Agronomy Research **16**(S2), 1351–1359, 2018
https://doi.org/10.15159/AR.18.118

Study of potential PCR inhibitors in drinking water for *Escherichia coli* identification

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Abstract. In the last few decades, the polymerase chain reaction (PCR) has become one of the most powerful molecular biological tools. However, the PCR is an enzymatic reaction and therefore sensitive to inhibitors which may occur in drinking water samples. In this work, the possible inhibition effect of chlorine, humic acids, and iron for real-time PCR (qPCR) efficiency was studied and the environmental sample from drinking water treatment system before iron removal was selected and analysed. The results demonstrated that the highest concentrations of humic acids (5 mg L^{-1} and 1 mg L^{-1}) and iron (4 mg L^{-1}) inhibited the PCR reaction while no effect of chlorine was observed. The analysis of the environmental sample with spiked *Escherichia coli* cells demonstrated reduction efficiency of the average threshold cycle (C_t) values compared with control dilution series determining the possible inhibition for qPCR assay.

Key words: Polymerase chain reaction, inhibitors, chlorine, humic acids, iron, *Escherichia coli.*

INTRODUCTION

While most microorganisms play an important role in nature, certain potentially harmful bacteria can contaminate food and water, and cause infectious diseases in both humans and animals (Leonard et al., 2003). Depending on the type of microorganism, conventional identification can take anything from a day to several weeks (Rossen et al., 1992). Therefore, the limitations of culture-based assays (e.g., inability to detect unculturable cells, time consumption) in analysis of microorganisms from oligotrophic environments, such as drinking water, have facilitated the introduction of molecular methods in routine tests for more relevant, rapid and 'real-time' identification (Kim et al., 2013; Fatemeh et al., 2014).

Quantitative real-time PCR (qPCR) has been recommended as a powerful alternative diagnostic tool due to its high sensitivity and specificity (Schriewer et al., 2011). Nevertheless, environmental samples may contain a high concentration of organic and inorganic substances like phenolic compounds, heavy metals and humic acids which may inhibit enzymatic reactions leading to the production of biased results and impact the sensitivity of both conventional and quantitative PCR (Green & Field, 2012; Schrader, 2012; Sidstedt et al., 2015). For instance, PCR inhibition for *E. coli* identification from water samples has been linked with the presence of inhibiotory substances (Walker et al., 2017).

Typical concentrations of dissolved iron in groundwater that is the primary source of drinking water in Nordic countries range from 0.5 to 10 mg L^{-1} (Klove et al., 2016) while other sources, mainly surface water, can be rich in humic substances (Juhna & Klavins, 2001). A major component of naturally occurring organic carbon in water is humic acid, as it can represent up to 90% of dissolved organic carbon in water (Sounthararajah et al., 2015). For instance, Tihomirova et al. (2010) have demonstrated that often the amount of organics directly after water treatment can exceed 5 mg L^{-1}. Finally, the disinfection of drinking water often involves the use of reactive chemical agents such as chlorine. For effective disinfection, the average concentration of free chlorine in the final treatment step might vary from 0.2 to 1 mg L^{-1} (World Health Organization, 2011). Thus, the application of molecular tests, e.g., PCR, in environmental sample, e.g., source waters, and drinking water analyses must be performed with caution to exclude the possibility of inhibition.

Traditionally, to determine the inhibitory effect of the PCR reaction, it has been suggested to carry out the control reactions (Schrader et al., 2012). For instance, serial dilution with variable concentration of possible inhibitors in analysed control samples might provide an excellent mean to determine inhibiting effects when a gene target is present in high copy numbers (Kontanis & Reed, 2006; Schriewer et al., 2011). Therefore, inhibition of qPCR can be measured as the increase in threshold cycle (C_t) relative to an uninhibited control (Huggett et al., 2008). Moreover, the analysis of the PCR product is possible through a measurement of the melt characteristics of the amplicons where the change in the melt curve demonstrates the modification of the PCR product (Opel et al., 2009).

