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Assessing PCR Inhibition from Humic Substances

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Abstract: Inhibition remains the greatest methodological challenge in molecular analysis of buried biological remains. Inhibitory compounds associated with soil environments comprise primarily of humic acids and fulvic acids, collectively referred to as humic substances. We examined the sensitivity of 13 DNA polymerases to both humic acids (11ng-110μg) and fulvic acids (9.4ng-94μg) and the concentration at which successful amplification can be achieved. This research identified that all 13 DNA polymerases tested exhibited inhibition with varying concentrations of humic acids and that 5 out of the 13 DNA polymerase tested exhibited inhibition with varying concentrations of fulvic acid. The most tolerant DNA polymerase to inhibition due to the presence of humic and fulvic acids is *pfu* DNA polymerase followed by KlenTaq® LA DNA polymerase and RealTaq DNA polymerase that were both only inhibited by 11μg and 110μg of humic acids. In addition, we present the use of size exclusion chromatography to remove small molecular weight humic substance, dramatically increasing the success of molecular analysis on material associated with burial. This research has implications to the fields of environmental microbiology, soil science, forensic science and archaeological science.

Keywords: Inhibition, PCR, Humic Substances, Size Exclusion Chromatography, Humic acids, Fulvic acids.

INTRODUCTION

Inhibition is only one of the problems facing the use of PCR amplification of DNA. The affects of inhibitory compounds on PCR is different depending on the inhibitory compound, the amplification conditions and the DNA polymerase being used [1-5]. Some substances will inhibit the enzymatic activity of the DNA polymerase while others will cause template inhibition. Template inhibition can be caused by the inhibiting substance binding to the template preventing it from being amplified. It has been indicated that humic substances can produce both types of inhibition [2, 5]. The compounds causing this inhibition are dependent on the source of the biological material. Humic substances are primarily the cause for inhibition when extracting DNA from soil or buried biological remains [1, 4-6]. Thus the inhibition from humic substances is cause for concern in the fields of environmental microbiology, soil science, forensic science and archaeological science. Here we present the differences observed from the inhibition of humic and fulvic acids on 13 DNA polymerases.

Humic substances are amorphous, dark-coloured organic compounds which are relatively resistant to chemical and biological degradation [6, 7]. The true nature of the dark brown and black substances formed by the decomposition of organic matter in the soil, and grouped together under the collective name humus, has long been a problem to both chemist and biologist, owing to the fact that the composition of these substances varies not only with the nature of the humus-producing materials, but also with temperature, moisture and pressure, and the methods of extraction [8]. Oxidative coupling of phenols is a significant reaction in the for mation of humic structures in the soil [9]. These substances are endowed with hormone-like activity that improves plant nutrition and growth [10, 11]. Humic substances can make up 5.0-7.63mg/g of soil but this is all dependent on the soil type [1]. Humic acids (HAs) comprise one of the major fractions of humic substances [11]. They are characterised by dark-coloured, alkali-soluble, acid-insoluble, and high molecular weight organic matter [7, 11]. Fulvic acids are another major fractions of humic substances [6]. These are characterised by light brown, water soluble compounds [6]. The capacity for soil to protect organic structures relies on the mineral composition of the soil, and predominantly involves weak non-covalent interactions between mineral surfaces and organic molecules [9]. According to Piccolo [12] and Booth *et al.* [9], protection can also occur within hydrophobic domains of humic substances. The chemical properties that help to stabilise humic structures include covalent bonding within, and non-covalent interactions between, structural units [9].

The failure of PCR reactions using soil and sediment DNA samples has usually been ascribed to inhibition by a brown substance contaminating DNA preparations [6, 13-16]. The inhibition of PCR when amplifying DNA from the soil has also been ascribed to humic substances [4-6, 13-22]. Approximately 0.7-3.3μg/μL of humic acids can be recovered in DNA extracted from soils depending on the type of soil and this represents 0.21-0.99% of total humc acids in the soil [1]. This inhibitor is difficult to remove using standard DNA purification procedures, and appeared to be universally present in organic soils and sediments [6]. Tsai and Olsen [20] observed that their standard PCR reaction was inhibited by the addition of as little as 10ng of humic acid [6]. Tebbe and Vahjen [1] found a similar degree of inhibition using commercial humic acid, and also showed that the brown in-

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hibitor from soil co-migrated with humic acid on agarose gels [6]. Sutlovic *et al*. [4] measured the inhibition that humic substances produced in ancient bone samples by real time PCR. The two mechanisms that have been considered is template cross-linking and enzymatic inhibition [2, 5]. While the inhibition of the PCR by template cross-linking is easily understood, the mechanism of enzymatic inhibition by humic substances remain unknown, due to the amorphic nature of humic substances [5, 18-19, 23].

