found that the absorption spectra of different HA preparations differed significantly in the UV range (Fig. 2A). Therefore and due to the lack of sensitivity, an accurate DNA quantification by UV spectrophotometry in the presence of HA was not feasible.

Quantification of DNA using fluorescent dyes is also hampered by HA (6). This may be a result of quenching processes or additional fluorescence due to interfering compounds. Quenching processes can be based on an inner filter effect and collisional effects. In addition, quenching may be due to the binding of compounds to DNA, SG or the SG/DNA

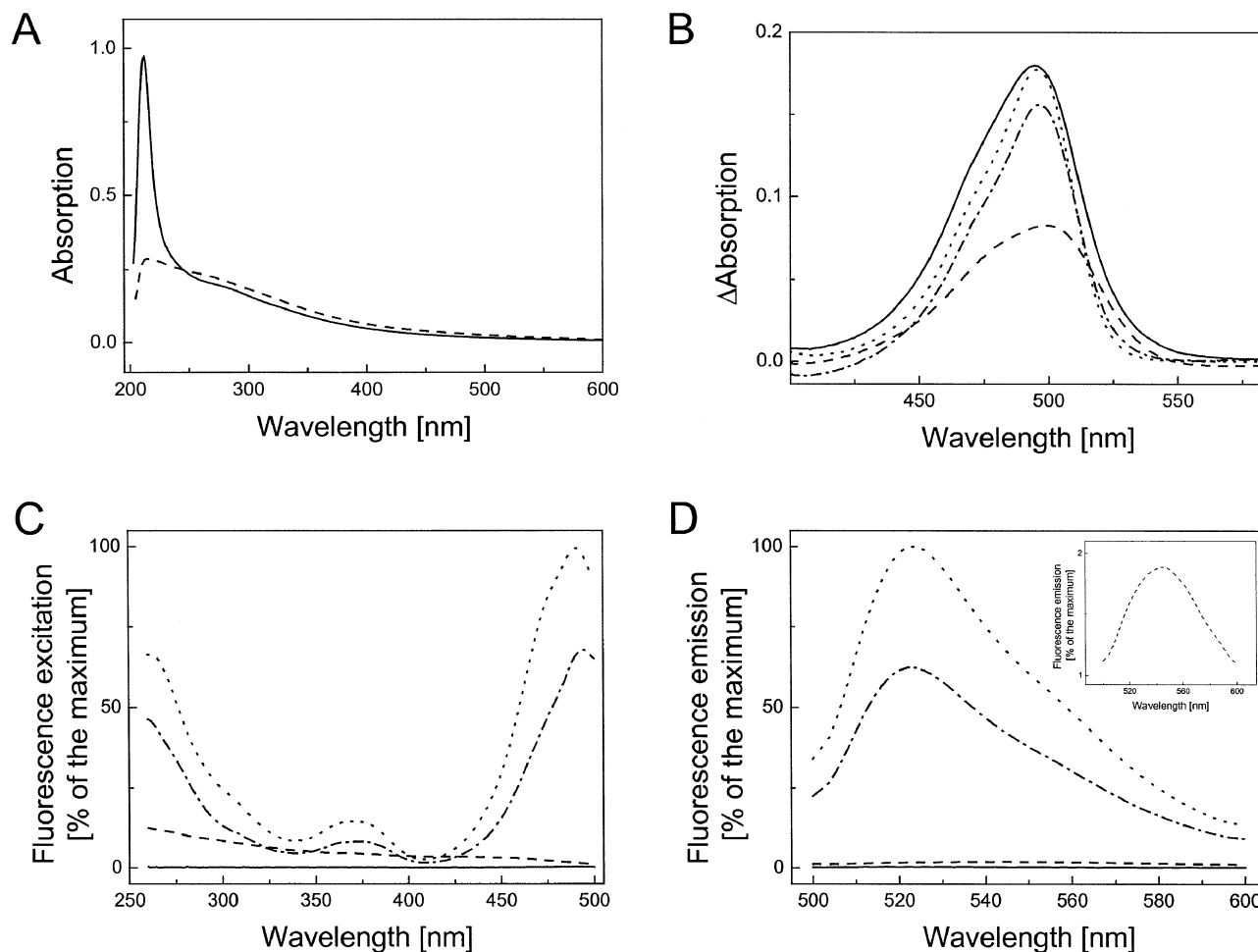


Figure 2. Absorption and fluorescence spectra of HA, SG, SG in the presence of HA, SG/DNA complex and the latter in the presence of HA. (A) Absorption spectra of HA from Fluka (6 µg/ml) and a 1600-fold dilution of HA obtained from an environmental soil sample. (B) Differential spectrophotometry studies on SG (—) diluted 3000-fold in the presence of 10 µg/ml λDNA (.....), 20 µg/ml HA from Fluka (- - -) and both (- · - ·). (C) Fluorescence excitation and (D) fluorescence emission spectra of HA (- - -, insert), SG (—), the SG/λDNA complex (.....) and the latter in the presence of HA (- · - ·). From the latter excitation spectrum, the contribution of HA was subtracted. The concentrations of HA and λDNA were 10 and 0.1 µg/ml, respectively. Registration of excitation spectra were performed by monitoring emission at 522 nm. Emission spectra were recorded by excitation at 490 nm.

complex. In contrast to fluorescence quenching, such binding events may also lead to fluorescence enhancement of complexes formed.

Differential spectroscopic scans strongly indicated that binding of HA to SG occurred. In the settings presented in Figure 2B, the presence of HA significantly reduced the absorbance of SG in the visible range and shifted the maximum of SG absorption from 495 to 500 nm. In addition, the shoulder at ~470 nm was reinforced. Therefore, the full width at half maximum of the complete visible absorption band broadened. In contrast, the binding of SG to λDNA resulted in a reduction in the bandwidth of the visible absorption band. Only a minor, yet significant, bathochromic shift from 495 to 496 nm was observed. Hypochromicity was also much less pronounced. When both HA and λDNA were present in the SG solution a superimposition of the effects was observed. Of note, in the absence of SG no alterations in the DNA spectra were observed when HA were present and vice versa (data not shown).

Contrary to the observations made in the visible range, the absorption bands of SG in the UV range were almost equally

altered in the presence of λDNA and HA. In both cases, the absorption bands of SG at around 370, 297 and 252 nm were bathochromically shifted by 5, 3 and 2 nm, respectively (data not shown). Such effects were also observed for ctDNA and stDNA (data not shown).

Fluorescence spectra

Apart from the changes observed in the absorption spectra, alterations in the fluorescence excitation and emission spectra of the SG/λDNA complex in the presence of HA were detected (Fig. 2C and D). However, the fluorescence emission and excitation spectra of SG in the presence of HA and vice versa did not change observably (data not shown). Upon binding to λDNA, SG exhibited characteristic fluorescence excitation and emission spectra that were not observed for the unbound dye (Fig. 2C and D). The excitation spectra of the SG/λDNA complex resembled those of the absorption spectra of the bound dye. Excitation maxima occurred at 490 nm, followed by a slight shoulder at ~470, 370 and 250 nm, preceded by an explicit shoulder at ~300 nm (Fig. 2C). Fluorescence excitation at 370 and 260 nm was 6.8 and 1.5