The aim of this study was to estimate if qPCR could be applied for the analysis of drinking water, which is rich in organic and inorganic compounds. The possible inhibition of chlorine, iron and humic acids was examined and evaluated in artificial and water samples. *Escherichia coli* was selected as a test organism because it is the most widely used faecal indicatororganism in microbiology analyses. The research included tests with real-time PCR assay and the results of the threshold cycle (C_t) values were compared in-between the standard curves constructed for *E. coli.*

MATERIALS AND METHODS

Bacterial strain and DNA extraction

Escherichia coli strain (ATCC®25922™) was grown aerobically at 37 °C for 24 h in Tryptone soya broth (Oxoid, UK). The commercial kit GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania) was used according to manufacturer's instructions for DNA extraction and preparation of control dilution series and water sample analyses.

Sample preparation

Inhibitor stock solutions were prepared and serially diluted to obtain final concentrations of 4, 0.8, 0.08 mg L^{-1} of chlorine (from NaClO, Sigma-Aldrich, Germany); 5, 1, 0.3 mg L^{-1} of humic acid (Sigma-Aldrich, Germany), and 4, 0.2, 0.1 mg L^{-1} of iron ($FeCO_3$, Sigma-Aldrich, Germany). All subsequent dilutions were prepared in water.

The water sample of 1 L was collected from a drinking water treatment plant (Dobele, Latvia) during iron removal process (iron concentration > 0.5 mg L^{-1}) in sterile glass bottle, stored at +4 °C and transported to the testing laboratory for further use. For the investigation of possible PCR inhibition from the water sample, a positive control (dilution series of *E. coli*), and negative control (water sample without *E. coli*) was prepared and compared in-between the water sample with spiked *E. coli* with the same DNA concentration as control dilution series. The water sample was analysed with real-time PCR (see below) and tested in triplicates.

Real-time PCR analysis of inhibitors

The amplification was performed in 7300 Real Time PCR System (Applied Biosystems, USA). PCR reaction mixture of 25 µl contained 5 µl template DNA, 12.5 µl SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific, Lithuania), 0.5 µl of primer pair EC_5 (5'-AAAGCCGTGGCACAGGCAAGCGT-3') and $EC8c_2$ (5'-TCAATTTGTTATCGCTATCCAGTTGG-3') (Spierings, 1993) and the required amount of PCR-nuclease free water (Thermo Scientific, Lithuania). To enhance the effect of the chlorine, humic acids and iron, the required concentration of inhibitor was added at the end to reach a final reaction volume of 25 µl. Control (without inhibitors) dilution series was performed using the same protocol, with an equivalent volume of PCR water used in place of the inhibitor.

In order to evaluate and compare the success and effectiveness of PCR inhibition, MS Excel 2013 was used for C_t average value and *t*-test statistical calculations. Each sample was tested in triplicates.

RESULTS AND DISCUSSION

Real-time PCR is becoming the method of choice for the detection of pathogenic microorganisms and other target organisms in the environmental samples (Schriewer et al., 2011). Although qPCR can provide more specific and accurate quantification than other molecular techniques, it does have limitations that must be considered when applying it in practice (Kim et al., 2013). Previous research has already demonstrated that water sample analyses with real-time PCR might be inhibited by organic compounds like polyphenol, fulmic and humic acids, metal ions and chlorine (Schrader et al., 2012). However, besides the substance class, there has not been much research on the relationship between the inhibitor concentration and effect caused to the qPCR efficiency. Therefore, within this study, the inhibition of qPCR was investigated and compared with uninhibited control as the increase in the threshold cycle (C_t), which represents the number of cycles required for the fluorescent signal to cross the threshold of background level.

Chlorine effect on qPCR

The treatment of drinking water typically involves a final disinfection process to prevent the discharge of pathogens. For instance, in Latvia, the drinking water treatment process is based on chlorination and the average concentration of free chlorine in the final treatment step might vary from 0.5 to 3 mg L^{-1} (Nescerecka et al., 2014). Based on these observations, three different concentrations (4, 0.8 and 0.08 mg L^{-1}) were investigated in this study.