Some DNA polymerases have a greater tolerance for inhibition. Recently, genetically engineered DNA polymerases have been shown to tolerate high concentrations of inhibitors [24]. Kermekchiev *et al*. [24] has not only identified that KlenTaq® DNA polymerase (Sigma) has a high tolerance for inhibition found in DNA extractions from blood and soil but has also identified the specific regions of the enzyme that has lead to this increase tolerance. It was shown to be a N-terminal deletion of 278 amino acids along with some other polymorphisms [24]. Eilert *et al*. [3] has shown that some DNA polymerases from *T. aquaticus* and other species can have a greater tolerance for inhibitory substances including humic acids.

The most common method used to overcome PCR inhibition by humic substances is to dilute the extract. By diluting the extract the concentration of the inhibitory compounds is reduced to a level where inhibition no longer occurs. Alternatively many chemists and biologists use more robust extraction methods that are able to remove these humic substances more often however there is not one extraction method that removes them entirely. Due to the amorphous nature of the humic substances there is not one purification method that can reliably remove these substances either. Dilution of the extract will also reduce the concentration of DNA which reduces the efficiency of PCR amplification. The PCR amplification success might be sporadic or less amplified product being generated. Alternatively adding more DNA polymerase can overcome the inhibition of humic substances [4]. Other methods that have been used to remove humic substance from DNA extracts to prevent PCR inhibition, these include gel electrophoresis [25, 26], size exclusion chromatography [20, 27], gel filtration chromatography [20, 27-28], chelation by proteins (like BSA) [29], chelation by EDTA [6], chelation with polyvinylpolypyrrolidone [17]. We propose a combination of size exclusion chromatography and gel filtration chromatography as a method to overcome this type of inhibition. Size exclusion chromatography was used here to remove the humic substance to demonstrate the removal of inhibition by removal of the humic substance. Size exclusion chromatography has also been used to remove metal ion inhibition in buried biological remains [30].

MATERIALS AND METHODS

Humic Substances

Humic substances were extracted from peat samples. These were collected from a peat bog (GG3) near Uppsala, Ontario from a commercial harvesting operation from a depth of 1.5 meters. The samples were air dried at room temperature in a fume hood. The large particles were removed and the dried peat sample was passed through a 2.0mm sieve. Peat was then crushed with mortar and pestle to a fine powder. 10 grams of the fine powder were weighed and placed into a 200mL flask. The sample was equilibrated to a pH1.0 with 1M HCl and made up to a final volume of 100mL at room temperature (10mL liquid/1g dry sample). The suspension was shaken for 1 hour and then separated by decantation after low speed centrifugation. The supernatant is retained for further processing (fulvic acid fraction 1).

The remaining pellet was neutralised with 1M NaOH to pH7.0 then 0.1M NaOH was added under an atmosphere of N_2 to give a final extract to soil ratio of 10:1 and resuspended under N_2 with intermittent shaking for 12 hours. The alkaline suspension was allowed to settle overnight and the supernatant collected by means of decantation after centrifugation. The supernatant was acidified with 6M HCl with constant stirring to pH1.0 and then allowed the settle for 16 hours. The supernatant was removed after centrifugation (fulvic acid fraction 2). The pellet (humic acids) was dried in an oven at 25°C ready for use. The two fulvic acids fractions were combined and dried in an oven set to 25°C.