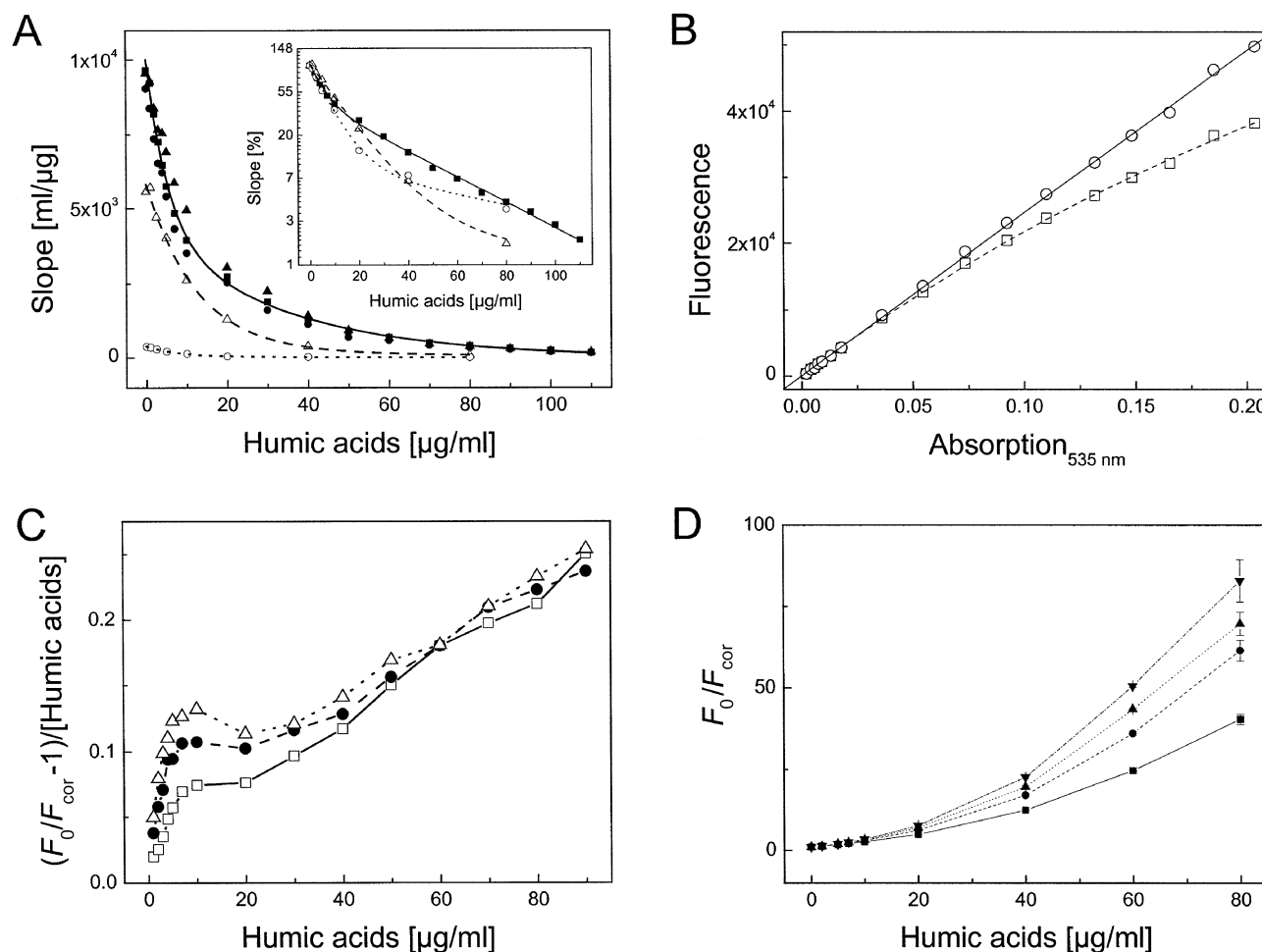


Figure 3. Quenching of dye/DNA complex fluorescence by HA. (A) Dependence of slopes of standard curves on HA concentrations. Slopes of standard curves exhibited by SG/DNA complexes (—) with ctDNA (solid square), λDNA (solid circle) and stDNA (solid triangle), by PG/ctDNA complexes (open triangle, —) and by H 33258/ctDNA complexes (open circle,). The insert shows a direct comparison of the appropriate dye/ctDNA complexes by setting the slopes of the standard curves without HA to 100%. (B) Dependence of HA fluorescence on the HA concentration. The observed fluorescence values (F_{obs}) (open square, - - -) of HA were plotted versus increasing HA concentrations given as absorbance at 535 nm. F_{obs} were transformed into corrected fluorescence values (F_{cor}) (open circle, —) by applying equation 2. Fluorescence excitation was performed at 485 nm, the emitted fluorescence was detected at 535 nm. (C) Modified Stern-Volmer plots on the superimposition of several quenching effects. SG at a dilution of 1:6250 and ctDNA at 0.7 (open square, —), 3 (solid circle, - - -) and 5 μg/ml (open triangle,). (D) Temperature dependence of fluorescence displayed by a Stern-Volmer plot. SG fluorescence in the presence of 5 μg/ml ctDNA and increasing HA concentrations was determined at 23 (solid square), 25 (solid circle), 27 (solid triangle) and 29°C (solid inverted triangle).

times lower, respectively, compared to excitation at 490 nm. The presence of HA generally led to a reduction in fluorescence excitation of the SG/λDNA complex and to a bathochromic shift (Fig. 2C). The decreases in fluorescence excitation values at 490 and 485 were about 1.6-fold and the decreases at 370 and 260 nm were around 1.7- and 1.4-fold, respectively, when fluorescence emission was registered at 522 nm. However, the fluorescence excitation values of HA alone at 490, 485, 370 and 260 nm were 1.5, 1.8, 4.6 and 12.6% for fluorescence excitation at 490 nm of the SG/λDNA complex. Consequently, the impact of HA fluorescence may be neglected at 490 and 485 nm. Considering a HA impact, fluorescence excitation values at 370 and 260 nm only decreased ~1.1-fold.

Of note, the maximum of fluorescence emission of the HA applied was around 545 nm, when excited at 490 nm (Fig. 2D insert). Excitation at shorter wavelengths led to maxima of

fluorescence emission with shorter wavelengths and higher intensities (data not shown). For example, excitation of HA fluorescence at 340 nm resulted in an emission maximum at ~470 nm. HA fluorescence at 522 nm after excitation at 340 nm was about twice as high when compared to excitation at 488 nm. This is of interest as upon excitation at 490 nm, the SG/λDNA complex exhibited maximal fluorescence emission at ~522 nm (Fig. 2D). The same was true for excitations in the UV range (data not shown). In the presence of HA fluorescence, emission of the SG/λDNA complex was significantly reduced, but the maximum remained at ~522 nm.

Quenching of dye/DNA complex fluorescence by HA

Increasing concentrations of HA continuously quenched the fluorescence exhibited by SG, PG and H 33258/DNA complexes. The slopes of the DNA standard curves in appropriate settings for each dye were significantly reduced in a fashion

Table 1. Comparison of the impact of increasing HA concentrations on the slopes of SG/st, λ and ctDNA calibration curves, of the PG/ctDNA calibration curves and of H 33258/ctDNA calibration curves

	SG stDNA	λ DNA	ctDNA	PG ctDNA	H 33258 ctDNA
k_1 (%·ml/ μ g)	7.9 ± 1.5	5.1 ± 0.7	5.3 ± 0.7	12.2 ± 4.5	7.9 ± 0.9
Slope (%·ml/ μ g)	-5.7 ± 0.5	-8.1 ± 0.5	-8.5 ± 0.4	-6.2 ± 1.3	-8.5 ± 0.5
k_2 (%·ml/ μ g)	30.2 ± 3.6	31.9 ± 3.7	33.9 ± 3.9	$52.4 \pm \text{n.d.}$	$83.6 \pm \text{n.d.}$
$A_1 + A_2$ (%)	102 ± 20	102 ± 12	104 ± 12	$104 \pm \text{n.d.}$	$102 \pm \text{n.d.}$
y intercept (%)	100.4 ± 1.6	99.2 ± 1.5	101.6 ± 1.1	103.0 ± 3.8	100.6 ± 1.3

The slopes of the dye/DNA complexes in the absence of HA were defined as 100% (Fig. 3A, insert) and equation 1 was applied. The sum of A_1 and A_2 yields the y intercept of the exponential decay which should ideally be 100%. The same applies to the linear regression analysis.

that could be fitted best by a second order exponential decay function in contrast to other curves, including first and third order exponential (Fig. 3A). The second order exponential decay was described according to:

$$y = A_1 e^{-x/k_1} + A_2 e^{-x/k_2} \quad 1$$

In order to compare the impact of quenching on the three dye/DNA complexes, the slopes of the calibration curves in the absence of HA were set as 100% (Fig. 3A insert) and parameters defining the second order exponential decay were calculated (Table 1). Two ranges became obvious, one at HA concentrations <5–10 μ g/ml and another between 10 and 110 μ g/ml. At HA concentrations <5–10 μ g/ml the impact of quenching was of equal magnitude for the three dye/DNA complexes, but the PG/DNA complex fluorescence appeared to be slightly less impaired than SG and H 33258. Significant differences occurred at HA concentrations >10 μ g/ml. Interestingly, the determined y intercepts were generally >100% (Table 1). This was likely due to a slight sigmoid dependence. This sigmoid characteristic became more obvious when quenching was followed with a single DNA concentration. Of note, decreasing DNA concentrations, i.e. increasing dye/base pair ratios, enhanced the sigmoidicity (data not shown).

