Metal–DNA interactions: Exploring the impact of metal ions on key stages of forensic DNA analysis

Dan Nana Osei Bonsu1,2  |  Denice Higgins2,3  |  Claire Simon4  |  Julianne M. Henry4  |  Jeremy J. Austin2

1Chemistry and Forensic Science, School of Environment and Science, Griffith University, Nathan, Queensland, Australia
2Forensic Research Group, Australian Centre for Ancient DNA (ACAD), School of Biological Sciences, The University of Adelaide, Adelaide, South Australia, Australia
3School of Dentistry, Health and Medical Sciences, The University of Adelaide, Adelaide, South Australia, Australia
4Forensic Science SA, Attorney-General’s Department, Adelaide, South Australia, Australia

Correspondence
Dan Nana Osei Bonsu, Chemistry and Forensic Science, Griffith University,170 Kessels Rd., Nathan, QLD 4111, Queensland, Australia.
Email: d.bonsu@griffith.edu.au

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Abstract
Forensic DNA analysis continues to be hampered by the complex interactions between metals and DNA. Metal ions may cause direct DNA damage, inhibit DNA extraction and polymerase chain reaction (PCR) amplification or both. This study evaluated the impact of metal ions on DNA extraction, quantitation, and short tandem repeat profiling using cell-free and cellular (saliva) DNA. Of the 11 metals assessed, brass exhibited the strongest PCR inhibitory effects, for both custom and Quantifiler Trio quantitation assays. Metal ion inhibition varied across the two quantitative PCR assays and the amount of DNA template used. The Quantifiler Trio internal PCR control (IPC) only revealed evidence of PCR inhibition at higher metal ion concentrations, limiting the applicability of IPC as an indicator of the presence of metal inhibitor in a sample. Notably, ferrous ions were found to significantly decrease the extraction efficiency of the DNA-IQ DNA extraction system. The amount of DNA degradation and inhibition in saliva samples caused by metal ions increased with a dilution of the sample, suggesting that the saliva matrix provides protection from metal ion effects.

KEYWORDS
DNA analysis, forensic science, metal ions, Quantifiler Trio, quantitative PCR

1 INTRODUCTION

Forensic DNA analysis of biological samples collected from metal objects presents many challenges due to the adverse impact of metal ions on DNA recovery, extraction, amplification, and profiling [1–3]. As efficient catalysts of redox reactions, metal ions mediate DNA damage via oxidative stress induced by free radicals from the reduction process of transition metals [4]. This has been linked with the limited quality and success of touch DNA retrieved from brass-made ammunition, especially from the copper component of the alloy [5, 6]. Co-extraction can also result in the binding of metal ions to DNA, which can either impede access to the DNA template [7, 8] or cause inhibition of DNA polymerase activity, leading to quantitative polymerase chain reaction (qPCR) and short tandem repeat (STR) profiling failure [5, 6, 9–11]. A competitive interaction of a more electropositive divalent metal cation with magnesium ions may also disrupt the optimal magnesium concentration for DNA polymerase activation, affecting PCR performance. Notably, the polymerase processivity of
metal ion–doped samples and the half-maximal concentration of the metal ion that causes inhibition of PCR (IC$_{50}$) is directly linked with the type of metal [7]. Metal ions may also indirectly affect DNA profiling outcomes via impacts on qPCR assays that are used to estimate DNA quantity in casework samples. Metal ions interacting with the qPCR assay via the inhibition of the polymerase, and interaction with target DNA, internal PCR control (IPC) or the passive reference dye can cause over [12] – or under-estimation of DNA concentration [7, 13] leading to sub-optimal DNA input into subsequent STR profiling reactions.

The impact of metals on forensic DNA quantitation has been evaluated in only two instances using commercial or custom qPCR assays. Earlier research [13] employed the Quantifiler Human DNA Quantification Kit from Thermo Fisher Scientific to evaluate the effect of six metal ion inhibitors (Al, Ca, Cu, Fe, Ni, and Pb) on qPCR. However, the kit has been replaced by more robust assays with improved buffer systems and inhibitor tolerance (personal communication—Goodwin Corey, Field Applications Specialist—Genetic Sciences, Thermo Fisher Scientific). Kuffe et al. [7] used an in-house assay to assess metal ion impact on qPCR efficiency, but their research had limitations, including the use of a higher amount of template DNA (5 ng) than recommended for qPCR and the fact that operational forensic labs typically use well-validated commercial qPCR kits.