A concentration gradient was prepared from the extracted humic and fulvic acids. The humic acid solution consists of 0.11g of humic acids mixed with 1.0mL of ddH_2O. The fulvic acid solution consists of 0.94 grams of fulvic acids dissolved in 10mL of ddH_2O. A series dilution was performed to generate five humic acids solutions these were $1.1x10^{-1}$g/mL, $1.1x10^{-2}$g/mL, $1.1x10^{-3}$g/mL, $1.1x10^{-4}$g/mL and $1.1x10^{-5}$g/mL. While the five fulvic acids solutions were $9.4x10^{-2}$g/mL, $9.4x10^{-3}$g/mL, $9.4x10^{-4}$g/mL, $9.4x10^{-5}$g/mL and $9.4x10^{-6}$g/mL also generated by serial dilution. The concentration of humic substances in the soil varies according to the type of soil. Humic substances have been shown to be in the milligram range [31, 32]. The final amounts tested here range from 110µg to 11ng of humic acids and 94µg to 9.4ng of fulvic acids encompassing the range of humic substances that could be extracted from the soil and comparable to previous work by Tebbe and Vahjen [1].

DNA Amplification

The inhibition study examined the effect that humic acids and fulvic acids has on PCR amplification using 13 DNA polymerases. All reagents and reactions were prepared using sterile conditions and all reactions were optimised prior to use based on the supplier's instructions. Each reaction was set up with a negative and positive control with the five different concentrations of humic substance added to the PCR in triplicate. All the amplifications were performed using human mtDNA as template and forward and reverse primers at the 5´ nucleotide positions 14724 and 15149 respectively. This amplification generates a 425bp product. An Eppendorf Mastercycler gradient thermocycler was used for all experiments. The DNA polymerases and their reaction mixes can be found in Table **1**. For the inhibition studies 1µL of the humic substances solutions were added to the PCR reactions. All PCR amplifications were performed at 10µL total volume. All of the PCR amplification conditions for the DNA polymerases (except Phire™ Hot Start DNA polymerase, $Vent_R$™ DNA polymerase and Deep $Vent_R$™ DNA polymerase) have an initial denaturation 94°C for 2 minutes followed by 24 cycles of denaturation 94°C for 30 seconds, annealing 50°C for 1 minute and extension for 1 minute with a final hold at 4°C. The conditions for Phire™ Hot Start DNA

Table 1. The DNA Polymerases and Their Reaction Mixtures

DNA Polymerase	Supplier/Manufacturer	Reaction
Pfu DNA polymerase	Fermentas	1X Buffer, 0.2mM dNTP, 0.2μM of each primer, 0.25U DNA polymerase
AccuTaq™ LA DNA polymerase	Sigma	1X Buffer, 0.5mM dNTP, 0.6 μM of each primer, 0.5U DNA polymerase
LongAmp™ *Taq* DNA polymerase	New England Biolabs	1X Buffer, 0.3mM dNTPs, 0.4 μM of each primer, 1U DNA polymerase
Crimson *Taq*™ DNA polymerase	New England Biolabs	1X Buffer, 0.2mM dNTPs, 0.2 μM of each primer, 0.25U DNA polymerase
Phusion® High-Fidelity DNA polymerase	Finnzymes	1X Buffer, 0.2mM dNTPs, 0.2 μM of each primer, 0.2U DNA polymerase
GoTaq® DNA polymerase	Promega	1X GoTaq® Green Master Mix, 0.2μM of each primer
KlenTaq® LA DNA polymerase	Sigma	1X Buffer, 0.2mM dNTPs, 0.2μM of each primer, 1U DNA polymerase
RealTaq DNA polymerase	RBC	1X Buffer, 0.1mM dNTPs, 0.2μM of each primer, 0.25U DNA polymerase
Extensor High Fidelity PCR Mix	ABgene	1X Buffer, 0.2mM dNTPs, 0.2μM of each primer, 0.5U DNA polymerase
Thermoprime Plus DNA polymerase	ABgene	1X Buffer, 0.2mM dNTPs, 0.2μM of each primer, 3.0mM $MgCl_2$, 0.25U DNA polymerase
Phire™ Hot Start DNA polymerase	Finnzymes	1X Buffer, 0.2mM dNTPs, 0.2μM of each primer, 0.2μL of DNA polymerase
$Vent_R$® DNA polymerase	New England Biolabs	1X Buffer, 0.2mM dNTPs, 0.1μM of each primer, 0.1U DNA polymerase
Deep $Vent_R$™ DNA polymerase	New England Biolabs	1X Buffer, 0.1mM dNTPs, 0.4μM of each primer, 4.0mM $MgSO_4$, 0.2U DNA polymerase