Apart from the observation that a sigmoid trait existed, the absolute values of the y intercepts of the slopes of the DNA standard curves also indicated the sensitivity of the different assays, with the SG assay exhibiting the highest sensitivity (Fig. 3A). According to the y intercepts, the SG assay was 1.7 and 25 times more sensitive than the PG and H 33258 assays, respectively. Considering that the impact of HA on the three assays was comparable, we concluded that the SG approach was the most promising one to determine soil or sediment DNA in the presence of HA. Consequently, the mechanisms underlying the quenching process of SG fluorescence were subsequently studied in more detail to gain information for the development of an assay appropriate for DNA determination from sediment and soil samples.

Inner filter effect. An inner filter effect can be one mechanism underlying a quenching process. As HA absorb radiation at the excitation and the emission wavelengths of the SG/DNA complex, it appeared plausible that parts of the incident excitation radiation and emitted fluorescence of the dye/DNA complex would be absorbed by HA. This inner filter effect was

detected by investigating the intrinsic fluorescence of HA subject to the absorption at the relevant excitation (data not shown) and emission wavelengths (Fig. 3B). The measured fluorescence intensity of the HA was proportional to an absorption value of approximately 0.05 for the emission wavelength (A_{em}) and 0.06 for the excitation wavelength (A_{ex}). At the settings used, this corresponded to ~20 μ g/ml HA in both cases. A further increase in absorption due to higher HA concentrations resulted in a non-linear increase in their intrinsic fluorescence. By applying equation

$$F_{cor} = F_{obs} \cdot e^{[A_{ex} + A_{em}/2]} \quad 2$$

according to Lakowicz (38), the observed fluorescence values (F_{obs}) were transformed into corrected fluorescence values (F_{cor}) that perfectly matched straight lines with correlation coefficients around 0.9998 for the setting where the absorption of the excitation wavelength was applied as well as for the one where the emission wavelength was used. This indicated that an inner filter effect also occurred in the SG assay as soon as HA were present (38). This was particularly so when the concentration of the HA exceeded 20 μ g/ml.

Collisional and static quenching. Apart from an inner filter effect, collisional and static quenching represent the most common quenching mechanisms. Collisional quenching results from diffusive encounters between the excited state fluorophore and the quencher. Static quenching is due to ground state complex formation between the fluorophore and the quencher resulting in a non-fluorescent complex (38). The mechanisms behind fluorescence quenching may be investigated by applying the Stern–Volmer equation:

$$F_0/F_{cor} = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad 3$$

where F_0 and F_{cor} are the corrected fluorescence intensities in the absence and presence of a quencher, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the excited state of the fluorophore in the absence of quencher, $[Q]$ is the concentration of the quencher and $K_{SV} = k_q \tau_0$, the Stern–Volmer quenching constant. To exclude an inner filter effect, the corrected fluorescence values were used instead of the observed fluorescence values.

Stern–Volmer plots of the quenching of the SG/DNA complex fluorescence in the presence of HA showed upward curvatures. The curvatures were concave toward the y-axis,

indicating that at least two quenching processes, e.g. static and collisional, had to be involved (Fig. 3D). No substantial differences in the extent of fluorescence quenching were detected for λ , ct and stDNA. Of note, F_0/F_{cor} values <1 were observed at HA concentrations below $\sim 2 \mu\text{g/ml}$. In particular, this effect became prominent when DNA concentrations $<0.2 \mu\text{g/ml}$ were applied (data not shown). The upward curvatures of the Stern–Volmer plots could be partially fitted to a quadratic equation. In order to clarify whether or not deviation from linearity of the Stern–Volmer plots was due to combined static and collisional quenching processes by HA, a modified Stern–Volmer equation, which is second order in $[Q]$, was applied (38):

$$F_0/F_{\text{cor}} = 1 + (K_D + K_S)[Q] + K_D \cdot K_S[Q]^2 \quad 4$$

where K_S represents the constant for static quenching that can be considered as the association constant for a potential complex formation between the quencher and the fluorophore, and K_D indicates the collisional quenching constant. For the linearisation of the data, in order to allow for a possible graphical evaluation of K_S and K_D , the modified Stern–Volmer equation 4 was rearranged to

$$F_0/F_{\text{cor}} = 1 + K_{\text{app}}[Q] \quad 5$$

where K_{app} is described by

$$K_{\text{app}} = (F_0/F_{\text{cor}} - 1) \cdot 1/[Q] = (K_D + K_S) + K_D \cdot K_S[Q]. \quad 6$$

Thus, K_D and K_S can be derived by plotting K_{app} versus $[Q]$.

Modification of the quenching data did not yield a linear curve in the modified Stern–Volmer plot. Instead, two distinct linear areas occurred (Fig. 3C). A first one appeared in the range between 5 and 10 $\mu\text{g/ml}$ HA, which was dependent on the DNA concentration applied; a second one was between 40 and 90 $\mu\text{g/ml}$ HA, which did not appear to be noticeably dependent on the DNA concentration applied. Unfortunately, determination of K_D and K_S by solving $I = K_D + K_S$ and $S = K_D \cdot K_S$ by rearranging to $K_S^2 - K_S I + S = 0$, with I being the y intercept and S the slope, was not possible. Solution of the resulting quadratic equations led to irrational numbers, because the slopes were orders of magnitude higher than the intercepts. This effect was probably due to the heterogeneity and high relative molecular weight of the HA, which ranged from 200 to 250 000 g/mol, offering multiple interaction sites.

As K_D and K_S could not be inferred from quenching data by applying the modified Stern–Volmer equation, the impact of potential collisional or static quenching had to be investigated by other methods. The most definitive approach to distinguish collisional and static quenching is to measure fluorescence lifetimes in the presence and absence of the quencher (38). However, this technique implies special equipment. Another, more simple method is based on the influence of rising temperature on the quenching of the fluorescence by a quencher (38). Higher temperatures typically result in the dissociation of complexes. In this case, the static quenching constant K_S is reduced and the resultant Stern–Volmer plot approaches the x-axis. In contrast, higher temperatures result in larger diffusion coefficients and the bimolecular quenching constants k_q in equation 3 should increase. Consequently,

collisional quenching becomes prominent and the resultant curves of the Stern–Volmer plot bend increasingly toward the y-axis with rising temperatures. Interestingly, this temperature effect was detected only for HA concentrations $>10 \mu\text{g/ml}$, whereas up to 10 $\mu\text{g/ml}$ HA no marked influence of temperature on the quenching data could be observed (Fig. 3D).

Interactions of HA with SG and the SG/DNA complex

Differential spectrophotometry. Although it was shown that collisional quenching occurred, the possibility of a contribution of complex formation between the components to the quenching needed to be further addressed. Differential spectrophotometry did not give any indication for the interaction of HA and DNA (see above). However, the spectral changes of SG in the presence of HA observed in differential spectrophotometry experiments indicated binding events (Fig. 2B). Following titration of SG with increasing HA concentrations, differential spectrophotometry revealed a biphasic dependence, which was altered in the presence of DNA (Fig. 4A). Of note, a similar biphasic dependence was also observed when SG was titrated with increasing DNA concentrations alone (data not shown). Due to the heterogeneity of HA, their diverse relative molecular masses and molecular properties, as well as due to the fact that the concentration of SG is unknown, dissociation constants could not be determined.

Gel mobility shift assays. Binding of HA to SG was further studied by gel mobility shift assays. These experiments revealed that a certain fraction of HA interacted with SG. The formed complexes were even stable under electrophoretic conditions (Fig. 4B, I). The intensity of the brown colour of the visible fraction of HA increased upon the addition of SG and the electrophoretic mobility was reduced. In contrast, the electrophoretic mobility and intensity of the UV-fluorescent fraction of HA was not altered in the presence of SG (Fig. 4B, II).