Custom (in-house, e.g. [2, 7]) qPCR assays consist of various PCR master mixes, polymerases and primers that must be constituted per user preference.

On the other hand, commercial DNA quantification kits such as Investigator Quantiplex Pro Kit (Qiagen) [14], InnoQuant HY (InnoGenomics Technologies) [15], PowerQuant System (Promega Corporation) [16] and Quantifiler Trio DNA Quantification Kit (Thermo Fisher Scientific) [17] are sold ‘ready-to-use’. Both custom and commercial assays include one or more human-specific PCR targets and an inert fluorescent passive reference dye such as ROX (carboxy-X-rhodamine) or Mustang Purple [14–16, 18]. The passive reference dye is required for normalisation between sample wells since its fluorescence is unaffected by the amplification cycles [19]. As a result, a cycle threshold (C$_T$) is defined as the cycle number at which there is a discernible difference between a sample fluorescence signal and that of the passive reference [20]. The quantification estimates of samples are determined by comparing their respective C$_T$ to an external calibration curve constructed from a dilution of reference standards [21]. Therefore, anything that alters the PCR conditions can affect the accuracy of DNA quantitation.

Commercial qPCR kits are more suited to operational forensic laboratories for casework. Apart from their advanced buffer systems that provide better tolerance of inhibitors [8], quantitative and qualitative assessment of total human DNA can be concurrently performed in a single reaction [22, 23]. The quality data enables the prediction of STR typing success and provides a streamlined and efficient forensic analysis workflow [23]. The Quantifiler Trio DNA Quantification Kit (Thermo Fisher Scientific) is the most common qPCR kit used in forensic DNA laboratories in Australia. The Quantifiler Trio kit is based on a four-target system: small (SA) and large (LA) human autosomal targets, male (Y) targets and an internal PCR control (IPC) to detect inhibition [17, 22, 23]. The test sample is amplified, and a degradation index (DI) is calculated from the resulting SA and LA target DNA concentrations [17, 24, 25]. The IPC enables the detection of PCR inhibitors, such as metal ions, that may have been co-extracted with the nucleic acids and, together with the DI, serves as a metric of the overall quality of the DNA in the extracted sample.

The specific effects of metal ions on DNA quantification remain unclear. For example, metal oxidation products and gunpowder residue present on fired brass casings were implicated in inconsistent trace DNA quantitation and STR profile data [26]. More recently, Forensic Science South Australia (FSSA) has observed that several strong Hemastix positive trace blood stains on metal objects (such as blades, jewellery, and tools) in operational casework have failed to give a DNA profile, indicating that the potential presence of metal ions in crime scene samples may prevent probative DNA evidence from being attained (Claire Simon, FSSA—personal communication).

It is critical to assess the direct and indirect effects of metal ions on each step of the DNA analysis workflow from sample collection, DNA extraction, quantitation and STR profiling and to identify synergistic effects of different procedures that may exacerbate or minimise the effects of metal ions on DNA profiling success. However, previous research (e.g. [7, 13]) has only evaluated the impact of metal ions on the amplification (qPCR) step, using purified DNA with known concentration and omitting impacts of the biological matrix (e.g. saliva, blood, and other body fluids), the DNA extraction process (which can itself remove many potential contaminants/inhibitors in case samples) and STR profiling steps. Consequently, such studies are limited in their scope and direct application to casework situations because the low success rate of DNA analysis of samples collected from metal objects is not only associated with inhibition at the PCR stage.

