Humic substances cause fluorescence inhibition in real-time polymerase chain reaction

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ABSTRACT

Real-time polymerase chain reaction (qPCR) is the cornerstone of DNA analysis, enabling detection and quantification of minute nucleic acid amounts. However, PCR-based analysis is limited, in part, by the presence of inhibitors in the samples. PCR inhibition has been viewed solely as failure to efficiently generate amplicons, that is, amplification inhibition. Humic substances (HS) are well-known inhibitors of PCR amplification. Here we show that HS from environmental samples, specifically humic acid (HA), are very potent detection inhibitors, that is, quench the fluorescence signal of double-stranded DNA (dsDNA) binding dyes. HA quenched the fluorescence of the commonly used qPCR dyes EvaGreen, ResoLight, SYBR Green I, and SYTO 82, generating lowered amplification plots, although amplicon production was unaffected. For EvaGreen, 500 ng of HA quenched nearly all fluorescence, whereas 1000 ng of HA completely inhibited amplification when applying Immolase DNA polymerase with bovine serum albumin (BSA). Fluorescence spectroscopy measurements showed that HA quenching was either static or collisional and indicated that HA bound directly to the dye. Fulvic acid did not act as a qPCR detection inhibitor but inhibited amplification similarly to HA. Hydrolysis probe fluorescence was not quenched by HA. Detection inhibition is an overlooked phenomenon that needs to be considered to allow for development of optimal qPCR assays.

Real-time polymerase chain reaction, also called quantitative PCR (qPCR),[1] has become the cornerstone of DNA analysis, enabling detection of minute amounts of nucleic acids [1]. However, the full potential of qPCR is still limited, in part, by the presence of qPCR inhibitors in the samples [2]. These inhibitors may be divided into two main groups depending on how they disturb the qPCR, namely, amplification inhibitors and detection inhibitors. Amplification inhibitors (i.e., conventional PCR inhibitors) act by directly affecting the DNA polymerase, by changing the buffer composition, or by binding to nucleic acids. Detection inhibitors have not previously been reported in qPCR, but it is assumed that some molecules may quench the fluorescence signal from dyes or probes or may alter background fluorescence.

Environmental samples, including soils and aqueous sediment, are known to contain PCR inhibitors [3,4], and in particular humic substances (HS) have been implicated as the main cause for amplification failure [5]. Previous studies on the effects of HS on qPCR have shown conflicting results, both in the amounts of HS tolerated and in the presumed mechanisms of inhibition. Humic acid (HA) has been found to directly disturb the DNA polymerase [6,7] and to impair amplification by binding to template DNA [8]. Studies of HS in other scientific fields may add some insights into their possible effects on PCR. For example, HA forms colloids in water and complexes with iron and calcium ions [9], meaning that they could affect the ion content in PCR, for example, by chelating magnesium ions.

In general, HS are divided into three major fractions: fulvic acid (FA), HA, and humin [10]. Humin is insoluble and, thus, will not

affect PCR; HA is soluble at neutral or alkaline pH; and FA is soluble in water at all pH levels. FA and HA are both dibasic weak acids with carboxyl and hydroxyl groups, with a similar content of phenol groups [11]. PCR inhibition is caused by water-soluble HS, which are not efficiently removed by classical purification principles such as phenol or column purification. Therefore, more advanced DNA purification methods have been developed, such as subjecting the extracts to high pressure applying pressure cycling technology (PCT) [12] and synchronous coefficient of drag alteration (SCODA), where DNA is focused into a small area on a gel through altering voltage [13]. However, extensive sample treatment and DNA purification inevitably lead to DNA loss, with recovery rates of approximately 10 to 80% depending on the chosen method [14].

Another approach is to enable amplification by finding a DNA polymerase–buffer system that tolerates a high level of impurities, a concept called pre-PCR processing [2,15]. This lowers the need for pure extracts, leading to simplified analysis processes and improved detection limits. Here we applied pre-PCR processing to systematically investigate the effects of HS on qPCR. A DNA polymerase–buffer system with high resistance to HS was used to ensure that relevant effects were studied. Different HS samples, as well as standardized HA and FA preparations, were analyzed using various fluorescent DNA binding dyes and fluorescent hydrolysis probes. We used sediment from lakes as reference material, mimicking the complex HS content appearing in the water phase in DNA extraction of environmental samples. In addition, surface water, landfill soil, and plant soil were analyzed, giving a broad range of relevant HS-containing samples. Fluorescence spectroscopy was used to pinpoint the inhibition mechanisms of HS, for the first time discriminating between amplification inhibition and detection inhibition in qPCR.

Materials and methods

PCR inhibitory samples

Sediment samples (sediment with water) were taken from six Swedish forest lakes and pooled to ensure a representative content of HS. The sediment HS were diluted in Super-Q water (Merck Millipore, Billerica, MA, USA) and used as a model of the HS found in DNA extracts when analyzing environmental samples. See Table S1 in the online supplementary material for further description of the sediment HS. The HA standard (product no. 53680) was obtained from Sigma–Aldrich (Taufkirchen, Germany), and the FA standard (Suwannee River fulvic acid standard II) was purchased from the International Humic Substances Society (IHSS). HA and FA were dissolved in TE buffer (10 mM Tris and 0.1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0) (Medicago, Uppsala, Sweden). Plant soil was purchased from a local Swedish supermarket. Landfill soil was collected from a waste dump in Albäck, Trelleborg, Sweden. The soils were mixed with TE buffer (20% [w/w] for plant soil and 25% [w/w] for landfill soil) through 1 h of shaking at room temperature using a VIBRAX VXR basic shaker at 1500 rpm (IKA, Staufen, Germany). Supernatants carrying water-soluble HS were used in the qPCR experiments. Surface water was taken from the same six Swedish lakes as the sediment above and pooled. qPCR template DNA was extracted from human blood from one male using Chelex-based (Bio-Rad, Hercules, CA, USA) extraction [16] and subsequently quantified with a BioDrop μLITE (BioDrop, Cambridge, UK). The resulting DNA extracts were diluted to 1 ng/μl in Super Q water and stored in aliquots at −20 °C. Amplicons for fluorescence spectroscopy experiments were prepared from *Staphylococcus aureus* enterotoxin D (SED) according to Márta and coworkers [17]. The resulting PCR product

was purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) followed by a GeneJet PCR Purification Kit (Thermo Scientific, Waltham, MA, USA). The amplicon DNA concentration was subsequently measured with a BioDrop μLITE.