polymerase begin with an initial cycle at 98°C for 30 seconds followed by 25 cycles of denaturing at 98°C for 5 seconds, annealing at 50°C and extending at 72°C, a final extension runs at 72°C for 1 minute and then holds at 4°C. The conditions for $Vent_R$™ DNA polymerase begin with an initial cycle at 94°C for 2 minutes followed by 24 cycles that denature at 94°C for 30 seconds, anneal at 60°C for 30 seconds and extend at 72°C for 1 minute with a final extension at 72°C for 1 minute and hold at 4°C. The cycling conditions for Deep $Vent_R$™ DNA polymerase begins with an initial denaturation at 95°C for 3 minutes followed by 24 cycles that denature at 95°C for 30 seconds, anneal at 60°C for 30 seconds and extend at 72°C for 1 minute with a final extension at 72°C for 5 minute and hold at 4°C.

The amplified products were analysed by 1% agarose gel electrophoresis using a 1X TBE run buffer. The agarose gels were stained with ethidium bromide and 3μL of a 100bp DNA ladder (Fermentas) was used for size comparison. Each well contained of 3μL of loading dye combined with 5μL of amplified product. The gels are run at 110 volts for approximately 1 hour.

Size Exclusion Chromatography

BioSpin P30 (BIORAD) size exclusion chromatography columns were used as an additional purification. BioSpin P30 (BIORAD) size exclusion chromatography columns are designed to remove small molecules. Occasionally two BioSpin P30 chromatography column purifications may be necessary to remove the visual discoloration (e.g. brown pigmentation) of the samples because the concentration of the humic substances exceeds the binding capacity of the column. The size exclusion chromatography purification protocol followed the recommended procedure by the manufacturer to ensure comparability of results. BioSpin P30 columns were autoclaved prior to use.

Assessment of Inhibition

The inhibition by humic substances was determined by using humic acid and fulvic acid extracts. A concentration series of these substances were added to an optimised PCR to determine at what concentration PCR failure may have occurred. This PCR failure was correlated to the degree of inhibition of that substance to a variety of DNA polymerases. The failure of the PCR was determined through gel electrophoresis.

RESULTS

The inhibition study using humic acids (Table **2**) resulted in PCR inhibition, for all DNA polymerases studied, at the highest concentrations of 110μg of humic acids.The fulvic acid inhibition study (Table **3**) resulted in variable PCR inhibition and amplification across the 13 different DNA polymerases. The gel electrophoresis most reliably detected full inhibition and no inhibition (Figs. **1** and **2**). While most of the PCRs showed inhibition at the higher concentrations of humic acids (example Fig. **1**) most showed no inhibition of fulvic acids at any concentration (example Fig **2**). The use of size exclusion chromatography removed the majority of humic substances to allow successful amplification. However the amount of humic substances removed was dependant on the binding capacity of the column in which using two columns removed more of the inhibiting substances. The use of one size exclusion chromatography column efficiently removed enough humic substances to observe a 100 to 1000-fold difference in humic substance concentrations. The PCRs containing Deep $Vent_R$™ DNA polymerase produced positive amplifications with between 11ng and 110ng of humic acids and between 9.4ng and 940ng of fulvic acids using the size exclusion chromatography, a result that was previously inhibited at all concentrations tested without the size exclusion chromatography (Fig. **3**).

Table 2. The Results of the Humic Acid Inhibition Study on the DNA Polymerases

DNA Polymerase	Species	11ng	110ng	1.1µg	11µg	110µg
Pfu DNA polymerase	*Pfu*	+/+/+	+/+/+	+/+/+	+/+/+	-/-/-
AccuTaq™ LA DNA polymerase[a]	*Taq*	+/+/+	+/-/+	-/-/-	-/-/-	-/-/-
LongAmp™ *Taq* DNA polymerase[b]	*Taq, Pyrococcus* sp. GB-D	+/+/+	+/+/+	-/-/-	-/-/-	-/-/-
Crimson *Taq*™ DNA polymerase[b]	*Taq, Pyrococcus* sp. *GB-D*	+/+/+	+/+/+	+/+/+	+/+/+	-/-/-
Phusion® High-Fidelity DNA polymerase	*Engineered Taq*	+/+/+	+/+/+	-/-/-	-/-/-	-/-/-
GoTaq® DNA polymerase	*Taq*	+/+/+	+/+/-	-/-/-	-/-/-	-/-/-
KlenTaq® LA DNA polymerase	*Taq*	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-
RealTaq DNA polymerase	*Thermus* sp.	+/+/+	+/+/+	+/+/+	-/-/-	-/-/-
Extensor Hi-Fidelity PCR mix[c]	*Taq*	P/-/P	-/-/-	-/-/-	-/-/-	-/-/-
Thermoprime Plus DNA polymerase	*Taq*	+/+/+	+/+/+	-/-/-	-/-/-	-/-/-
Phire™ Hot Start DNA polymerase	*Taq*	-/-/+	+/+/+	-/-/-	-/-/-	-/-/-
$Vent_R$® DNA polymerase	*Tli*	+/+/+	+/+/-	-/-/-	-/-/-	-/-/-
Deep $Vent_R$™ DNA polymerase	*Pyrococcus* sp. *GB-D*	-/-/-	-/-/-	P/-/-	-/-/-	-/-/-