It is common for inhibitors/contaminants to interfere also with cell lysis required for DNA extraction [10, 27] and DNA profiling [23]. Further, the use of purified DNA alone enhances the success of metal ion nucleic acid interaction, discounting the effect of other components of the cellular matrix [8]. Hence, the current study aimed to examine the impact of ions from metal exhibits frequently encountered
as evidence from crime scenes on sample purification, qPCR amplification and DNA profiling. Specifically, we probed whether specific metal contaminants persist through the DNA extraction step and the effect of sample matrix on inhibitor activity. Additionally, the influence of metal ions on Quantifiler Trio and a custom (in-house) assay quantification of different sample types (purified DNA and saliva extracts) and consequent GlobalFiler STR profiles were examined.

2 MATERIALS AND METHODS

2.1 Metal selection

Most metals of forensic interest are often found in the built environment as common household objects and weapons [8]. For example, the alloys brass (copper and zinc), steel (iron and carbon) and stainless steel (steel plus chromium) are routinely used in the construction of the built environment and the manufacturing of tools, wires, firearms and ammunition [28, 29]. Tin finds various applications, including food packaging, beverage containers [30], toothpastes (Stannous fluoride, SnF2) for its antibacterial properties and tooth sensitivity prevention [31, 32], and ammunition components like bullet jackets, lead alloys (Pb) and primers [33, 34]. Copper is a significant component of most ammunition and improvised explosive devices encountered at crime scenes [8, 29, 35]. Nickel-plated ammunition casings are preferred over brass casings for use in self-defence and law enforcement guns due to their greater corrosion resistance when stored in leather holders [36]. Lead is one of the main elements of gunshot residue frequently found on surfaces of discharged firearms and/or cartridges, bullets and casings [37] potentially harbouring touch DNA. Hard tissues (teeth and bones) are sources of calcium, whereas aluminium and brass are frequently used for door/window frames and knobs and household items, among others [38]. Therefore, ions of these metals were selected because they are representative of everyday items frequently encountered at crime scenes and/or submitted to forensic laboratories as exhibits for trace DNA testing.

2.2 Metal ions

Stock solutions (50 mM) of 10 metal ions (all purchased from Sigma-Aldrich) and brass were prepared in DNA-free water and then diluted to working stocks of 10 mM using DNA-free water. These were aluminium sulphate hydrate (≥99.99% trace metal basis) (Al), calcium chloride (anhydrous, powder ≥97%) (Ca), chromium(III) chloride hexahydrate (puris p.a. ≥98.0% (RT)) (Cr), copper(II) sulphate (puris p.a., anhydrous ≥99.0% (RT)) (Cu), lead(II) nitrate (≥99.99% trace metal basis) (Pb), iron(III) chloride hexahydrate (puris p.a., ≥99%) (Fe(III)), iron(II) sulphate hydrate (99.999% trace metal basis) (Fe(II)), nickel(II) sulphate hydrate (≥99.99% trace metal basis) (Ni), tin(II) chloride (≥99.99% trace metal basis) (Sn), zinc chloride (reagent grade ≥98%) (Zn). To simulate brass, equal amounts of Cu and Zn stock solutions of the same concentration were mixed to allow for a balanced comparison.

2.3 Inhibitory effects of metal ions on DNA quantitation

We first tested PCR inhibition by directly adding metal ions to qPCR reactions immediately before thermocycling. This approach aimed to minimise opportunities for DNA degradation. We measured the impact of each metal ion at six concentrations (final concentration of 0, 0.1, 1, 1.5, 3 and 5 mM in the PCR) on two DNA input amounts (0.5 and 0.2 ng of ‘Human Male Genomic DNA, Promega, cat#: G1471’ in two different qPCR assays (in-house custom assay and Quantifiler Trio, see below for details). The 0.5 and 0.2 ng of template DNA were used as these are the optimal input amount for GlobalFiler STR profiling as validated in our laboratory and approach trace levels compared to the 5 ng or higher used in previous research [7]. Each metal ion concentration/DNA input/qPCR assay was run in triplicate. The metal ions (1 µL) were directly added to the PCR reaction mixture using an automated Tecan Liquid Handling Platform (LHP) (Tecan Group Ltd., see below for details). The 0.5 and 0.2 ng of template DNA were used as these are the optimal input amount for GlobalFiler STR profiling as validated in our laboratory and approach trace levels compared to the 5 ng or higher used in previous research [7]. Each metal ion concentration/DNA input/qPCR assay was run in triplicate. The metal ions (1 µL) were directly added to the PCR reaction mixture using an automated Tecan Liquid Handling Platform (LHP) (Tecan Group Ltd.), and the selected concentrations were based on previous research [7, 13]. For example, a previous study reported inhibition (IC50) values between 0.26 and 2.79 mM in the PCR for Al, Ca, Fe, Ni, Cu, Pb, and Zn metal ions [7]. Further, the chosen range is a more realistic semblance of ‘trace level’ metal ion concentrations that are expected to be present in casework samples following DNA extraction [39].