The following HA amounts were used in qPCR: 10, 20, 50, 100, 200, 300, 500, 600, 700, 800, 900, and 1000 ng (analyzed in triplicates). The following FA amounts were used in qPCR: 500, 750, 1000, 1250, and 2000 ng (analyzed in duplicates). Sediment dilutions corresponding to 0.02, 0.06, 0.10, 0.20, and 1 μl of sediment were used in qPCR (analyzed in triplicates). Supernatants of plant soil, landfill soil, and surface water were added to qPCR experiments in different amounts (specified in Table S3 of the supplementary material; analyses performed in triplicates).

qPCR analysis

All qPCR experiments were carried out on a LightCycler Nano Instrument (Roche Diagnostics, Basel, Switzerland) with a reaction volume of 20 μl. LightCycler Nano Software version 1.1 was used for determination of quantification cycle (C_q) values and amplicon melting temperature (T_m). Unless otherwise specified, the following reagents were included: 1× Immobuffer (Bioline Reagents, London, UK), 0.2 mM deoxynucleoside triphosphate (dNTP, Roche Diagnostics), $MgCl_2$ (4 mM for hydrolysis probe and 2.5 mM for double-stranded DNA [dsDNA] binding dye EvaGreen [EG], Roche Diagnostics), 0.3 μM of each primer (RB1_80F and RB1_235R, Life Technologies, New York, NY, USA) [18], 10 μg of bovine serum albumin (BSA, Roche Diagnostics), 1 U of Immolase (Bioline Reagents), and 2 ng of template DNA. For detection, either an EG dye (Biotium, Hayward, CA, USA) or a 6FAM probe (RB1, Life Technologies) [18] was used unless otherwise specified. The same qPCR conditions were used for all tests with a heat-activating step at 95 °C for 15 min, followed by 55 cycles of 10 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C. When a dye-based chemistry was used, melt curve analysis was included to determine the specificity of the formed product. Melt curve analysis was performed with 60 °C as starting temperature and 0.1 °C/s temperature increase up to 97 °C. Correct amplicon length (156 bp) was verified with 1% agarose gel electrophoresis stained with 1× GelRed (Biotium). Gel bands were visualized using BioOne Quantity (Bio-Rad). For dsDNA binding dye, end-point fluorescence intensity for the analyses containing inhibitors was normalized against the average value of the positive controls. This normalized fluorescence intensity was used as a measure of detection inhibition. For the hydrolysis probe analyses, C_q value shift was used as a measure of amplification inhibition. In the experiments with different dyes, 1× EG, 0.5× SYBR Green I (Life Technologies), 2 μM SYTO 82 (Life Technologies), and 1× ResoLight (Roche Diagnostics) were used in the qPCR experiments. In addition, 4 mM $MgCl_2$ was used for all dyes except EG, where 2.5 mM was used. Here duplicates were analyzed.

Fluorescence spectroscopy measurements

CLARIOstar (BMG Labtech, Ortenberg, Germany) was used for the fluorescence spectroscopy measurements, applying 6.0 mM focal height and a gain of 1800. Samples were analyzed in a total volume of 50 μl in a Costar 96-well plate (Corning, New York, NY, USA) and incubated 10 min before measuring at 37 °C. The fluorescence spectra were analyzed with excitation at 487 nm and emission spectra from 510 to 700 nm. Spectra for analyses containing HA and FA are presented with fluorescence intensities normalized against the values of the positive controls containing Super Q water. The reactions contained 1× PCR Buffer (15 mM $MgCl_2$, Roche Diagnostics), 1× EG, and 5 ng of SED amplicons. The following HA amounts were applied in fluorescence spectroscopy: 10, 20,

50, 100, 200, 300, and 500 ng (analyzed in triplicates). The following FA amounts were applied: 300, 500, 1000, and 2000 ng (analyzed in triplicates).

Results

Humic substance-tolerant DNA polymerases

Initially, we applied pre-PCR processing to find a DNA polymerase–buffer system with high tolerance to HS. Sixteen DNA polymerase–buffer systems were screened in hydrolysis probe qPCR for tolerance against different amounts of standardized aqueous sediment samples (see Tables S1 and S2 in supplementary material). The tested DNA polymerases showed a wide range of qPCR performance, some being completely inhibited by 0.025 µl of HS (data not shown) and one tolerating 3 µl of HS (Table S2). The addition of BSA improved the performance of most DNA polymerase–buffer systems. Among the top four, Immolase DNA polymerase with BSA was found to be the most robust alternative. Thus, Immolase with 10 µg of BSA was chosen for characterization of qPCR inhibition mechanisms.