[a] Mixture of *Taq* DNA polymerase and a proprietary proof-reading enzyme.
[b] Mixture of *Taq* DNA polymerase and Deep $Vent_R$® DNA Polymerases.
[c] Mixture of Thermoprime Plus DNA Polymerase and a proprietary proof-reading enzyme.
+ Indicates the band was present on the gel with a similar intensity to the amplification control.
- Indicates inhibition, no band was present on the gel.
P Partial inhibition indicates that there was a feint band on the gel, weaker than the amplification control.
Experiments performed in triplicate.

Table 3. The Results of the Fulvic Acid Inhibition Study on the DNA Polymerases

DNA Polymerase	Species	9.4ng	94ng	940µg	9.4µg	94µg
Pfu DNA polymerase	*Pfu*	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
AccuTaq™ LA DNA polymerase[a]	*Taq*	+/+/+	+/+/+	P/+/P	+/+/+	+/+/+
LongAmp™ *Taq* DNA polymerase[b]	*Taq, Pyrococcus* sp. GB-D	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Crimson *Taq*™ DNA polymerase[b]	*Taq, Pyrococcus* sp. *GB-D*	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Phusion® High-Fidelity DNA polymerase	*Engineered Taq*	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
GoTaq® DNA polymerase	*Taq*	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
KlenTaq® LA DNA polymerase	*Taq*	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
RealTaq DNA polymerase	*Thermus* sp.	+/+/+	+/+/+	+/P/P	P/P/P	P/-/P
Extensor Hi-Fidelity PCR mix[c]	*Taq*	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
Thermoprime Plus DNA polymerase	*Taq*	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
Phire™ Hot Start DNA polymerase	*Taq*	+/+/+	P/P/+	+/+/+	+/+/+	+/+/+
$Vent_R$® DNA polymerase	*Tli*	+/+/+	+/+/+	+/+/+	+/+/+	+/+/+
Deep $Vent_R$™ DNA polymerase	*Pyrococcus* sp. *GB-D*	+/+/-	-/-/-	+/-/-	-/+/+	-/-/-

[a] Mixture of *Taq* DNA polymerase and a proprietary proof-reading enzyme.
[b] Mixture of *Taq* DNA polymerase and Deep $Vent_R$® DNA Polymerases.
[c] Mixture of Thermoprime Plus DNA Polymerase and a proprietary proof-reading enzyme.
+ Indicates the band was present on the gel with a similar intensity to the amplification control.
- Indicates inhibition, no band was present on the gel.
P Partial inhibition indicates that there was a feint band on the gel, weaker than the amplification control.
Experiments performed in triplicate.