Furthermore, it could be shown that SG strongly reduced the electrophoretic mobility of DNA, whereas the presence of HA did not (Fig. 4C). Of note, electrophoretic mobility of DNA approached a minimum when the amount of SG was increased. For example, when a 6250-fold dilution of the SG reagent was applied together with 2.5 $\mu\text{g/ml}$ pACYC, the minimum in electrophoretic mobility was reached. Increasing amounts of SG did not further reduce the electrophoretic mobility (data not shown).

In contrast to this observation, we observed an unexpected increase in the electrophoretic mobility of the SG/DNA complex, even when only small amounts of HA were added (Fig. 4C). The electrophoretic mobility of the SG/DNA complex increased continuously with increasing amounts of HA up to a point where the electrophoretic mobility of pure DNA was reached (Fig. 4C). Furthermore, a significant loss in fluorescence intensity only occurred over a HA concentration of $\sim 10 \mu\text{g/ml}$. This was not caused by inadequate loading of the samples as shown by subsequent EtBr staining (Fig. 4C, I versus II).

Competition studies. The results of the electrophoretic mobility assays suggested competition of DNA and HA for binding

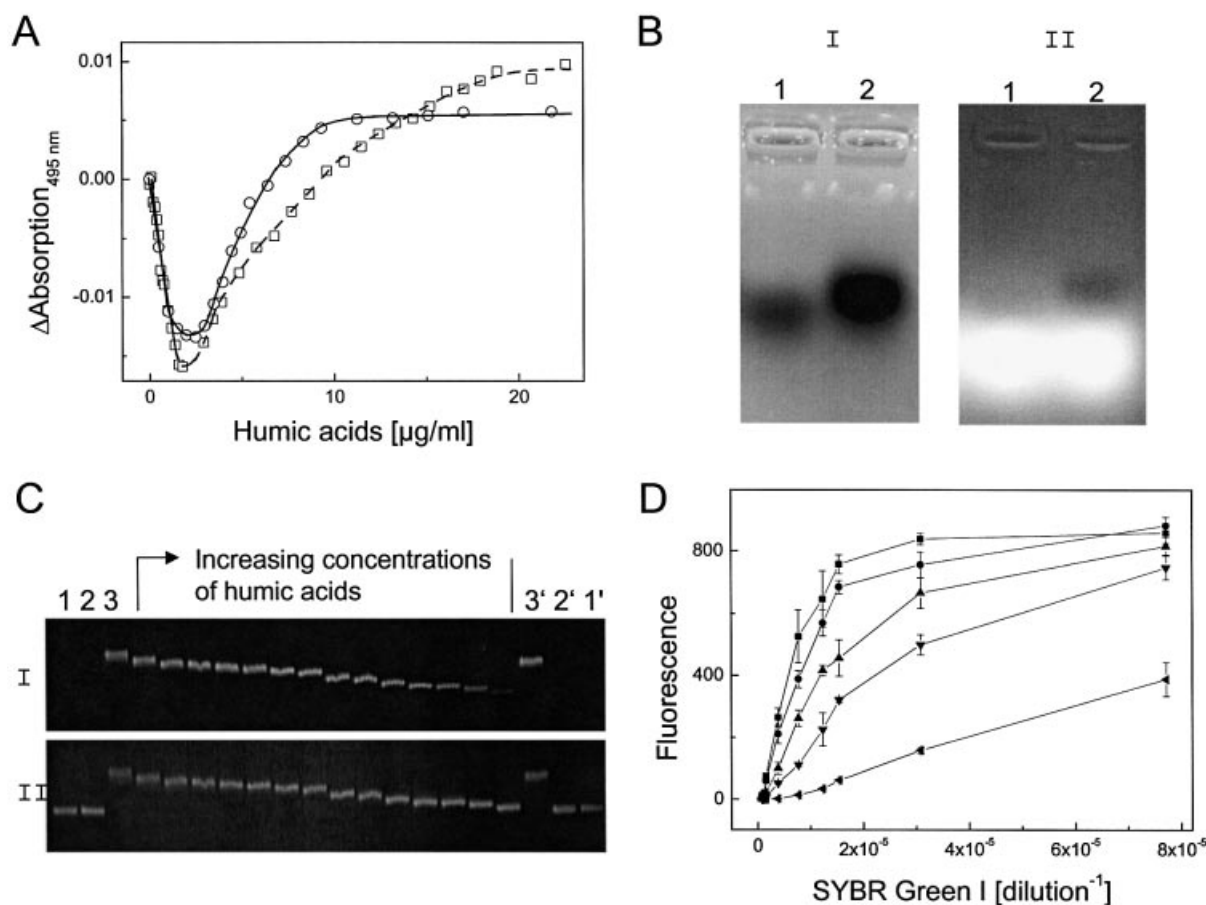


Figure 4. Interaction of HA with SG, DNA and the SG/DNA complex. (A) Differential spectroscopy on the binding of HA to SG in the absence (square) and presence of 1 $\mu\text{g/ml}$ λDNA (circle). 3000-fold diluted SG was titrated with HA. The setting with and without DNA covered HA concentration ranges between 0.5 and 22 $\mu\text{g/ml}$ HA as well as 0.1 and 23 $\mu\text{g/ml}$, respectively. The experiments were performed in TE buffer pH 7.5 at 23°C. The displayed curves were drawn manually through the data. (B) Agarose gel electrophoresis on the binding of the coloured fraction of HA to SG. Solutions containing 30 $\mu\text{g/ml}$ HA were loaded in the absence (1) or presence of 0.5 μl undiluted SG reagent (2). Detection of the bands occurred by daylight (I) and by UV radiation at 312 nm (II). (C) Studies on the electrophoretic mobility of the SG/DNA complex. Solutions of *EcoRI*-linearised plasmid pACYC 184 at a concentration of 2.5 $\mu\text{g/ml}$ were stained with 6250-fold diluted SG (lanes 3 and 3'). Unstained plasmid DNA served as a reference (lanes 1 and 1'). Mixing of unstained plasmid with 50 $\mu\text{g/ml}$ HA is shown in lanes 2 and 2'. In the remaining lanes SG/DNA complexes at the cited conditions were incubated with the following HA concentrations from left to right: 0.25, 0.5, 1.0, 1.5, 2, 3, 4, 8, 10, 14, 18, 22, 30 and 50 $\mu\text{g/ml}$. Fluorescence was monitored before (I) and after EtBr staining (II). (D) Dependence of the fluorescence intensity of the SG/DNA complex on the SG dilution in the presence of HA. Aliquots of 0.1 $\mu\text{g/ml}$ λDNA were titrated with SG in the presence of different HA concentrations (square, 0; circle, 0.1; triangle, 0.5; inverted triangle, 1.0; triangle left, 5.0 $\mu\text{g/ml}$).

of SG. Therefore, the dependence of the fluorescence of the SG/DNA complex on the SG dilution in the presence of different HA concentrations was investigated (Fig. 4D). It appeared that the weak but significant sigmoidal character of the dependence became more distinct with increasing HA concentrations. Also, maximal fluorescence values were obtained at higher SG concentrations. The double reciprocal plot of the data resulted in a competitive pattern, which was distorted by the sigmoidal description of the dependence (data not shown). To exclude the possibility of a non-competitive mechanism, i.e. HA display similar dissociation constants when bound to both SG and the SG/DNA complex, the slopes in the appropriate range of the double reciprocal diagram were plotted against the HA concentrations (data not shown). This secondary diagram showed a linear dependence with a correlation coefficient of 0.99862, which strongly indicated a competitive binding mode (31). Thus, HA bound to SG but not to the SG/DNA complex. The x intercepts of such

secondary diagrams usually depict the dissociation constant of this binding event. In this case, however, the absolute value of the x intercept of 154 ng/ml may only indicate that this competition process has a detectable impact on DNA quantification by SG at the settings applied. Again, the heterogeneity of HA, in particular with respect to their relative molecular masses and structures, influencing the number of binding sites, does not allow for a determination of the dissociation constant.