qPCR inhibition caused by humic substances

Applying the dsDNA binding dye EG, the addition of sediment HS led to lowered qPCR fluorescence signals (Fig. 1A). The addition of 0.02 µl of HS lowered the normalized end-point fluorescence intensity to approximately 0.6 compared with positive controls without HS. When 1 µl of HS was added, there was no visible fluorescence signal. This would normally be interpreted as complete inhibition of amplification (i.e., that no PCR product was formed). However, when running the qPCR products on agarose gel electrophoresis, clear bands with similar intensities were seen for 0 to 1 µl of HS (Fig. 1B). Seemingly, the inhibition seen in EG qPCR was due to quenching of fluorescence rather than to inhibition of amplification. Comparing EG results with hydrolysis probe qPCR runs verified this notion given that the probe run C_q values were unaffected by 1 µl of sediment HS (C_q of 29.18 ± 0.04 without HS and C_q of 29.16 ± 0.07 for 1 µl of HS). The 6FAM dye used in the probe system has similar excitation/emission spectra as EG but was not affected by fluorescence quenching. Landfill soil, plant soil, and surface water were also applied in qPCR (Table S3). Both soil types quenched EG fluorescence. For landfill soil, 1 µl gave nearly complete detection inhibition with a normalized end-point fluorescence intensity of 0.02. For plant soil, the effect was less dramatic; the addition of 8 µl resulted in normalized end-point fluorescence intensity of 0.54. For the soils, verification with gel electrophoresis showed that amplicon production was unaffected (data not shown). Surface water did not show any fluorescence inhibition. Subsequently, HA and FA standards were applied separately to investigate their respective roles in amplification inhibition and detection inhibition.

Detection inhibition caused by humic acid

Different amounts of HA standard were added to EG qPCR, quenching fluorescence in a fashion similar to the sediment HS (Fig. 2A). The addition of 50 ng of HA gave a quenching effect comparable to 0.02 µl of HS (normalized end-point fluorescence of 0.6). The addition of 500 ng of HA gave a nearly flat amplification plot. However, as in the case of HS, gel electrophoresis verified that the amounts of generated amplicons were the same for 0 to 500 ng of HA (data not shown). In hydrolysis probe qPCR, no quenching was observed and up to 600 ng of HA had no effect on the C_q values (Fig. 2B). For FA, no fluorescence quenching was noted in EG qPCR (Fig. 3).
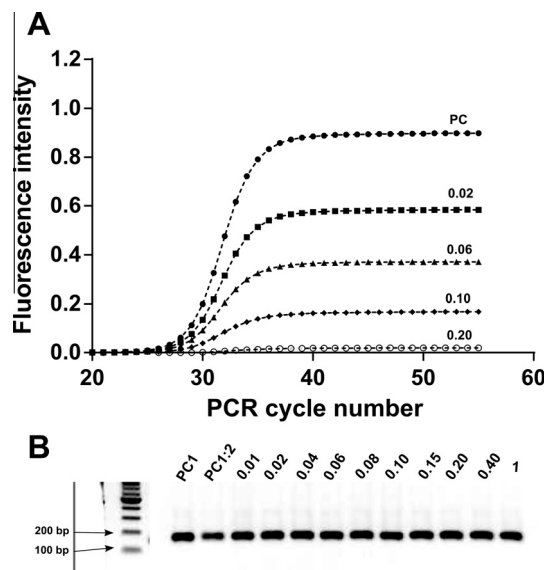

Fig.1. Inhibition effect of sediment HS on EG dye qPCR. (A) Amplification plots for the addition of different amounts of sediment HS, applying 2 ng of human DNA in all analyses: positive control (PC) with 0 µl of sediment HS (filled circles), 0.02 µl of sediment HS (filled squares), 0.06 µl (filled triangles), 0.10 µl (open diamonds), 0.20 µl (open circles), and 1.00 µl (open triangles). Here 0.20 µl of sediment HS shows nearly total inhibition, and 1.00 µl gives total inhibition (i.e., no amplification plot is visible). (B) Agarose gel electrophoresis results for the qPCR products in panel A. Up to 1.00 µl of sediment HS gave amplicon amounts in the same range as samples without HS (PC). For PC 1:2, half of the PCR product volume was taken, clearly showing the weakest amplicon band on the gel picture. Thus, the inhibitory effect of sediment HS seen in panel A is related to detection of amplicons (i.e., fluorescence quenching).

Amplification inhibition caused by humic acid and fulvic acid

In hydrolysis probe experiments, 1000 ng of HA caused nearly complete amplification inhibition (mean C_q of 48.5 ± 7.5) (Fig. 2B). EG qPCRs with at least 750 ng of FA showed impaired amplification, that is, elevated C_q values. When comparing qPCR data with gel electrophoresis results, the level of end-point fluorescence reflected the intensity of the gel bands; with 1500 ng of FA in the reactions, there was nearly no fluorescence signal (Fig. 3A) and nearly no visible amplicons on the gel (Fig. 3B). Contrary to HA, FA gave a similar negative effect on EG dye and hydrolysis probe qPCR, causing amplification inhibition leading to increased C_q values (Fig. 4).

To investigate the mechanisms of amplification inhibition, three DNA polymerases were evaluated using hydrolysis probe qPCR. BSA (10 µg) was used as facilitator in all runs. For all DNA polymerases (i.e., *Ex Taq* Hot Start, Immolase, and Tempase), more HA could be tolerated by doubling the amount of enzyme (see Table S4 in supplementary material). For example, adding 2 U instead of 1 U rescued amplification for *Ex Taq* Hot Start and Tempase with 500 ng of HA, although still generating partial inhibition. Immolase tolerated more HA than *Ex Taq* Hot Start and Tempase; the latter two were completely inhibited by 500 ng of HA (1 U of DNA polymerase), whereas Immolase was unaffected by 500 ng and only partially inhibited by 1000 ng (Table S4). Immolase (1 U) performed better than 2 U of the other two DNA polymerases for all HA amounts tested. Adding more $MgCl_2$ (6 mM instead of 4 mM) or more DNA (20 ng instead of 2 ng) to HA and FA qPCR hydrolysis probe runs did not counteract the inhibitory effect (data not shown).