2.4 Effects of metal ions on DNA after extraction and purification

We tested the impact of selected metal ions on DNA amplification when genomic DNA (Human Male Genomic DNA, Promega, cat#: G1471) was first mixed with metal ions and put through a standard DNA extraction process. This examined how metal ions may negatively interact with DNA before extraction (e.g. via DNA degradation) but also how efficiently the DNA extraction process can remove the inhibitory effects of metal ions. In quintuplicate, we mixed DNA samples by adding 3 µL of 5 mM
or 1 mM of Al, Cu, Zn or Fe(II) metal ion solution with 3 µL of 0.5 ng/µL single source Human Male Genomic DNA (Promega, cat#: G1471). The resulting DNA–metal sample was incubated for 1 h at room temperature and extracted using the DNA IQ system (Promega) on a Hamilton AutoLyS LHP with a final elution volume of 60 µL. This validated automated extraction protocol does not require Proteinase K. Each DNA IQ extraction batch included two reagent blanks and one positive control (2 µL of whole human blood spotted on a 5 mm × 5 mm square of FTA card (Whatman, GE Healthcare) according to the standard operating procedure at FSSA. DNA extracts were stored at −20°C prior to QuantiFiler Trio or in-house quantitation.

# 2.5 Matrix effect on metal ion DNA damage and PCR inhibition

The matrix entails the entirety of the material wherein the analyte of interest is embedded [40]. It is well documented that non-targeted analytes of biological samples (other matrix components) may impact the ability to detect, identify or quantify a targeted analyte, hence, the need to establish the matrix effect on the analytical process [40–42]. The impact of sample matrix on metal–DNA interaction, PCR inhibition and STR profiling was assessed with neat and diluted saliva samples, as a representative forensic sample, using six selected metals with the lowest IC₅₀ (brass, chromium, copper, iron(II), tin and zinc). Except for tin (Sn) in dentifrices that may be found in buccal/saliva samples, biomaterials encountered in forensic casework as a matrix potentially contaminated with metals are normally blood traces on a substrate or epithelial cells deposited via contact [8, 38]. Saliva was chosen as convenient, non-invasive sample with consistent matrix constituents that is amendable to serial dilution. We added 10 µL of a 5 mM metal ion solution to a 10 µL aliquot of neat, 1:20 and 1:50 diluted saliva sample obtained from a single consenting volunteer, in triplicate. The samples were extracted using the DNA IQ System and quantified using QuantiFiler Trio. The impact of metals on the quantity and quality of recovered DNA from saliva was assessed using the SA target yield, DI and the cycle threshold (Cₕ) of the SA and IPC data. STR profiling of the extracts was performed using the GlobalFiler PCR amplification kit as previously described [38].

# 2.6 DNA quantification and STR profiling

A custom assay and a commercial DNA quantification kit were used to determine the concentration of DNA in the samples.

## 2.6.1 Custom assay

The custom assay (in-house) qPCR assay using SYBR green chemistry targeting a small (67 bp) (forward: GGGCAGTGTTCACCCCTGAGGAAA ACT; reverse: GAGACACAGGGGTGTTTA) human-specific nuclear DNA amplicon was performed as previously described [43] on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific). The 10-µL reaction volumes consisted of 1× Brilliant III Ultra-Fast SYBR Green Low ROX qPCR Master Mix (Agilent Technologies), 0.15 µM forward primer, 0.15 µM reverse primer, 16 ng/µL Rabbit Serum Albumin and 1 µL template DNA. Thermal cycling conditions were 95°C denaturation step for 4 min, followed by 45 cycles of 95°C for 10 s, 58°C for 20 s and 72°C for 15 s. DNA concentration was determined using the comparative Cₕ method by comparing unknown samples to a standard curve using the QuantStudio 6 Flex Real-Time PCR Software v1.3 and applying ROX as the passive reference as described in our previous study [43]. A zero value for DNA concentration was assigned to samples with no detectable amplification, reported as undetermined (UNDET) by the QuantStudio 6 Flex Real-Time PCR Software.