Impact of the SG/dsDNA ratio

The determined competitive scenario together with the observation of the sigmoidal quenching of the SG/DNA complex fluorescences by HA, which was enhanced at low DNA concentrations (see above), prompted us to apply higher amounts of SG than those used in the standard assay. A 1000-fold dilution of SG reagent led to a complex dependence of the quenching of SG/ctDNA complex fluorescence by HA, in

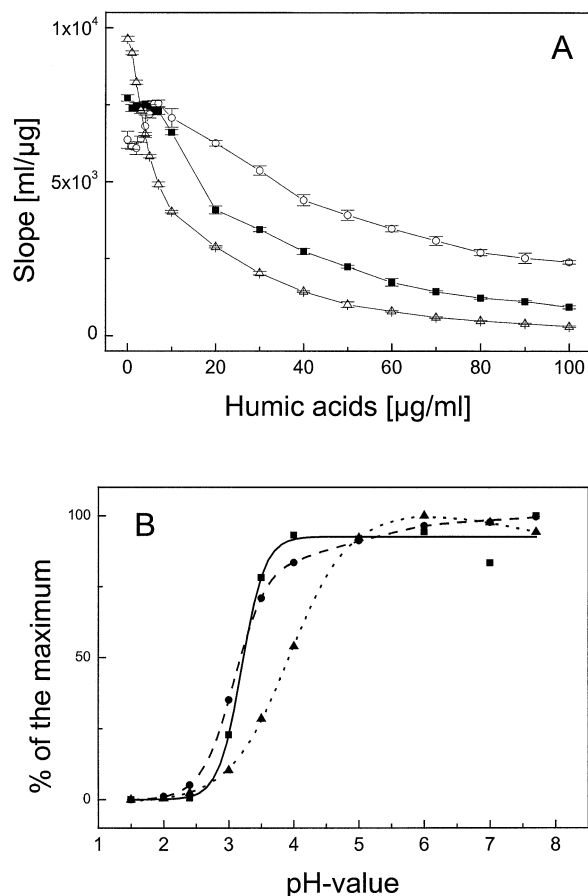


Figure 5. Impact of the dilution of the SG reagent (A) and influence of acidic pH values (B) on the assay in the presence of HA. (A) Effect of HA on the slopes of calibration curves and the role of the dilution of SG. The 6250-fold (open triangle), the 2000-fold (solid square) and the 1000-fold (open circle) dilutions of SG were incubated with ctDNA calibration curves in the presence of increasing HA concentrations. (B) Dependence of fluorescence intensity of the SG/DNA complex on the pH value. The influence of various pH values on 2000-fold diluted SG (solid triangle,), and on 30 μg/ml HA (solid circle, - - -) were measured at 495 and 260 nm, respectively. Fluorescence intensities of the SG/DNA complexes in the presence of HA (solid square, —) were performed with 1 μg/ml ctDNA. After incubation, samples were centrifuged and measurements were performed with the supernatant.

contrast to the findings obtained for the standard 6250-fold dilution (Fig. 5A). However, application of a 2000-fold dilution of the SG reagent resulted in an obvious sigmoidal pattern. The overall impact of HA was reduced, when compared to the settings with 6250-fold diluted SG reagent (Fig. 5A). The slopes of the standard curves at HA concentrations <7 μg/ml were hardly affected when 2000-fold diluted SG was applied. Linear regression within this range revealed a slope value that was about 22 times lower compared to that of the 6250-fold diluted SG reagent. Of note, a 1.3-fold reduction in sensitivity was observed.

Influence of the pH value

In addition to the effects observed for the dilution of the SG reagent, an influence of acidic pH values on the assay was investigated to find out whether protonation of carboxylic groups of HA, that may account for the binding of SG, has an

Table 2. Recoveries of the novel SG assay in comparison to the PG assay, when ctDNA standards containing 0.5 mg/ml HA were applied

ctDNA standard (μg/ml)	Recovery (%)			
	PG I	SG I	II	III
3.0	102 ± 2	96 ± 1	93 ± 1	95 ± 1
5.8	92 ± 2	91 ± 4	82 ± 2	91 ± 4
11.3	94 ± 2	96 ± 4	93 ± 6	96 ± 5
24.0	94 ± 9	105 ± 1	99 ± 3	104 ± 1
Mean recovery (%)	96 ± 4	97 ± 5	92 ± 6	97 ± 5

The standard addition technique applying evaluation methods I, II and III was used. Standard deviations were based on triplicate measurements of the same ctDNA standard.

effect. This could alter the pH dependence of the standard assay, which is not significantly affected by pH variations between 4 and 10 and has a pH optimum of 7–9 (15). Also, fluorescence of the SG/ctDNA complexes in the presence of HA was more or less constant at pH values >4 (Fig. 5B). Of note, significant precipitation of HA occurred when the pH was <4 (Fig. 5B). Sigmoidal fits resulted in a pH_(50%) value of 3.15 ± 0.04 for the precipitation of HA with a width of 0.32 ± 0.04 . In comparison, the pH_(50%) values for the sigmoidal pH dependence of the fluorescence of the SG/ctDNA complex in the absence and presence of HA were 2.82 ± 0.12 and 3.20 ± 0.01 with widths of 0.11 ± 0.07 and 0.18 ± 0.01 , respectively. The sigmoidal pH dependence of SG absorption at 495 nm led to a pH_(50%) value of 3.92 ± 0.01 with a width of 0.45 ± 0.01 (Fig. 5B). Of note, ctDNA hyperchromicity due to denaturation gave a pH_(50%) value of 1.97 ± 0.19 with a width of 0.24 ± 0.14 , which indicated that the decrease in fluorescence of the SG/ctDNA complex was not a consequence of DNA denaturation. In addition, this could be a clue to devise a two-step procedure for determining DNA in environmental samples. After precipitation of HA at around pH 3 and their removal without damage or removal of DNA, the latter could be determined at the appropriate pH by the SG assay with less interference.

DNA quantification of environmental samples

As the pH dependence of the fluorescence of the SG/DNA complex in the absence and presence of HA did not differ markedly, the pH value of 7.5 of the standard assay was applied for DNA quantification in environmental samples. Selective removal of HA from crude extracts, e.g. by acidic precipitation or DNA purification, was not necessary as the sensitivity of the SG assay allowed for a simple and fast one-step procedure by simply diluting the samples extensively to reach absorption values far below 0.05 at 485 nm. Apart from minimising the quenching effects, the linear range of the assay settings with 2000-fold diluted SG was reached (see above).

Recoveries of standard samples containing 0.5 mg/ml HA from Fluka and known amounts of ctDNA were determined by the modified SG assay and compared to recoveries obtained with the PG assay. The PG assay may be considered as a benchmark for fluorimetric DNA quantification in solution (15,25,39). The mean recovery of the SG assay ($97 \pm 5\%$) was comparable to the mean recovery of the PG benchmark assay ($96 \pm 4\%$) when the standard addition technique applying evaluation method I was used (Table 2). With the exception of

Table 3. Recoveries of the novel SG assay in comparison to the PG assay, when soil and sediment samples were investigated

Sample	No.	SG		Recoveries (%)			
		Single dilution	Dilution series	PG Standard addition technique	I	II	III
Marine	1	80 ± 4	103 ± 5	104 ± 2	92 ± 4	101 ± 2	n.d.
	2	93 ± 3	112 ± 5	105 ± 2	103 ± 3	105 ± 2	n.d.
Flatland	3	100 ± 2	126 ± 6	103 ± 3	100 ± 6	105 ± 3	n.d.
	4	90 ± 4	125 ± 4	102 ± 6	102 ± 8	105 ± 6	n.d.
	5	98 ± 5	125 ± 3	107 ± 5	106 ± 1	91 ± 1	102 ± 9
	6	100 ± 6	128 ± 9	100 ± 3	100 ± 4	99 ± 4	99 ± 7
Estuarine	7	93 ± 6	96 ± 3	93 ± 3	97 ± 8	99 ± 3	n.d.
	8	89 ± 2	98 ± 4	99 ± 2	94 ± 4	94 ± 5	n.d.
	9	87 ± 1	87 ± 2	95 ± 1	93 ± 4	97 ± 2	89 ± 8
	10	90 ± 3	89 ± 3	95 ± 1	99 ± 4	95 ± 1	89 ± 1
Limnic	11	95 ± 7	80 ± 8	84 ± 5	92 ± 11	95 ± 9	n.d.
	12	89 ± 1	76 ± 1	95 ± 2	95 ± 1	98 ± 4	102 ± 1
	13	87 ± 1	78 ± 2	97 ± 3	97 ± 1	99 ± 4	87 ± 4
	14	95 ± 3	99 ± 2	95 ± 5	96 ± 3	96 ± 5	n.d.
Soil	15	94 ± 3	96 ± 6	104 ± 2	103 ± 2	102 ± 3	n.d.
	16	89 ± 1	97 ± 5	94 ± 4	91 ± 1	93 ± 1	86 ± 7
	17	94 ± 1	92 ± 5	95 ± 4	94 ± 6	95 ± 4	101 ± 2
Mean recovery (%)		92 ± 5	100 ± 17	98 ± 6	97 ± 4	98 ± 4	94 ± 7