The inhibitor-relieving properties of BSA were investigated by adding various amounts of the protein to hydrolysis probe runs
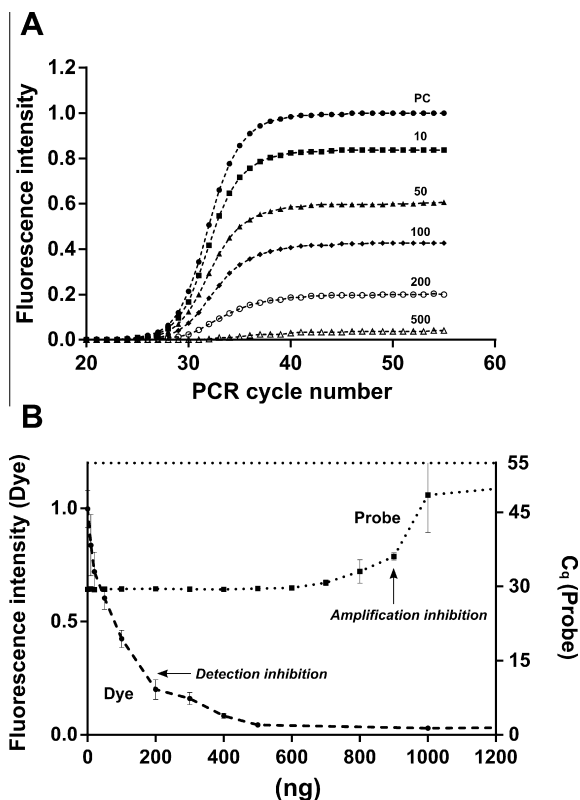
A

B

Fig.2. Inhibition of qPCR detection and amplification by HA standard. (A) Amplification plots for the addition of different amounts of HA to EG dye qPCR, applying 2 ng of human DNA in all analyses: positive control (PC) with 0 ng of HA (filled circles), 10 ng (filled squares), 50 ng (filled triangles), 100 ng (open diamonds), 200 ng (open circles), and 500 ng (open triangles). (B) Normalized end-point fluorescence intensities using EG dye detection (circles) and C_q values for hydrolysis probe detection (squares) are shown, with different amounts of HA standard. Probe fluorescence is not quenched by HA, meaning that probe C_q shifts signify amplification inhibition. The lowered normalized end-point fluorescence with EG is a detection inhibition effect.
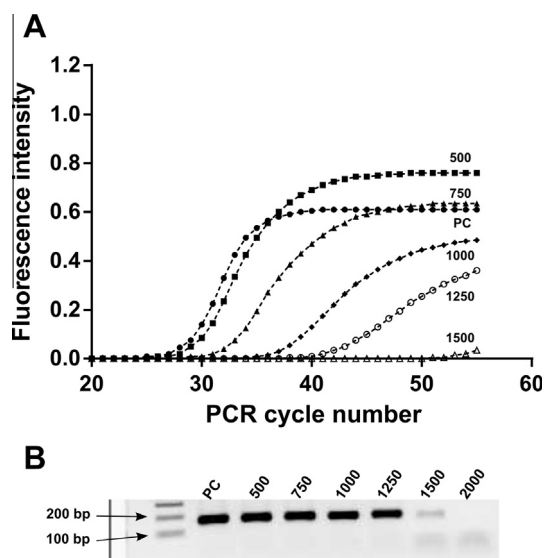

A

B

Fig.3. Inhibition of qPCR amplification by FA standard. (A) Amplification plots for the addition of different amounts of FA to EG dye qPCR, applying 2 ng of human DNA in all analyses: positive control (PC) with 0 ng FA (filled circles), 500 ng (filled squares), 750 ng (filled triangles), 1000 ng (open diamonds), 1250 ng (open circles), and 1500 ng (open triangles). (B) Agarose gel electrophoresis results for the qPCR products in panel A. Gel band intensity conforms with amplification plot fluorescence intensity (i.e., FA causes amplification inhibition), not detection inhibition.
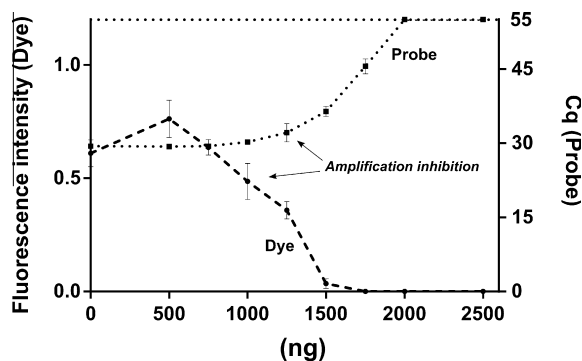

Fig.4. Similar effect of FA on EG dye and hydrolysis probe detection qPCR. Normalized end-point fluorescence intensities using EG dye detection (circles) and C_q values for hydrolysis probe detection (squares) are shown, with different amounts of FA standard. No detection inhibition was seen; EG dye and probe detection showed similar amplification inhibitory effects.

containing HA. Using Immolase without BSA, qPCR was completely inhibited by 100 ng of HA, but with 10 µg of BSA up to 500 ng of HA had no negative effect (see Table S5 in supplementary material). The tolerance to HA improved with increasing amounts of BSA, from 0.25 to 20 µg. For 20 µg of BSA, the amplification baseline was slightly skewed in some cases, possibly causing problems when analyzing qPCR data and indicating that high BSA amounts should be used with caution. When evaluating various PCR facilitators in the presence of HS—including proteins, biologically compatible solutes, and non-ionic detergents—the osmoprotectant trehalose and the protein gp32 gave promising results, although not as good as BSA (data not shown). For example, 0.2 M trehalose relieved some of the amplification inhibition by HS, but not as efficiently as 2 µg of BSA (data not shown). Mixing BSA, Trehalose, and gp32 in different combinations did not improve results compared with BSA alone.