The results showed that there was no significant difference ($P > 0.05$) in the average crossing threshold (C_t) values of the inhibitors when compared to the control dilution series (Table 1).

Table 1. The comparison of different chlorine concentrations based on the average crossing threshold (C_t) values

Dilution series	C_t			
	Control	4 mg L^{-1}	0.8 mg L^{-1}	0.08 mg L^{-1}
10^1	12.61 (± 0.13)	12.39 (± 0.03)	12.50 (± 0.22)	12.32 (± 0.20)
10^2	16.11 (± 0.07)	16.05 (± 0.12)	15.83 (± 0.10)	15.89 (± 0.06)
10^3	19.57 (± 0.06)	19.53 (± 0.22)	19.46 (± 0.11)	19.59 (± 0.16)
10^4	23.19 (± 0.06)	23.82 (± 1.35)	23.03 (± 0.05)	23.05 (± 0.04)
10^5	nd	26.69 (± 0.14)	nd	nd

nd – C_t was not detected; (±) standard deviation of the average values from 3 replicates.

Previous research (Aken & Lin, 2011) showed that high concentrations of the disinfecting agent, e.g., chlorine, UV, and silver, significantly inhibited the amplification of DNA for *E. coli* bacteria. Furthermore, Delgado-Viscogliosi et al. (2009) demonstrated that the doses of tested chlorine concentration above 0.5 mg L^{-1} are able to bind to DNA molecules, resulting in the inhibition of qPCR. However, here the chlorine did not demonstrate any effect on PCR efficiency. This could be linked to the fact that the incubation time was too short to destruct the DNA molecule and amplification process. Nevertheless, the sensitivity of the qPCR method can depend on the volume of analysed water affecting the final concentration of free chlorine. For instance, the cell number of the target microorganism in drinking water may be small and DNA could be detected by PCR amplification only after concentration of large volumes of drinking water. Thus, the internal controls could be used in order to avoid false-negative results due to chlorine inhibitory effect.

Humic acid effect on real-time PCR

Humic substances are the most commonly reported group of PCR inhibitors in the environmental samples (Filion, 2012). Tihomirova et al. (2010) have indicated that even after drinking water treatment the concentration of humic substances can exceed 5 mg L^{-1}, therefore, great suspicion on possible inhibition of PCR reaction arise. According to this, three different concentrations, 5, 1 and 0.3 mg L^{-1}, of humic acid were evaluated.

The inhibition by humic acids was observed at two concentrations – 5 mg L^{-1} and 1 mg L^{-1} ($P < 0.05$). The efficiency of the average crossing threshold (C_t) values for each dilution series decreased when compared to control, 65% and 16%, respectively. At the same time, no significant change was observed at 0.3 mg L^{-1} humic acid ($P > 0.05$) (Table 2).

Table 2. The comparison of different humic acid concentrations based on the average crossing threshold (C_t) values

Dilution series	C_t			
	Control	5 mg L^{-1}	1 mg L^{-1}	0.3 mg L^{-1}
10^1	12.22 (± 0.03)	19.66 (± 0.07)	14.29 (± 0.43)	12.36 (± 0.14)
10^2	15.68 (± 0.02)	23.16 (± 0.43)	17.45 (± 0.05)	15.90 (± 0.11)
10^3	19.68 (± 0.14)	27.60 (± 0.38)	21.41 (± 0.05)	19.90 (± 0.19)
10^4	23.44 (± 0.02)	31.68 (± 0.28)	25.32 (± 0.10)	23.60 (± 0.02)
10^5	27.24 (± 0.09)	36.33 (± 0.57)	29.41 (± 0.24)	27.53 (± 0.06)

nd – C_t was not detected; (±) standard deviation of the average values from 3 replicates.