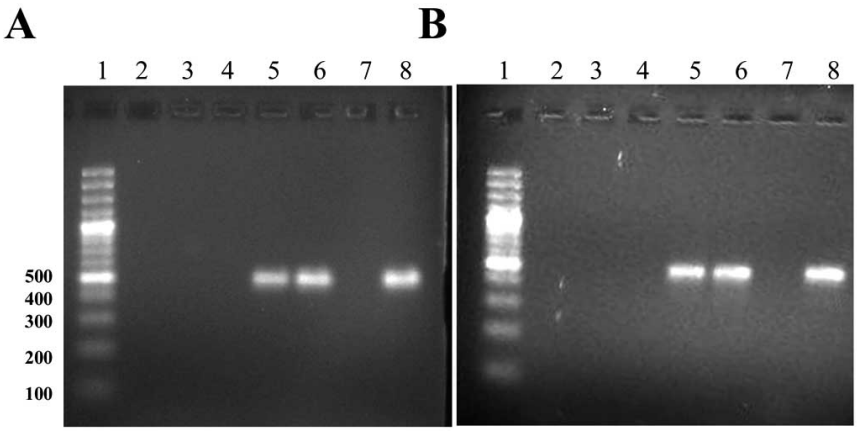

Fig. (1). Agarose gel electrophoresis examples of humic acid inhibition. Gel A is an example using LongAmp™ *Taq* DNA polymerase and Gel B is an example using $Vent_R$® DNA polymerase. Both gels are set up with the same conditions in each lane; lane 1, 100bp DNA ladder; lane 2, Amplification spiked with 11ng of humic acids; lane 3, Amplification spiked with 110ng of humic acids; lane 4, Amplification spiked with 1.1µg of humic acids; lane 5, Amplification spiked with 11µg of humic acids; lane 6, Amplification spiked with 110µg of humic acids; lane 7, negative PCR control; and lane 8, positive control.

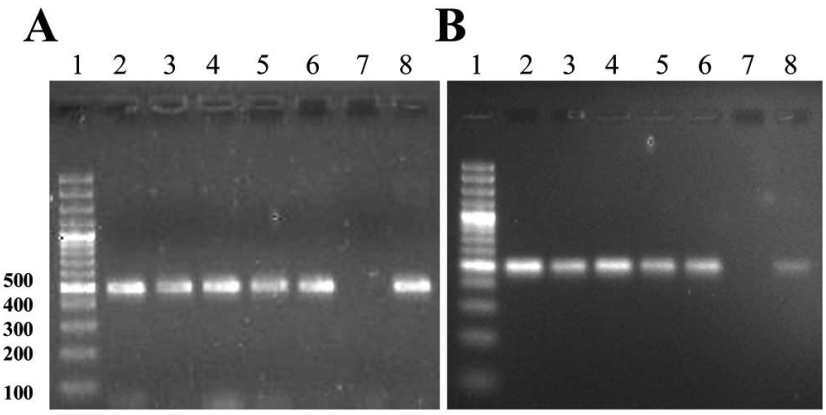

Fig. (2). Agarose gel electrophoresis examples of fulvic acid inhibition. Gel A is an example using Phusion® High-Fidelity DNA polymerase and Gel B is an example using *Pfu* DNA polymerase. Both gels are set up with the same conditions in each lane; lane 1, 100bp DNA ladder; lane 2, Amplification spiked with 9.4ng of fulvic acids; lane 3, Amplification spiked with 94ng of fulvic acids; lane 4, Amplification spiked with 940ng of fulvic acids; lane 5, Amplification spiked with 9.4µg of fulvic acids; lane 6, Amplification spiked with 94µg of fulvic acids; lane 7, negative PCR control; and lane 8, positive control.