Fluorescence quenching mechanisms

To investigate the generality of the fluorescence inhibition phenomenon, other dsDNA binding dyes were assessed in qPCR experiments (Table 1). The degree of quenching differed to some extent among the dyes, with the greatest effect on EG and ResoLight, where the normalized end-point fluorescence intensity was less than 0.1 for 500 ng of HA. SYBR Green I and SYTO 82 showed normalized fluorescence intensities of approximately 0.3 to 0.4 for 500 ng of HA. Notably, the addition of HA slightly lowered the T_m of the amplicons for all dyes except for SYTO 82. For EG, ResoLight, and SYBR Green I, 500 ng of HA lowered the T_m by approximately 0.6 to 1 °C. For SYTO 82, the T_m was unaffected by HA. Gel electrophoresis verified that the correct amplicon was formed irrespective of dye type (data not shown).

Fluorescence spectroscopy was used to investigate the mechanisms of HA-induced quenching (Fig. 5). The addition of HA lowered the fluorescence intensity for free EG dye and EG bound to dsDNA. For both free and bound dye, 50 ng of HA lowered the normalized fluorescence to approximately 0.6 compared with positive controls, and 100 ng of HA lowered the normalized fluorescence to approximately 0.3. HA did not affect the emission spectra of the EG–DNA complex (see Fig. S1 in supplementary material), with EG showing maximum fluorescence at 525 nm (excitation = 487 nm) in both free and bound form and in the presence and absence of HA. Free dye and dye bound to dsDNA have similar emission spectra, although the amplitude of fluorescence increased

Table 1
Comparison of four qPCR dyes in terms of HA quenching effect.

Dye	Measurement	Humic acid (ng)				
		0	20	100	300	500
EvaGreen	Relative intensity	1.00	0.67;0.85	0.47;0.48	0.15;0.19	0.03;0.03
	T_m	81.89;81.85	81.58;81.62	81.18;81.23	81.16;81.14	81.03;80.99
ResoLight	Relative intensity	1.00	0.79;0.77	0.38;0.55	0.10;0.12	0.06;0.06
	T_m	82.76;82.73	82.69;82.67	82.42;82.50	82.18;82.15	82.09;82.09
SYBR Green I	Relative intensity	1.00	1.16;1.02	0.81;0.89	0.58;0.46	0.34;0.37
	T_m	83.31;83.07	83.23;83.22	82.85;82.86	82.46;82.46	82.23;82.19
SYTO 82	Relative intensity	1.00	1.05;0.99	0.58;1.00	0.40;0.44	0.36;0.42
	T_m	81.97;81.95	81.96;81.92	81.88;81.84	81.95;81.97	81.90;81.93

Note. End-point fluorescence intensities, normalized to reactions without HA and amplicon melting temperatures (T_m) are shown for different HA amounts. All dyes show the HA quenching effect, with EvaGreen and ResoLight being more affected than SYBR Green I and SYTO 82. T_m is slightly lowered by HA for all dyes except SYTO 82, where T_m shows no HA effect. Data obtained from two experiments unless indicated otherwise.
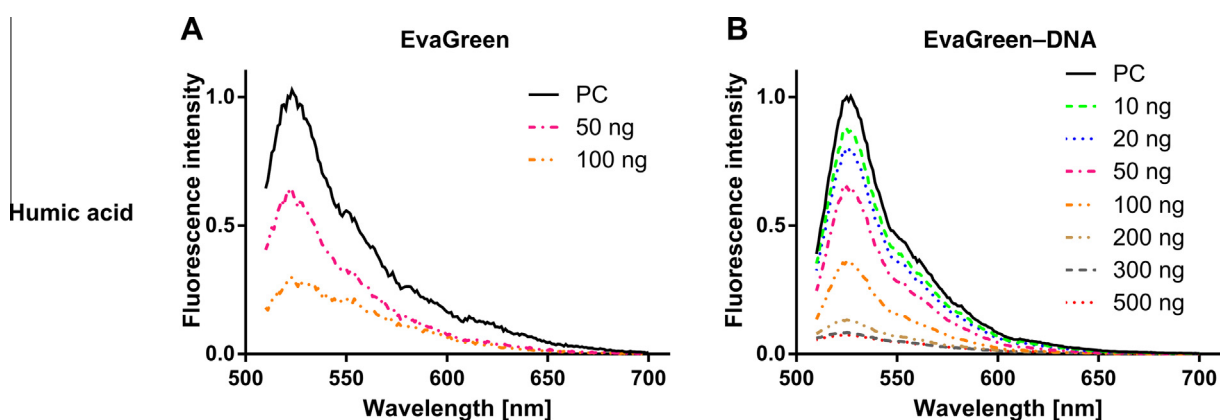

Fig.5. HA quenching effect on free and dsDNA-bound EG dye. (A) Fluorescence emission spectra for free EG with different amounts of HA: 0 ng of HA (black), 50 ng (pink), and 100 ng (orange). Fluorescence values are normalized to the fluorescence intensity of analyses without HA. (B) Fluorescence emission spectra for EG bound to dsDNA (5-ng amplicons), with different amounts of HA: 0 ng of HA (black), 10 ng (green), 20 ng (blue), 50 ng (pink), 100 ng (orange), 200 ng (brown), 300 ng (gray), and 500 ng (red). Fluorescence values are normalized to the fluorescence intensity of analyses without HA. HA quenches fluorescence in a similar fashion for both EG dye without DNA and with DNA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
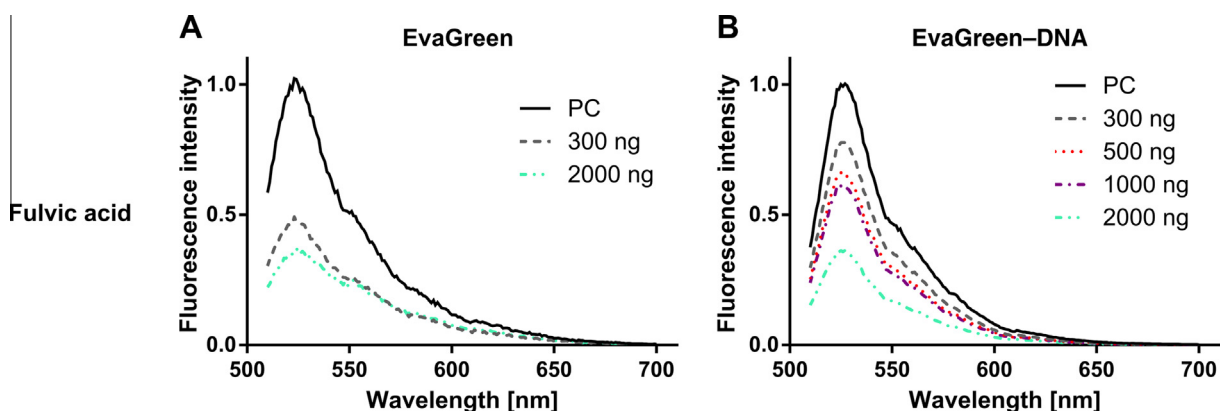