Additionally, the same results of PCR inhibition were presented at amplification plot curves with 5 and 1 mg L^{-1} concentrations of humic acids. All inhibitor dilution series did not coincide with non-ihibitory amplification plots (Fig. 1) and demonstrated the influence of humic acids on the PCR efficacy.

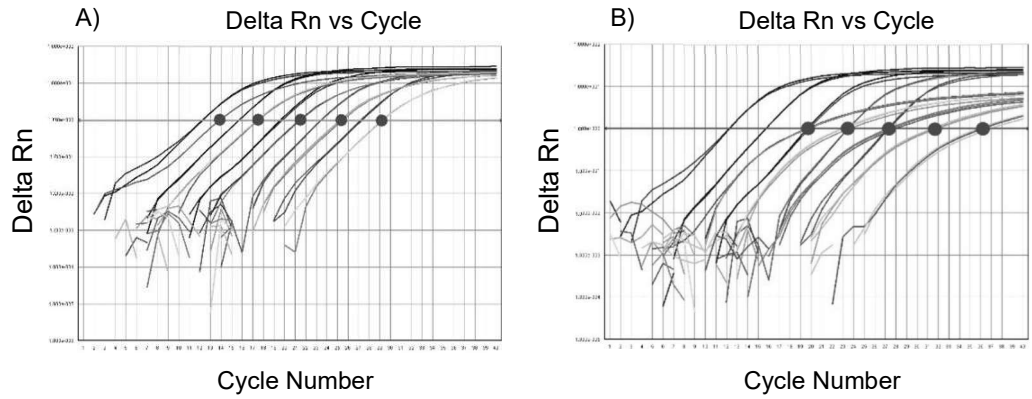

Figure 1. Investigation of humic acid inhibitory properties in real-time PCR amplification with *E. coli* standard curves. **A)** Amplification plot curves from 1 mg L^{-1} chlorine dilution series (red marks at threshold (C_t) point) when compared with control dilution series; **B)** Amplification plot curves from 5 mg L^{-1} chlorine dilution series (marked as **x** at threshold (C_t) point) when compared with control dilution series.

Sidstedt et al. (2015) demonstrated that humic acids from environmental samples are very potent inhibitors that can quench the fluorescence signal of double-stranded DNA binding dyes, including SYBR Green. Furthermore, humic acids have been found to directly disturb the DNA polymerase and form colloids in water and complexes with iron ions, meaning that they could affect the ion content in PCR, for example, by chelating magnesium ions (Sidstedt et al., 2015). As humic substances are amorphous, dark-colored organic compounds, which are relatively resistant to chemical and biological degradation, it is difficult to remove using standard DNA purification procedures (Watson & Blackwell, 2000). Therefore, 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 (Matheson et al., 2010). The results in this paper also indicated that by decreasing the concentration of humic acids from 5 mg L^{-1} to

0.3 mg L^{-1}, increased the efficiency of qPCR. However, dilution of the sample also reduced the concentration of DNA, which can decrease the efficiency of PCR amplification (Filion, 2012). Therefore, Schriewer et al. (2011) have demonstrated absorbent DAX-8 technology to remove humic acids permanently from nucleic acid extracts. This method has a potential to significantly increase the reliability of reported non-detects and measured results obtained by qPCR in environmental monitoring.

Iron effect on real-time PCR

Another group of inhibitors include heavy metals. Generally, the mechanisms of PCR inhibition by heavy metals are still not very well understood. However, one possible explanation could be the variable resistance of DNA polymerases to heavy metal ions (Filion, 2012). The inhibition is attributed to various metals, like mercury, zinc, lead, and also iron (Filion, 2012; Chowdhury et al., 2016) that is often found in water sources. Groundwater can contain > 3 mg L^{-1} iron and not always it is removed sufficiently during the water treatment process. According to the World Health Organization, the concentrations of iron in drinking water is normally below 0.3 mg L^{-1} but may be higher in places where various iron salts are used as coagulating agents in drinking water treatment plants and where iron pipes are used for water distribution (World Health Organization, 2008). The results of the C_t data from 4 mg L^{-1} samples showed a 100% reduction of qPCR efficiency when compared to the control indicating no DNA amplification. At the same time no significant change was observed for 0.2 and 0.1 mg L^{-1} samples ($P > 0.05$) (Table 3).