Standard deviations were based on triplicate measurements of the same extract of a soil or sediment sample.

the comparatively low recovery of the 5.8 µg/ml ctDNA standard, determined with evaluation method II, recoveries of the SG assay were comparable for all evaluation methods applied. Generally, recoveries were high, within the range of 91–105%.

Further, the performance of both assays was tested in extracts of environmental samples from various origins by the use of the standard addition technique applying evaluation method I (Table 3). Here, the mean recovery of the SG assay (98 ± 6%) was slightly higher than the mean recovery of the PG benchmark assay (94 ± 7%). However, it has to be considered that only eight recoveries were taken into consideration to determine the mean recovery of the PG assay, in contrast to 17 with respect to the mean recovery of the SG assay.

Apart from the evaluation techniques based on standard addition, the SG assay also showed acceptable recoveries for the simple and quick evaluation methods applied, i.e. triplicates of a single sample dilution or dilution series were assayed (Table 3). However, the mean recoveries of most environmental samples tested with a single dilution suggest a tendency to underestimate the DNA content. This tendency was also prominent for the limnic samples using the dilution series method. On the other hand, intriguingly high recoveries were found for flatland samples with this method. Consequently, a high standard deviation of the mean recovery for the dilution series method was determined.

These findings would favour standard addition. With the exception of one limnic sample (no. 11) determined by standard addition applying evaluation method I, good recoveries were obtained for the SG assay with all evaluation methods based on standard addition (Table 3).

Because of the high reliability of the standard addition technique, the absolute DNA concentrations of the environmental samples were determined by calculating the mean

Table 4. DNA concentrations of soil and environmental samples, determined by the PG and the novel SG assay

Sample	No.	DNA concentration (µg/g dry weight)	
		SG	PG
Marine	1	0.6 ± 0.1	n.d.
	2	0.3 ± 0.1	n.d.
Flatland	3	1.0 ± 0.1	n.d.
	4	1.5 ± 0.1	n.d.
	5	1.3 ± 0.2	1.3 ± 0.2
	6	2.1 ± 0.4	2.1 ± 0.7
Estuarine	7	8.2 ± 0.4	n.d.
	8	4.2 ± 0.3	n.d.
	9	8.5 ± 0.4	10.5 ± 0.5
	10	8.1 ± 0.5	9.1 ± 0.7
Limnic	11	3.5 ± 0.2	n.d.
	12	28.4 ± 0.8	29.0 ± 2.5
	13	16.3 ± 0.5	18.6 ± 2.4
	14	22.3 ± 1.4	n.d.
Soil	15	19.4 ± 0.7	n.d.
	16	14.8 ± 0.2	17.2 ± 0.2
	17	12.7 ± 0.6	13.8 ± 0.4

DNA concentrations were determined by calculating the mean DNA concentrations of the three evaluation methods based upon standard addition.

DNA concentrations using the results of the three evaluation methods (Table 4). Generally, marine and flatland sediments contained low amounts of DNA, ~0.3–2.1 µg/g. In contrast, estuarine sediments showed higher DNA contents, from 4.2 to 8.5 µg/g. DNA contents of the limnic sediments were between 3.5 and 28.4 µg/g. On average, higher DNA contents between 12.7 and 22.3 µg/g were observed for soil samples.

In contrast to the SG assay, attempts to determine DNA by UV spectroscopy were not successful. DNA concentrations were enormously overestimated by factors ranging from 10 to 30 (data not shown). Although the H 33258 assay was more

specific than UV spectroscopy, sensitivity was not sufficient to gain valuable data when crude extracts of environmental samples were assayed directly (data not shown).

DISCUSSION

Quantification of DNA is of importance for the investigation of microbial communities in soils and sediments. However, co-extracted HA markedly impair DNA quantification. Due to the spectral properties of HA, DNA quantification in crude extracts by UV spectrophotometry is strongly impaired. Actually, this may even occur after extensive DNA purification (23). The observed heterogeneity of the absorption spectra of different HA preparations indicates that the correct DNA concentration may not be deduced by determining the effect of HA with the aid of defined absorption coefficients at given wavelengths. Only a decrease in the $A_{260/230}$ or $A_{260/280}$ ratios may be used as an indicator for humic or other impurities.

In contrast to UV spectrophotometry, adequate fluorescent assays are much more sensitive and specific for DNA. However, it was shown that the standard H 33258, PG and SG assays were also strongly influenced by the presence of HA.

In this study, we have characterised the effects of HA on the SG assay on the basis of molecular mechanisms. Spectroscopic investigations and gel mobility shift assays on the influence of HA on the SG assay indicated that at least the visible fraction of HA impaired the assay by binding SG.

Binding of SG to HA probably changed the polarity of the environment of SG and may have consolidated its structure, which led to the observed bathochromic and hypochromic shifts of the visible and UV absorption bands of SG. SG, a monomeric non-symmetrical cyanine dye, is composed of a *N*-alkylated benzazolum ring linked by a single methine bridge to a substituted 4-quinolinium ring carrying an exocyclic amino group (39–41). Consolidation of the methine bridge of SG probably did not occur upon binding to HA as it is supposed to be responsible for the enormous increase in fluorescence upon binding to DNA (39–41). The latter may be the reason for the observed reduction in the bandwidth of the visible absorption band of SG upon binding to DNA. Of note, the hypochromic shift of the SG visible band when bound to DNA is only marginal but conspicuous when SG interacts with HA, whereas the UV shifts of SG binding to DNA and HA are low and comparable. Taking into account the core structure of SG, a possible explanation may be that a $n \rightarrow \pi^*$ transition or a $l \rightarrow \pi^*$ transition based on an exocyclic amino group is impaired (42). Carboxylic groups of HA could closely interact with the positively charged, alkylated nitrogen of the benzazolum ring. Consequently, the electrophilic aromatic character would be reduced. This will also reduce the possibility of electron donation of unshared electron pairs from an exocyclic amino group that is lying almost parallel to the π orbitals of the aromatic system (39–41). Consequently, electron delocalisation is reduced and the high absorption coefficient of SG, which is in the range between 60 000 and 80 000/M/cm at 495 nm (39–41), decreases.

It may also be possible that HA interact with the unshared electron pairs, which would reduce the electron donating properties and consequently result in a decline in absorption.