## 2.6.2 Quantifiler Trio assay

Quantification with the commercial kit employed Quantifiler Trio DNA Quantification Kit (Thermo Fisher Scientific) on an Applied Biosystems 7500 Real-Time PCR System with the HID Real-Time PCR Analysis software v1.2 (Thermo Fisher Scientific), as per the manufacturer’s instructions. All samples were run in triplicate in a total reaction volume of 20 µL, 18 µL of master mix, 1 µL of DNA template and 1 µL of metal ion or water. Samples reported by the HID Real-Time PCR Analysis software as showing undetectable amplification were assigned an SA target yield value of zero and an IPC value of 40.

## 2.6.3 DNA profiling

STR profiling was performed with the GlobalFiler PCR Amplification Kit (Thermo Fisher Scientific) at the input DNA concentration on a ProFlex thermocycler (Thermo Fisher Scientific) for 29 cycles. PCR fragments were separated on an Applied Biosystems 3500xl Genetic Analyzer (Thermo Fisher Scientific). GeneMapper ID-X Software v1.6 (Thermo Fisher Scientific) was used to determine fragment size and allele calls using an analytical threshold of 50 relative fluorescence units (RFU).
TABLE 1 IC₅₀ values for quantitative polymerase chain reaction (qPCR) inhibition for two DNA template input amounts (0.5 and 0.2 ng) for an in-house assay utilising SYBR green chemistry and ROX as the passive reference.

<table>
<thead>
<tr>
<th>Metal</th>
<th>IC₅₀ (0.5 ng) ± SD (mM)</th>
<th>IC₅₀ (0.2 ng) ± SD (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brass</td>
<td>0.050 ± 0.042</td>
<td>0.029 ± 0.015</td>
</tr>
<tr>
<td>Ni</td>
<td>0.058 ± 0.018</td>
<td>0.036 ± 0.025</td>
</tr>
<tr>
<td>Cr</td>
<td>0.064 ± 0.020</td>
<td>0.038 ± 0.022</td>
</tr>
<tr>
<td>Pb</td>
<td>0.064 ± 0.018</td>
<td>0.029 ± 0.030</td>
</tr>
<tr>
<td>Zn</td>
<td>0.099 ± 0.045</td>
<td>0.041 ± 0.020</td>
</tr>
<tr>
<td>Al</td>
<td>0.124 ± 0.031</td>
<td>0.030 ± 0.028</td>
</tr>
<tr>
<td>Fe(III)</td>
<td>0.437 ± 0.037</td>
<td>0.144 ± 0.024</td>
</tr>
<tr>
<td>Cu</td>
<td>0.497 ± 0.044</td>
<td>0.099 ± 0.013</td>
</tr>
<tr>
<td>Ca</td>
<td>1.302 ± 0.064</td>
<td>0.055 ± 0.020</td>
</tr>
<tr>
<td>Sn</td>
<td>1.541 ± 0.030</td>
<td>0.914 ± 0.021</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>1.671 ± 0.037</td>
<td>0.631 ± 0.021</td>
</tr>
</tbody>
</table>

Abbreviation: SD, standard deviation.

2.7 Data analysis

DNA quantity was estimated using the SA DNA yield. DNA quality was assessed using the DI, calculated as the ratio of the small to the LA target concentrations. A degradation value of <1 indicates no degradation, 1–10 indicates slight-to-moderate degradation, and >10 indicates severe degradation [25]. Samples where no LA target was amplified were considered severely degraded. PCR inhibition was assessed using the internal PCR control (IPC) cycle threshold (Cₜ) values, with values above 30 indicating inhibition. The level of PCR inhibition by metal ions was determined using the SA target yield and internal PCR control (IPC) in the presence of added metal ions over the established concentration range.