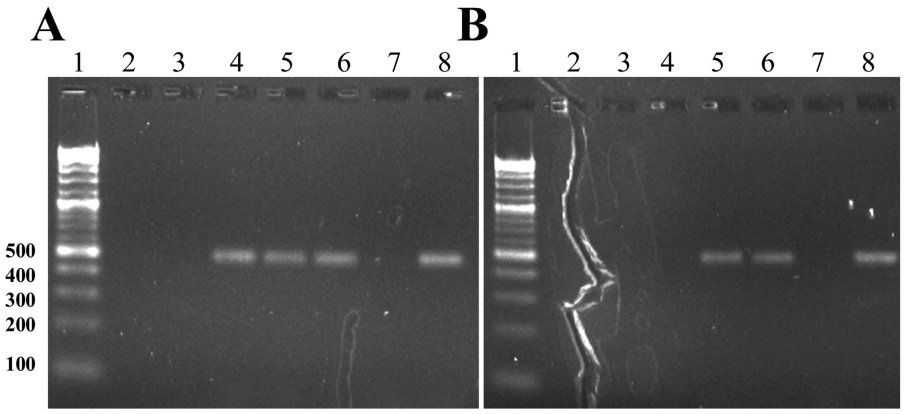

Fig. (3). Gel electrophoresis examples of the efficiency of the size exclusion chromatography in removing some of the inhibition by humic acids (Gel A) and fulvic acids (Gel B) from amplification with Deep $Vent_R$™ DNA polymerase. Gel A: lane 1, 100bp DNA ladder; lane 2, Amplification spiked with 11ng of humic acids; lane 3, Amplification spiked with 110ng of humic acids; lane 4, Amplification spiked with 1.1µg of humic acids; lane 5, Amplification spiked with 11µg of humic acids; lane 6, Amplification spiked with 110µg of humic acids; lane 7, negative PCR control; and lane 8, positive control. Gel B: lane 1, 100bp DNA ladder; lane 2, Amplification spiked with 9.4ng of fulvic acids; lane 3, Amplification spiked with 94ng of fulvic acids; lane 4, Amplification spiked with 940ng of fulvic acids; lane 5, Amplification spiked with 9.4µg of fulvic acids; lane 6, Amplification spiked with 94µg of fulvic acids; lane 7, negative PCR control; and lane 8, positive control.

DISCUSSION

Humic substances are one of the biggest problems faced when extracting DNA from biological material recovered from the soil. The results of the inhibitory affects of humic acids in this research indicate that all of the PCRs and respective DNA polymerases have a tolerance threshold at 110µg of humic acids. The failure of the PCRs could be due to enzymatic inhibition of the respective DNA polymerases, it could represent the concentration at which the binding of humic acids to the DNA template becomes ubiquitous preventing any template from being amplified or a combination of both. However some of the DNA polymerases (Extensor Hi-Fidelity DNA polymerase, Phire™ Hot Start DNA polymerase and Deep Vent$_R$™ DNA polymerases) are intolerant to humic acids at all concentrations tested suggesting both mechanisms may be present in the PCR. There are currently such a diverse range of DNA polymerases available that it is difficult to identify which DNA polymerases might be adversely affected by the presence of humic substances. Many researchers [1, 4-5, 15, 18, 20, 23-24, 27-29, 33] have shown that different *Taq* DNA polymerases are sensitive to humic substances while Kermekchiev *et al.* [24] showed some *Taq* DNA polymerases and some engineered *Taq* DNA polymerases have been highly resistant to the affects of humic acids. The research presented here reflects the same trend with variation between the different *Taq* DNA polymerases. Kermekchiev *et al.* [24] has also shown that other non-*Taq* species of DNA polymerases are less affected by humic acids as has this research with *pfu* DNA polymerase but some pyrococcus species DNA polymerases do not share the same degree of tolerance like the Deep Vent$_R$™ DNA polymerase. There has been very little research that has isolated fulvic acids to determine the contribution to humic substance inhibition they provide. The outcome indicates that DNA polymerases are more tolerant of fulvic acids than humic acids. However there was the same variability between the DNA polymerases affected by fulvic acids. Three of the DNA polymerases (LongAmp™ *Taq* DNA polymerase, Crimson *Taq*™ DNA polymerase and Extensor Hi-Fidelity DNA polymerase) were inhibited by fulvic acid at every concentration while all of the PCRs using the other enzymes showed no inhibition at any of the concentrations tested.

Overall the DNA polymerase least affected by inhibition from humic and fulvic acids is *pfu* DNA polymerase, suggesting there might be a greater tolerance in the DNA polymerases of this species, followed by KlenTaq® LA DNA polymerase and RealTaq DNA polymerase. Some of the polymerases were inhibited by one humic substance and not the other (only humic acids - Phire™ Hot Start DNA polymerase). The blended DNA polymerases were varied and most likely dependant on each of the enzymes used in the mixture. One of the least successful DNA polymerases was the Extensor Hi-Fidelity DNA polymerase this is a blended DNA polymerase containing Thermoprime Plus DNA Polymerase and a proprietary proof- Reading enzyme. Thermoprime Plus DNA Polymerase was tested on its own and performed generally the same as the *Taq* DNA polymerases so the failure observed in the Extensor Hi-Fidelity DNA polymerase mixture could be due to the second enzyme in the mixture or the amount of template inhibition that might have been present. The two DNA polymerases that were the most sensitive to the inhibitory compounds were Extensor Hi-Fidelity DNA polymerase and Deep Vent$_R$™ DNA polymerase which were inhibited at every concentration with both humic and fulvic acids (excluding the sporadic success of Deep Vent$_R$™ DNA polymerase). However all of the DNA polymerases tested produced better results after size exclusion chromatography.