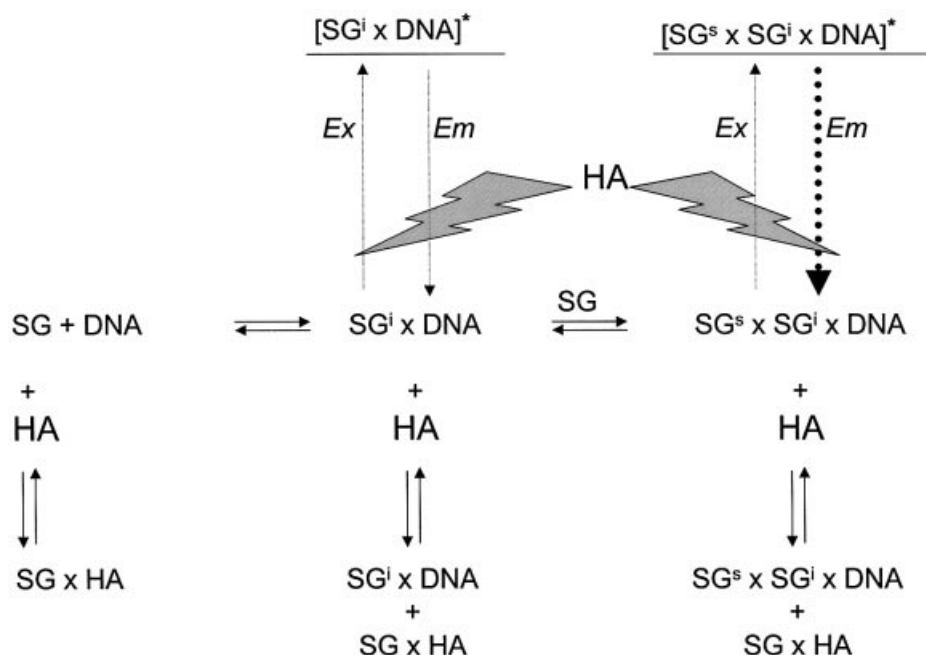
The fact that humic and fulvic acids are polydisperse mixtures of natural organic compounds containing a large number of aliphatic and C-substituted aromatic structures and different functional groups (17,43) supports these notions.

Intriguingly, the asymmetry of the visible band of SG represented by a slight shoulder at ~470 nm was less affected by the presence of HA in comparison to the maximum at 495 nm. Therefore, we would propose that the asymmetry of the visible band is not due to a 0–0 transition (44), but rather the result of another $n \rightarrow \pi^*$ or a $\pi \rightarrow \pi^*$ transition that is influenced by HA to a minor extent. However, to elucidate possible mechanisms responsible for the observed spectral properties exhaustive studies would have to be performed, which are beyond the scope of this work.

The fluorescence excitation spectra reflected the results of the absorption spectra and revealed the quenching impact of HA on SG/DNA complex fluorescence. In addition, it became clear that the excitation of the SG/DNA complex should not be performed in the UV range but rather at ~485 nm, because HA fluorescence is more intense upon excitation in the UV range. Consequently, interferences by HA fluorescence will be less prominent when the SG or the PG assay are applied where excitation is preferentially performed at 485 nm and emission determined at ~522 nm, in contrast to the H 33258 assay where excitation takes place at 360 nm and emission is detected at 465 nm. Due to the observed fluorescence of HA at ~470 nm upon UV excitation, also reported by Piana and Zahir (45), quenching of H 33258/DNA complex fluorescence may be underestimated. Of course, HA fluorescence does not necessarily make up for the quenching of SG/DNA complex fluorescence. Therefore, we think that it is advantageous to minimise the influence of HA to allow for a reproducible DNA quantification of environmental samples. Considering this aspect, it may be of interest to apply DNA-specific dyes with an excitation at even longer wavelengths than PG or SG.

In our studies, we observed that at low HA concentrations the PG assay was quenched to a slightly lesser extent than the SG and the H 33258 assays. Although, Bachoon and co-workers (6) reported that the H 33258 assay is much less affected by HA than the PG assay. This may be due to the use of different HA sources or because of the impact of HA fluorescence (see above). Furthermore, we think that the DNA concentrations applied were at least about an order of magnitude too high for the chosen dye concentrations (25). Consequently, the dye/base pair ratios were too low and the impact of HA overestimated, in particular for PG (15,25).

Apart from general considerations with respect to the dye/base pair ratio, e.g. the influence of the dilution of a dye for a given DNA concentration range (15), we propose that the presence of HA alters the dye/base pair ratio by competitively binding SG (Scheme 1), which would lead to diminished fluorescence only if the amount of dye bound to DNA is reduced. This scenario would account for the observed deviations in the modified Stern–Volmer plots at low HA concentrations and the sigmoid character of the dependence of dye/DNA complex fluorescence or slopes of calibration on HA concentrations. The reduction in DNA-bound SG by competition of HA could be partially obviated by providing more SG, e.g. by applying a 2000-fold dilution of SG reagent. However, excessive amounts of SG, e.g. a 1000-fold dilution of the SG reagent, probably led to prominent quenching by SG



Scheme 1. Simplified model of the mechanisms underlying the impact of HA on the SG assay. The following aspects are displayed: competition of HA and DNA for binding SG with respect to intercalation or semi-intercalation of SG (SG^i) followed by surface binding of SG to DNA (SG^s); fluorescence quenching of the excited SG/DNA complexes $[SG^i \times DNA]^*$ and $[SG^s \times SG^i \times DNA]^*$ by HA due to collision and the inner filter effect (sparks). As we suppose that $[SG^s \times SG^i \times DNA]^*$ expresses a much higher fluorescence than $[SG^i \times DNA]^*$, the impact of HA on the SG assay is mainly based on the quenching of $[SG^s \times SG^i \times DNA]^*$ (dotted arrow). For reasons of clarity stoichiometric coefficients and fluorescence excitation and emission of HA are not shown. Furthermore, a possible quenching sphere of action by HA has not been considered in this model. *Ex* stands for fluorescence excitation and *Em* for fluorescence emission.

itself (15), which may have been reduced by increasing HA concentrations. This scenario would explain the more complex dependence of the quenching of SG/ctDNA complex fluorescence by HA when a 1000-fold dilution of SG reagent is used in contrast to a 2000- or 6250-fold dilution (Fig. 5A).

Yet, competition of HA and DNA for binding SG appears to be even more complex, as SG probably expresses two binding modes; intercalation or semi-intercalation as well as surface binding (15,39). Actually, this model may be used to explain the observed deviations from linearity toward the *x*-axis in the Stern–Volmer plots, because intercalated and surface-bound SG are probably differentially accessible to the quencher (38,46). However, these quenching experiments could not be performed at constant ionic strength due to the heterogeneity of the HA applied. Consequently, such interpretations have to be treated with great care. Nevertheless, it can be justified to interpret the sigmoidal dependence of the binding of SG to DNA (Fig. 4D) and the electrophoretic mobility shift assays (Fig. 4C) as a high affinity intercalation or semi-intercalation followed by a cooperative surface binding of SG. Then, it may be argued that the SG/DNA complex with intercalated dye can be treated separately from the complex with intercalated and surface-bound SG (Scheme 1). According to this scenario, the influence of the competition of HA and DNA for SG on the assay will mainly be due to the withdrawal of surface-bound SG by HA as surface-bound SG expresses much higher fluorescence than intercalated SG (Fig. 4D).

Although competitive binding events are responsible for the quenching of the fluorescence of the assay, direct binding of HA to the SG/DNA complex, which would lead to static quenching, could not be shown. To further address this aspect,

we would propose performing measurements of fluorescence lifetimes, as this method would definitely allow discrimination between static and collisional quenching (38). However, we revealed the impact of collisional quenching on the fluorescence of the SG/DNA complex by examining the influence of temperature on the Stern–Volmer plot (Fig. 3D). As indicated, the degree of collisional quenching was negligible at HA concentrations $<10 \mu\text{g/ml}$. Consequently, the impact of collisional quenching processes can be minimised by simply diluting the HA-contaminated DNA extract.

It may be argued that static quenching is rather unlikely anyhow, as a close interaction of negatively charged HA (17,18) and negatively charged DNA would be unrealistic. The spectrophotometric and gel mobility shift assays performed fully support this notion. However, it may be envisioned that positively charged SG (39) could act as a mediator, in particular when SG is bound to the surface of the DNA. This concept could explain the increase in electrophoretic mobility of the SG/DNA complex with rising HA concentrations without significant loss in fluorescence up to a critical concentration (Fig. 4C). The positive charges of SG tightly bound to DNA are shielded by the negative charges of associated HA that co-migrate to the anode with the SG/DNA complex. This would speak for a quenching sphere of action, where the quencher is adjacent to the fluorophore at the moment of excitation, but does not actually form a ground state complex (38). A quenching sphere of action would also explain the deviation from linearity in the Stern–Volmer plots.

In summary, the characterisation of the molecular mechanisms underlying the impact of HA from Fluka on the SG assay revealed an inner filter effect, collisional quenching and

competitive binding of SG by HA to be responsible for the extensive quenching of the SG/DNA fluorescence by HA. The influence of a quenching sphere of action remains to be verified by further investigations.