The concentration of the metal that gave 50% inhibition of PCR (IC₅₀) values was determined for each metal ion using non-linear regression (four parameters) of data using GraphPad Prism version 9.3.1 (350) (GraphPad Software). STR profiles were assessed qualitatively and quantitatively for any indications of inhibition (e.g. allele dropout and profile (interlocus) balance). Profile intensity/strength was determined utilising the average peak heights (RFU) of all observed STR alleles across the triplicate of each metal-treated saliva sample. Profile balance was determined using the coefficient of variation (CoV) of RFU across the STR profiles as previously described [44]. The Kruskal–Wallis test was used to assess any differences in mean RFUs, percentages of detected alleles and mean CoV of metal-treated saliva samples compared to the non-treated control samples, followed by Dunn’s multiple comparison test for p < 0.05. The relative impact of metal ion interference on the performance of DNA-IQ in relation to DNA yield, IPC Cₜ, DI, and sample matrix was assessed with the Mann–Whitney U test. For all analyses, significance was reported at p < 0.05.

2.8 Ethical consideration

The study was approved by the Human Research Ethics Committee of the University of Adelaide (Ethics approval no.: H-2016-218) in accordance with the National Health and Medical Research Council: National Statement of Ethical Conduct in Human Research [45]. In addition, written informed consent was obtained from the single donor of the saliva samples.

3 RESULTS

3.1 Impacts of metal ions on qPCR estimation of DNA concentration

3.1.1 Custom assay

All 10 metal ions and brass inhibited the custom qPCR assay leading to substantial underestimates of DNA concentration, at both DNA input amounts (0.5 and 0.2 ng). The IC₅₀ results show that brass, nickel, chromium, lead and zinc were the strongest PCR inhibitors (IC₅₀ all less than 0.1 mM and no DNA detected at metal ion concentrations above 1 mM). In contrast, iron(II), tin and calcium were the least inhibitory. The inhibitory effects of metal ions were influenced by the amount of DNA input. For all metals, the IC₅₀ was 1.6–23 times higher for 0.5 ng, compared to 0.2 ng of input DNA, indicating that higher concentrations of DNA necessitate more metal ions to bind and hinder PCR, or that higher DNA concentrations chelate more metal ions before reaching inhibitory levels for DNA polymerase (Table 1).

3.1.2 Quantifiler Trio assay

The 10 metal ions and brass also produced inhibition of Quantifiler Trio. Inhibition was either similar to the custom assay (i.e. brass, Cr, Zn, Al, Fe(III)) or substantially higher (Fe(II)) or lower (Ni, Pb, Cu, Ca) than the custom assay (Figure 1). The IC₅₀ results show that brass, chromium, zinc, and aluminium were stronger PCR inhibitors (IC₅₀ values less than or equal to 0.1 mM, Table 2), whereas calcium, copper and nickel were the least inhibitory. As with the custom assay, the inhibitory effect of metal ions (as measured by the IC₅₀) was influenced by DNA input amount—IC₅₀ was 1.5–6.5 times
higher for 0.5 ng, compared to 0.2 ng of input DNA. At 0.1 mM Sn, the estimated DNA concentration was only 24% of the true input amount (Table 2), indicating strong inhibition equivalent to that of brass and/or chromium. However, at higher concentrations of tin, DNA concentration was overestimated, making it impossible to accurately measure the IC\textsubscript{50}.

For both custom and Quantifiler Trio assays, the inhibition strength of the tested metals was consistent for brass and chromium, irrespective of the quantity of the template.
DNA. An exception is, however, seen with the Quantifiler Trio assay where chromium shows slightly more enhanced inhibition potential than brass at 0.2 ng template (IC$_{50}$: 0.046 ± 0.020 vs. 0.020 ± 0.013), whereas the reverse is observed when the starting amount of DNA is 0.5 ng/µL. The preceding outcome implies that chromium is a slightly more potent inhibitor than brass at trace DNA levels, such as those found in touch samples. Copper inhibited qPCR less than expected when tested using the Quantifiler Trio assay (IC$_{50}$: 1.8 ± 0.07 mM). Similarly, the inhibition of Ni and Pb was more noticeable with the custom assay than with the Quant Trio assay (Figure 1, Tables 1 and 2). The latter kit was also found to be more resistant to inhibition by calcium, with DNA amplifications detected in the presence of approximately 2.6 mM of the metal. Differences in the chemistry and components (e.g., DNA polymerase type) of these assays may account for the observed disparities.