The removal of humic substances and subsequent successful PCR amplification was achieved with size exclusion chromatography. This research supports previous research by Miller [27] and Tsai and Olsen [20] on the removal of humic substances. Size exclusion chromatography column will trap small molecules within the gel beads allowing just the larger molecules to pass through which would include the DNA. Not all humic substances will consist of small molecules as humic acids are amorphous. So size exclusion chromatography simply removes a sufficient portion of the humic substances that would allow it to be amplified. Compared to the tradition method of simply diluting the extract to dilute out the inhibitor the removal of some of the inhibiting compounds would have a similar effect while retaining the same or very similar concentrations of DNA. Other methods like using a chelator (EDTA, BSA) can be an effective way of resolving the problem but the efficiency might vary between different DNA polymerases. Many manufacturers include BSA in their buffers for PCR which can aid in the amelioration of humic substance inhibition. Increasing the quantity of DNA polymerase can be more expensive depending on the DNA polymerase and can produce sporadic results. The purification with size exclusion chromatography allowed the amplification of a 100 to 1000-fold increase in concentration of the humic substances by the removal of these substances to within ranges of DNA polymerase tolerance. This could be explained by three possibilities, 1) the larger humic substances that will not be removed by size exclusion are still present to inhibit the amplification, 2) the binding capacity of the size exclusion chromatography columns has been reached and a second purification with these columns will remove more humic substances to allow the amplification to be successful or 3) the inhibition is template inhibition, where the humic substance has bound to the template and cannot be removed by size exclusion chromatography.

Due to the amorphous nature and the size range of humic substances we propose that gel filtration chromatography and size exclusion chromatography can be used in conjunction with each other for a more reliable removal of humic substances. The gel filtration chromatography can be used to remove the larger humic substances as indicated by various researchers [20, 23, 27-28] as it filters the molecules by size allowing the smaller molecules to pass through the column faster. The gel filtration column allows the ability to recover any size molecules from the smaller molecules upwards by increasing either the speed or the time of centrifugation. In this application the larger molecules would be retained within the gel matrix of the column thus removing large humic substances. However the use of gel filtration may remove some of the large fragments of DNA but through controlled centrifugation this problem can be evaluated. While size exclusion chromatography can be used to remove the smaller humic substances by trapping small molecules

within the gel beads allowing just the larger molecules to pass through including the DNA.

The limitations of this research are the use of gel electrophoresis as the detection system and the binding capacity of the size exclusion chromatography column. Gel electrophoresis can be reliably used to indicate full inhibition and very little or no inhibition but does not assess the degree of partial inhibition. The use of real time PCR would be more reliable for the identification the varying degree of partial inhibition [33]. While it is possible to remove both humic acids and fulvic acids using size exclusion chromatography columns the removal is constrained by the binding capacity of the column which will limit how much of the smaller humic substances are able to be removed, this can be overcome by running the sample through two size exclusion chromatography columns.

CONCLUSIONS

The inhibitory nature of humic substances on *in vitro* molecular analysis, specifically PCR amplification has been explored with 13 DNA polymerases. In addition this research uses both constituents of humic substances, humic acids and fulvic acids, extracted directly from soil material rather than synthetic sources providing a more reliable assessment. The results indicate that humic acids are far more of a concern than fulvic acids in regards to inhibition and that the effects on the PCRs of each of the DNA polymerases vary greatly. The DNA polymerase most tolerant of these inhibitory substances was the *Pfu* DNA polymerase. KlenTaq® LA DNA polymerase and RealTaq DNA polymerase were the next most tolerant enzymes against both fulvic and humic acids. Even though the PCR containing the Deep $Vent_R$™ DNA polymerase proved to be the most sensitive polymerase to these two substances the Deep $Vent_R$™ DNA polymerase still has the highest temperature tolerance of all these enzymes. It must be noted that each of these enzyme are exceptional enzyme that have been designed and prepared for specific molecular uses and their sensitivity to humic and fulvic acids does not imply their sensitivity to other inhibitors or that they are less reliable enzymes. However for the extraction of DNA from soil bacteria or from buried biological remains this research may provide information for the researcher to determine which DNA polymerase might be the most appropriate. This research emphasises the importance of testing the preferred DNA polymerase with these humic substances to determine the inhibitory affect they may have in the PCR amplification. In addition this research provides the use of size exclusion chromatography as a method to purify extractions from buried biological material to specifically remove humic and fulvic acids.

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