On the basis of these findings, the next step was to develop a strategy to minimise the negative impacts of HA on the DNA quantification by SG in general. The inner filter effect and collisional quenching as well as a possible quenching sphere of action could be reduced by diluting the environmental extract with TE buffer. However, the dilution procedure was restricted by the sensitivity of the assay. Of note, the detection limit for the quantification of DNA by SG applying a standard microplate reader and a sample volume of 250 μ l is \sim 50 pg/ml and up to 2-fold higher than the quantification of DNA by PG (15,26). Also, the increase in the SG concentration by applying a 2000-fold dilution of the SG reagent instead of a 6250-fold dilution was feasible to reduce the competitive impact of HA. The drawback of this approach was a loss of sensitivity, which was around 1.3-fold. But the interfering effect of HA at concentrations <7 μ g/ml could be reduced up to 22-fold.

Apart from diluting the sample and increasing the SG concentration, the reduction in the pH value appeared to be another possibility to reduce the impact of HA. However, a possible quenching sphere of action as well as the unwanted binding events impairing the SG assay could not be eliminated by applying an acidic pH, in order to protonate HA and thereby reduce their affinity for SG. Of note, even though SG absorption at 495 nm was reduced \sim 2-fold around pH 4, the fluorescence of the SG/DNA complex did not markedly decrease. This could indicate a shift in dye protolytic equilibria upon binding to DNA, a mechanism that is also reported for other interactions between dyes and macromolecules, e.g. the binding of Coomassie brilliant blue G 250 to proteins (47).

After setting up the novel SG assay, we assessed its reliability in comparison to the PG assay by determining the recoveries of ctDNA standards containing known amounts of DNA and 0.5 mg/ml HA as well as environmental extracts. The PG assay was chosen because it is considered to be a benchmark assay for DNA quantification in solution (15,25,39). Our data showed that both assays revealed good and comparable DNA recoveries for the ctDNA standards when the standard addition technique applying evaluation method I was used.

When environmental samples were tested, the recoveries determined by evaluation method I of the PG assay were somewhat lower than those of the SG assay. This disadvantage of the PG assay may be overcome by applying higher PG concentrations in accordance with the changes made with respect to the SG assay. However, if the reagent costs are taken into consideration this does not appear to be feasible. Cost evaluations may be based on Molecular Probes momentary European Union price when 1–4 units of reagent are ordered. The PG reagent costs 290 €/ml and the SG reagent 418 €/ml. If the necessary increase in the PG concentration is about equal to that used for the SG assay, the reagent costs of the PG assay would be 17.3 times higher compared to the novel SG assay. Final dilutions of PG and SG reagents of 80- and 2000-fold, respectively, would have to be applied. Applying the final 250-fold dilution of the standard PG assay,

costs of the PG assay would still be 5.6 times higher than the costs for the novel SG assay.

With respect to the evaluation methods I, II and III following the standard addition technique, no marked differences in the mean recoveries and their standard deviations were observed for environmental samples (Table 3). With one exception (see above), the same was true for ctDNA standards (Table 2). In contrast, applying single dilutions resulted in a markedly lower mean recovery. Although the dilution series showed a mean recovery of 100%, its standard deviation of 17% was very high. This means that the application of time-consuming standard addition techniques pays off, when it comes to accuracy. Still, for a simple and quick DNA estimation the use of single dilutions or dilution series appears to be feasible.

Apart from recoveries, DNA concentrations were determined in order to further assess the reliability of the assay. DNA concentrations determined were reasonable for the environmental samples investigated. Some issues have to be addressed before judging the established novel SG assay. DNA concentrations may vary from sample to sample as the extracted amount of DNA depends on the soil and sediment type, soil biology, seasonal climate and on the content of water, oxygen and organic matter (34,48). Further, DNA concentrations depend on the extraction and purification method used and the storage of the sample (9,48,49). Thus, all these parameters should be considered when comparing DNA concentrations.

An important aspect for the assessment of the DNA concentrations determined may be derived from estimations of total numbers of prokaryotes present in soils or sediments, if the prokaryotes are supposed to be the main supplier of DNA (34). For soils like grasslands and cultivated soils prokaryotic cells numbers were estimated to be 2×10^9 cells/g soil (50). More *et al.* enumerated 3.8×10^9 cells/g in sediment samples by direct acridine orange counts (34). Based upon a single stationary phase genome weighing 5×10^{-15} g for each cell (34,51), based on data for *E.coli*, then 1 g of sediment would contain \sim 10–20 μ g DNA. In fact, DNA concentrations of forest soils have been determined to be in the range 13.7–26.9 μ g/g (23). For pasture and arable soils prokaryotic numbers up to 2.1×10^{10} cells/g soil have been reported (5). This implies that up to 105 μ g DNA/g soil may be extracted. The DNA concentrations obtained could even be higher when other than prokaryotic organisms are lysed by the extraction procedure.

According to data from aquatic environments a genome weighing 1×10^{-15} g DNA/cell appears plausible as well (11). Consequently, values around 2 μ g/g sediment may be expected as well. Actually, Lovell and Piceno reported a DNA concentration of aquatic sediments ranging between 1.3 and 23.3 μ g/g (33). However, they determined DNA concentration by fluorimetry using H 33258 and based their values on the wet weight of their samples. Still, these data reflect the possible range of DNA concentration from aquatic sediments. With respect to estuarine samples, it should be stated that Ogram *et al.* determined the DNA concentration by agarose gel densitometry to be \sim 27 μ g/g (dry weight) (52).

The data outlined above are in agreement with our findings that also reflect a broad range of DNA concentrations. Marine samples including flatland were in the range 0.3–2.1 μ g/g. Soil

and limnic samples were in the range 12.7–28.4 µg/g, with the exception of the limnic sample 11. This exception may be due to the texture of the samples, e.g. limnic sample 11 consisted mainly of small roots and leaves that had not yet decomposed, in contrast to a finer texture of the samples with higher DNA concentrations. Intriguingly, estuarine samples revealed DNA concentrations around 4.2 and 10.5 µg/g. This was right in between the data for limnic and marine samples. It would be interesting to follow up by further studies if the DNA concentrations reflect the intermediary position of estuarine environments and the consequences for habitats, fauna and flora.

Of note, UV spectra revealed DNA concentrations of 11.4 and 5 µg/g for marine samples 1 and 2, respectively (data not shown). Taking into account a 10–30-fold overestimation of DNA concentrations when UV spectroscopy is used for DNA quantification in environmental extracts (see above), then the DNA concentrations should be ~0.4–1.1 and 0.2–0.5 µg/g for marine samples 1 and 2, respectively. The DNA yields determined by the modified SG assay were within these ranges (Table 4).

Apart from the general consistency of the DNA concentrations determined by the novel SG assay with respect to the data found in the literature and the theoretical considerations, the high recoveries indicate the reliability of the novel SG assay.

In conclusion, the standard and the modified SG assays proved to be more sensitive than the PG, the H 33258 and the UV absorbance assays. The influences of HA on the standard SG, the PG and the H 33258 assays were comparable, but were significantly reduced when the modified SG assay was applied, in particular at low HA concentrations. This observation may be explained by the mechanisms determined to be responsible for the impact of HA from Fluka on DNA quantification by SG. Beside fluorescence quenching, competitive binding mechanisms seemed to account for the influence of HA on the SG assay. This competition appeared to be based on the withdrawal of intercalated or semi-intercalated and surface-bound SG from DNA. We believe that the latter strongly impairs the SG assay, as surface-bound SG mainly accounts for fluorescence, in contrast to intercalated or semi-intercalated SG. By applying a 2000-fold dilution of the SG reagent, the influence of competition was found to be reduced in the novel SG assay. Consequently, the assay became more robust while maintaining its high sensitivity. Due to the lack of sensitivity, the H 33258 and the UV absorbance assays were not applicable for the determination of DNA in crude sample extracts. Although the PG assay appeared to be robust, higher sensitivity, markedly lower costs and a better reliability, with respect to the recoveries observed, favour the modified SG assay. Therefore, the novel SG assay should be very useful for the investigation of DNA directly extracted from soils and sediments.

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