### 3.1.3 Quantifiler Trio IPC C$_T$ variability and impact on DNA quantification

In addition to inhibition of DNA quantification, we examined the impact of metal ions on the internal PCR control (IPC). The IPC C$_T$ values for all untreated control DNA samples ranged between 27.6 and 27.9, close to the expected value (27.5), indicating no inhibition. The inhibitory impact of metal ions on the IPC varied widely across the 10 metal ions and brass and for the two different DNA input amounts (Figure 2). Aluminium had no impact on the IPC C$_T$ which stayed within the general Quantifiler Trio threshold of 20–30 (Figure 2) for all levels of inhibitor concentration, despite a marked reduction in estimated DNA concentration. In the presence of increasing metal ion concentrations and with 0.5 ng input DNA, the IPC C$_T$ values exceeded the upper threshold (30) at 5 mM (Ca), 3 mM (Cr, Cu, Fe(III), Ni) and 1.5 mM (Pb, Zn, brass).

The increase in the average IPC C$_T$ values of the DNA treated with these metals was significant (at least two cycles) and likely to indicate PCR inhibition or degradation of the DNA template. Fe(II) had almost no impact on the IPC C$_T$, whereas concentrations of Sn at 1 mM or higher caused the IPC C$_T$ to fall at least 12 C$_T$ units lower than the average of controls and below the lower limit of Quant Trio IPC (20), irrespective of the amount of template DNA (Figure 2). This outcome was not observed in tin-treated samples quantified with the custom assay. At 0.2 ng input DNA, similar patterns were observed—no impact on IPC C$_T$ with Al, IPC C$_T$ > 30 at 5 mM (Cr, Cu and Fe(II)), 3 mM (Ca, Fe(III), Pb, Ni and Zn) and 1.5 mM (Zn, brass). In most cases, evidence of inhibition of human DNA quantification was apparent at a much lower metal concentration than detected by the IPC for all metals except zinc, tin and brass.

### 3.2 Impact of metal ions on DNA extraction and qPCR

When a known quantity of DNA was added to metal ions and then subjected to DNA extraction and qPCR, DNA recovery and quantification were contingent on the metal ion and its initial concentration.

For all samples treated with 1 mM of metal ions, 60%–80% of input DNA was recovered, the highest yield being Al (1.20 ± 0.04 ng) (Figure 3A), with no indication of DNA degradation (DI ≤ 1) (Figure 3B). For samples treated with 5 mM of Cu, Fe(II) or Zn, only 16%–32% of DNA was recovered, compared to 69% recovery for 5 mM Al (Figure 3D). Moderate degradation (2.92 ± 1.64) was only observed with 5 mM Fe(II)-treated DNA, with one sample reaching a DI of 5.8 (Figure 3E), whereas all others for both metal concentrations were within the range (DI ≤ 1) of undegraded DNA (Figure 3B,E). In all instances, irrespective of metal ion concentration, IPC C$_T$ was within the expected range (~27–28).

### 3.3 Matrix effect on metal ion inhibition and STR genotyping

Detectable amounts of DNA were observed over the range of saliva dilutions for non-treated control samples, consistent with the dilution factor (Figure 4A). The mean ± SD of DNA yield was 1.57 ± 0.15 ng (neat saliva), 0.07 ± 0.02 ng (1:20 saliva dilution) and 0.03 ± 0.02 ng (1:50 saliva dilu-
**FIGURE 2** Quantifier Trio of IPC C<sub>T</sub> results for different DNA input amounts ((A) 0.5 ng and (B) 0.2 ng) for 10 metal ions and brass. Three quantification runs per treatment. Green dots: control DNA sample with no metal ions added; blue and red dots: DNA sample with 0.1, 1, 1.5, 3 or 5 mM metal ion. The general IPC C<sub>T</sub> threshold for Quantifier Trio of 20–30 is indicated. Red dots indicate IPC C<sub>T</sub> values significantly higher (>30) or lower (<20) than the threshold.