Table 3. Comparison of different iron concentrations based on the average crossing threshold (C_t) values

Dilution series	C_t			
	Control	4 mg L^{-1}	0.2 mg L^{-1}	0.1 mg L^{-1}
10^1	12.07 (± 0.07)	nd	12.09 (± 0.19)	11.97 (± 0.09)
10^3	19.97 (± 0.14)	nd	20.10 (± 0.01)	20.06 (± 0.03)
10^5	28.17 (± 0.12)	nd	28.30 (± 0.12)	28.34 (± 0.38)

nd – C_t was not detected; (±) standard deviation of the average values from 3 replicates.

The results of this study apparently demonstrated that elevated concentration of iron above 4 mg L^{-1} inhibited the PCR reaction, therefore when analysing the groundwater samples directly, first the inhibition should be tested.

The water sample from drinking water treatment plant was taken in the middle of iron treatment step with iron concentration > 5 mg L^{-1} and analysed for possible PCR inhibition. The results demonstrated no detectable *E. coli*. Meanwhile, the significant difference in the average crossing threshold values in-between *E. coli* control dilution series and the water sample with the same concentration of spiked *E. coli* DNA was observed (Table 4). The results of the water sample with *E. coli* DNA indicated on higher C_t values (14%) when compared to control determining the possible inhibition of uncovered environmental compounds, e.g., iron from DNA extraction.

Table 4. The comparison of control *E. coli* dilution series with the water sample with/without *E. coli* based on the average crossing threshold (C_t) values

	C_t		
Dilution series	Control	Environmental sample with *E. coli*	Environmental sample without *E. coli*
10^7	12.97 (± 0.09)	14.64 (± 0.06)	nd
10^6	16.57 (± 0.12)	18.41 (± 0.03)	nd
10^5	20.35 (± 0.08)	22.24 (± 0.14)	nd

nd – C_t was not detected; (±) standard deviation of the average values from 3 replicates.

According to these investigations, real-time PCR assays must be carefully designed, conducted, and validated with an understanding of the methods limitations and possible inhibitors. Several approaches have attempted to reduce the inhibitory impact of sample quantification with qPCR. For instance, nucleic acid extraction methods that increase DNA yield while removing most qPCR inhibitors have been developed specifically for troublesome water samples (Green & Field, 2012). However, it cannot be guaranteed that the preparations will extract DNA free of PCR inhibitors. Therefore, further analysis of environmental samples should include a pretreatment step before/after the DNA extraction to remove possible inhibitory compounds, e.g., iron, and all reactions should be analysed for the presence of inhibitory effects.

CONCLUSIONS

Three possible inhibitors were examined for the efficiency of drinking water sample analyses with qPCR. The results indicated that humic acids at concentration of 5 and 1 mg L^{-1} reduced the PCR reaction efficiency for 65% and 16%, respectively, while concentration of 4 mg L^{-1} from iron fully inhibited amplification of DNA. The analysis of the environmental sample with spiked *E. coli* cells demonstrated the reduction efficiency of the average C_t values for 14% compared with the control dilution series determining the possible inhibition for qPCR assay. The findings of the research demonstrate that prior PCR analyses, the evaluation of potential inhibitors must be performed.

ACKNOWLEDGEMENTS. This work has been supported by Riga Technical University funding for doctoral students and National Research Programme No. 2014.10-4/VPP-3/21 'MultIfunctional Materials and composItes, photonicS and nanotechnology (IMIS^2)', Project No. 4 'Nanomaterials and nanotechnologies for medical applications', Subproject No. 4.7. (2014–2017). The authors gratefully acknowledge Kristina Tihomirova and Viktorija Denisova for their assistance in humic acid and chlorine concentration measurements.

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