Fig.6. FA quenching effect on free and bound EG dye. (A) Fluorescence emission spectra for free EG with different amounts of FA: 0 ng of FA (black), 300 ng (gray), and 2000 ng (turquoise). Fluorescence values are normalized to the fluorescence intensity of analyses without FA. (B) Fluorescence emission spectra for EG bound to dsDNA (5-ng amplicons), with different amounts of FA: 0 ng of FA (black), 300 ng (gray), 500 ng (red), 1000 ng (purple), and 2000 ng (turquoise). Fluorescence values are normalized to the fluorescence intensity of analyses without FA. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in the bound state (∼10-fold). In addition, FA quenched EG fluorescence with and without dsDNA present (Fig. 6). However, the amount of FA needed to lower the normalized fluorescence to below 0.5 was at least 1000 ng, whereas less than 100 ng of HA had the same quenching effect. In qPCR (Figs. 3 and 4), FA showed no fluorescence inhibition effect. This is due to the fact that the amounts of FA needed to quench the fluorescence also inhibit amplification, masking any FA quenching effect.
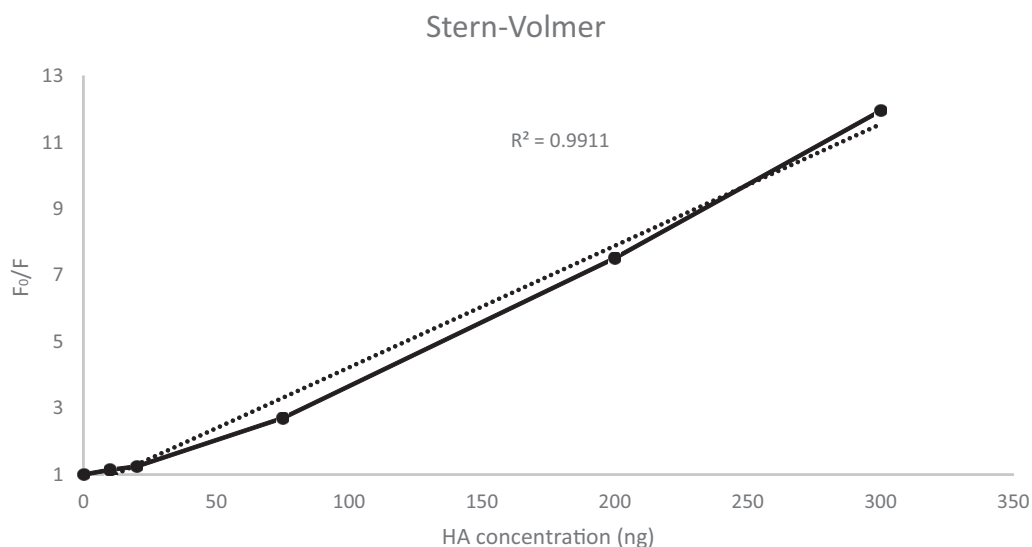
Fig.7. Stern–Volmer plot for HA quenching. Fluorescence intensity of EG bound to dsDNA without the quencher (F_0) divided by the fluorescence intensity with the quencher (F) is plotted against the concentration of the quencher (HA). Displayed is the R^2 of the best fit of linear regression (0.9911).

A Stern–Volmer plot was generated to identify the quenching mechanism when HA acts as quencher of EG (Fig. 7). The linear relationship between the ratios of the fluorescence intensity without quencher (F_0) and the fluorescence intensity with quencher (F), against the concentration of the quencher, indicates that static quenching or collisional quenching is the major mechanism. Static quenching occurs when the quencher forms a ground state complex with the fluorophore in its unexcited state, and collisional quenching occurs when the quencher encounters the fluorophore in its excited state [19].

Discussion

HS in general, and HA in particular, are known to disturb PCR-based nucleic acid analysis [3,4]. This has previously been viewed solely as an issue of amplification inhibition, that is, failure to efficiently generate amplicons [6,7]. However, by using an HS tolerant DNA polymerase, we have shown that HA from sediment and soil can also act as detection inhibitors in qPCR, that is, quench the fluorescence signal of dsDNA binding dyes.

HA, but not FA, causes fluorescence inhibition in qPCR. FA also quenches EG fluorescence, but to a much lower extent than HA. Free EG dye and DNA-bound EG were similarly quenched by HA, indicating that there is a direct interaction between HA and the dye. If HA instead bound to DNA, hindering EG binding, a specific negative effect on DNA-bound EG would be expected. The linear relation in our Stern–Volmer plot (Fig. 7) pointed toward static or collisional quenching, of which static quenching fits better with HA's similar effect on free and bound EG. Thus, we hypothesize that HA binds to the dye, forming a ground state complex and/or hindering the dye from binding to DNA. We did not correct our data for a possible inner filter effect because we see no absorbance of HA at approximately 500 nm but instead see a strong peak at 320 nm (data not shown), meaning that the absorption spectra of fluorophore and quencher do not overlap. This is consistent with previous findings concerning HA fluorescence spectra [20]. Although this is the first report of fluorescence quenching leading to detection inhibition in qPCR, HS have previously been shown to negatively affect fluorometric DNA quantification using the DNA binding dyes PicoGreen [21] and SYBR Green I [22]. In the latter

study, SYBR Green I was found to generate both collisional and static quenching.

We observed fluorescence quenching for four of the most commonly used qPCR dyes. We also noted differences between dyes, with EG and ResoLight seeming to be relatively more quenched than SYBR Green I and SYTO 82. Considering that $1 \times$ EG has a concentration of 1.33 μM and $0.5 \times$ SYBR Green I equals 0.34 μM [23], and that we used 2 μM SYTO 82 (ResoLight has an unknown concentration), there is no correlation between the effect and the dye concentration. The differences between dyes could be caused by their different structures and affinities for HA, for example, based on ionic charge. Cyanine dyes generally have positive charges, and HA is negatively charged in qPCR (pH 8.3). SYBR Green I is a positively charged unsymmetrical cyanine dye [24]. EG is likely a positively charged symmetrical cyanine dye [25], although the exact structure is a trade secret.

Increasing the amount of DNA polymerase is a way of overcoming HA amplification inhibition (Table S4) [7]. HA amplification inhibition effects also differ among different types of DNA polymerases (Table S4). *Ex Taq* Hot Start and Tempase are derived from *Thermus aquaticus* (*Taq*), whereas Immolase is isolated from a novel unspecified organism. *Taq*-derived DNA polymerases have previously been found to be more susceptible to inhibition by, for example, blood and bone than DNA polymerases derived from other organisms such as *Thermus thermophilus* (*Tth*) [26]. HA is a chelating agent [9], and binds DNA under certain circumstances [27]. However, because neither increased Mg^{2+} amounts nor increased DNA amounts could relieve amplification inhibition, neither of these properties is a likely reason for qPCR amplification inhibition. We conclude that HA-induced amplification inhibition is caused by a direct effect on the DNA polymerase rather than on Mg^{2+} ions or template DNA.

Other than choosing a robust DNA polymerase, inhibitor tolerance can be improved by adding amplification facilitators. A comprehensive list of facilitators that have been shown to counteract inhibition can be found in Hedman and coworkers [2]. BSA is a large protein active in the bloodstream with a good binding capacity for known PCR inhibitors such as bilirubin and fatty acids [28] (accession no. P02769). BSA has previously been shown to relieve inhibition from HA and FA in PCR [5,6]. We have shown that BSA has a great impact on amplification inhibition caused by HA, likely

sheltering DNA polymerase molecules by binding to HA (Table S5). Even the most robust DNA polymerase in our screening, Immolase, performs poorly without BSA. However, we see no effect of BSA on HA-induced fluorescence quenching. The same EG fluorescence inhibition was seen with 2 and 10 µg of BSA (data not shown). BSA has a net charge of approximately −8.4 at pH 6.8 and −17.2 at pH 10.5 [29]. The cyanine dyes generally have positive charges in qPCR, and HA is negatively charged, possibly explaining why HA–dye interactions may be preferred over HA–BSA interactions.

Environmental samples contain complex mixtures of HS, and the content of HA and FA differs between different soils and waters. Because FA is more soluble in water than HA [10], we would expect our surface water samples to contain more FA and the soil samples to contain more HA. The soil and sediment samples were brownish, like HA, and the surface water was yellow, like FA. Sediment HS and soil caused significant quenching of qPCR fluorescence, whereas surface water did not (Table S3), fitting with the assumption that the former contain predominately HA and the latter contain predominately FA. FA and HA are heterogeneous substances with structural similarities given that they are both "quilts" of aromatic groups with hydroxyl and carboxyl groups attached [10]. Furthermore, there are differences that may explain why HA molecules are stronger quenchers than FA. HA contains more carbon than FA, in particular aromatic carbon. In addition, HA molecules are generally larger, with molecular weights up to approximately 100,000 Da compared with 1000 to 10,000 Da for FA [9]. Thus, it is likely the amount of aromatic groups and/or the greater molecule size that make HA the better fluorescence quencher. FA and HA cause amplification inhibition at similar levels, with HA being somewhat more potent when applying Immolase with 10 µg of BSA (1000 ng of HA gives an effect similar to 2000 ng of FA; Figs. 2B and 4). FA and HA have similar amounts of phenol groups, indicating that phenol is the likely cause for amplification inhibition directly affecting the DNA polymerase [6].

By determining that HS cause fluorescence quenching as well as amplification inhibition, it is possible to overcome qPCR inhibition by using different strategies. For example, because the fluorescence quenching problem affects dsDNA binding dyes but not the hydrolysis probe, applying a probe-based system is a way of overcoming detection inhibition. Furthermore, by using a DNA polymerase–buffer system, including amplification facilitators such as BSA and even a DNA polymerase blend [30], a system that tolerates a high amount of HS can be developed. We emphasize that there is also the possibility to use different purification strategies to remove HS from the DNA extracts, but common for all of these strategies is that they result in a significant DNA loss. Therefore, it is beneficial to design a PCR that can tolerate some HS and thereby avoid the extra work and loss of target nucleic acids associated with sophisticated DNA purification methods.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.07.002.

References

[1] C.T. Wittwer, M.G. Herrmann, A.A. Moss, R.P. Rasmussen, Continuous fluorescence monitoring of rapid cycle DNA amplification, Biotechniques 22 (1997) 130–138.

[2] J. Hedman, R. Knutsson, R. Ansell, P. Rådström, B. Rasmusson, Pre-PCR processing in bioterrorism preparedness: improved diagnostic capabilities for laboratory response networks, Biosecur. Bioterror. 11 (2013) S87–S101.

[3] Y.L. Tsai, B.H. Olson, Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction, Appl. Environ. Microbiol. 58 (1992) 2292–2295.

[4] C.C. Tebbe, W. Vahjen, Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast, Appl. Environ. Microbiol. 59 (1993) 2657–2665.

[5] C.A. Kreader, Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein, Appl. Environ. Microbiol. 62 (1996) 1102–1106.

[6] C.N. Albers, A. Jensen, J. Bælum, C.S. Jacobsen, Inhibition of DNA polymerases used in Q-PCR by structurally different soil-derived humic substances, Geomicrobiol J. 30 (2013) 675–681.

[7] D. Sutlovic, M.D. Gojanovic, S. Andelinovic, D. Gugic, D. Primorac, *Taq* polymerase reverses inhibition of quantitative real time polymerase chain reaction by humic acid, Croat. Med. J. 46 (2005) 556–562.

[8] K.L. Opel, D. Chung, B.R. McCord, A study of PCR inhibition mechanisms using real time PCR, J. Forensic Sci. 55 (2010) 25–33.

[9] J.I. Kim, G. Buckau, G.H. Li, H. Duschner, N. Psarros, Characterization of humic and fulvic acids from Gorleben groundwater, Fresen. J. Anal. Chem. 338 (1990) 245–252.

[10] F.J. Stevenson, Humus Chemistry: Genesis, Composition, Reactions, John Wiley, New York, 1982.

[11] C.N. Albers, G.T. Banta, O.S. Jacobsen, P.E. Hansen, Characterization and structural modelling of humic substances in field soil displaying significant differences from previously proposed structures, Eur. J. Soil Sci. 59 (2008) 693–705.

[12] P.L. Marshall, J.L. King, N.P. Lawrence, A. Lazarev, V.S. Gross, B. Budowle, Pressure cycling technology (PCT) reduces effects of inhibitors of the PCR, Int. J. Legal Med. 127 (2013) 321–333.

[13] S. Schmedes, P. Marshall, J.L. King, B. Budowle, Effective removal of co-purified inhibitors from extracted DNA samples using synchronous coefficient of drag alteration (SCODA) technology, Int. J. Leg. Med. 127 (2013) 749–755.

[14] D.N. Miller, J.E. Bryant, E.L. Madsen, W.C. Ghiorse, Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples, Appl. Environ. Microbiol. 65 (1999) 4715–4724.

[15] P. Rådström, R. Knutsson, P. Wolffs, M. Lövenklev, C. Löfström, Pre-PCR processing: strategies to generate PCR-compatible samples, Mol. Biotechnol. 26 (2004) 133–146.

[16] P.S. Walsh, D.A. Metzger, R. Higuchi, Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material, Biotechniques 10 (1991) 506–513.

[17] D. Márta, N. Wallin-Carlquist, J. Schelin, E. Borch, P. Rådström, Extended staphylococcal enterotoxin D expression in ham products, Food Microbiol. 28 (2011) 617–620.

[18] H. Niederstätter, S. Köchl, P. Grubwieser, M. Pavlic, M. Steinlechner, W. Parson, A modular real-time PCR concept for determining the quantity and quality of human nuclear and mitochondrial DNA, Forensic Sci. Int. Genet. 1 (2007) 29–34.

[19] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Springer, New York, 2006. p. 954.

[20] Y. Wang, T. Fujii, Evaluation of methods of determining humic acids in nucleic acid samples for molecular biological analysis, Biosci. Biotechnol. Biochem. 75 (2011) 355–357.

[21] D.S. Bachoon, E. Otero, R.E. Hodson, Effects of humic substances on fluorometric DNA quantification and DNA hybridization, J. Microbiol. Methods 47 (2001) 73–82.

[22] H. Zipper, C. Buta, K. Lämmle, H. Brunner, J. Bernhagen, F. Vitzthum, Mechanisms underlying the impact of humic acids on DNA quantification by SYBR Green I and consequences for the analysis of soils and aquatic sediments, Nucleic Acids Res. 31 (2003) e39.

[23] F. Mao, W.Y. Leung, X. Xin, Characterization of EvaGreen and the implication of its physicochemical properties for qPCR applications, BMC Biotechnol. 7 (2007) 76.

[24] H. Zipper, H. Brunner, J. Bernhagen, F. Vitzthum, Investigations on DNA intercalation and surface binding by SYBR Green I, its structure determination, and methodological implications, Nucleic Acids Res. 32 (2004) e103.

[25] I. Kavanagh, D. Leake, G. Ball, Dye blends, patent US20130052650 A1 (2013).

[26] W. Abu, P. Al-Soud, Rådström, Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples, Appl. Environ. Microbiol. 64 (1998) 3748–3753.

[27] D. Sutlovic, S. Gamulin, M. Definis-Gojanovic, D. Gugic, S. Andjelinovic, Interaction of humic acids with human DNA: proposed mechanisms and kinetics, Electrophoresis 29 (2008) 1467–1472.

[28] The UniProt Consortium, Activities at the Universal Protein Resource (UniProt), Nucleic Acids Res. 42 (2014) D191–D198.

[29] U. Boehme, U. Scheler, Effective charge of bovine serum albumin determined by electrophoresis NMR, Chem. Phys. Lett. 435 (2007) 342–345.

[30] J. Hedman, A. Nordgaard, C. Dufva, B. Rasmusson, R. Ansell, P. Rådström, Synergy between DNA polymerases increases polymerase chain reaction inhibitor tolerance in forensic DNA analysis, Anal. Biochem. 405 (2010) 192–200.