**FIGURE 3** Autosomal DNA (SA) yield, degradation index and SA cycle threshold (SA C<sub>T</sub>) of DNA samples (3 µL of 0.5 ng/µL) treated with 3 µL of 1 mM (top panel, A, B and C) and 5 mM (bottom panel, D, E and F) of Al, Cu, Fe(II) and Zn metals, (n = 5) and incubated for 1 h at room temperature. Mean values represented by ‘+’.
FIGURE 4  Effect of sample matrix on (A) autosomal DNA yield, (B) degradation and (C) SA $C_T$ of neat, 1:20 and 1:50 saliva samples treated with brass, Cr, Cu, Fe(II), Sn and Zn.

The average DI was ≤1, and no significant differences in the values between the three saliva samples (Figure 4B). The SA and IPC $C_T$ values for these control samples ranged between 27.2 and 27.7 and were also not affected by the sample matrix dilution. Notably, whereas the IPC data for all metal-treated samples were not different from the non-treated sample (Table S1), the SA $C_T$ (Figure 4C) increased with increasing dilution factor to undetectable (i.e. UNDET, scored 40) levels with no SA target amplification (see asterisks for 1:50 dilutions in Figure 4A). Except for Fe(II) ($p = 0.011$), the DNA yield from neat saliva samples treated with metal ions (~1.0–1.2 ng) did not show any statistically significant difference compared to the untreated control sample ($p = 0.086$) for all other metals tested (Figure 4A). The mean DI, which was below 2.5, was also statistically similar to the control ($p = 0.165$) (Figure 4B) and, in conjunction with the normal IPC $C_T$ values, indicated that the quality of the extracted DNA was high and downstream quantification was not impacted. In contrast, only 20%–71% of DNA was recovered for 1:20 diluted saliva samples treated with metal ions (Figure 4A).

Brass (0.05 ± 0.02 ng) and Cr (0.014 ± 0.002 ng) showed the highest and lowest yield of the treated 1:20 saliva samples, respectively (Figure 4A). SA yield was determined for triplicate Cu, Fe(II), Sn and Zn-treated 1:20 saliva samples and was significantly different from the control ($p = 0.034$) (Figure 4A). However, DNA was recovered in only two replicates each of brass (0.05 ± 0.02 ng) and Cr (0.014 ± 0.002 ng)-treated 1:20 saliva samples (see asterisks in 1:20, Figure 4A). Some samples treated with brass or Sn, and all the triplicate Cr-treated samples gave a detectable SA target signal but failed to give a signal for the LA target (Table S1). The failure of the SA and/or LA targets to amplify in these 1:20 samples meant that DI values were only available for just one sample each of brass (DI = 2.0) and Sn (DI = 2.0) (see asterisked bars in Figure 4B) but none in Cr-treated samples (asterisked space in Figure 4B) indicating either severe DNA degradation or PCR inhibition [25]. Apart from two replicates of Cu-spiked samples that gave a detectable SA target signal (Table S1) but failed to give a signal for the LA target (SA $C_T = 38.8$, IPC $C_T = 27.7$), no DNA recoveries were observed for all metal-treated samples of the 1:50 saliva dilution (see asterisks in Figure 4A).

3.3.1  STR genotyping and profile balance on saliva treated with metal ions

STR profiling was not successful for all metal-treated 1:20 saliva dilutions. Profiling was not performed for 1:50 saliva extracts due to the extensive inhibition and/or degradation seen from the quantification data. For neat saliva samples, the highest average peak height was 5384 RFU for the non-treated saliva samples compared to saliva spiked with metal–ions. Excepting Cu-treated samples with an average of 4100 RFU ($p = 0.324$), the signal intensity of the control samples was statistically significantly different from all other metal-treated neat samples (Table 3,
TABLE 3 Summary of quantitative short tandem repeat (STR) data for metal-treated neat saliva samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak height (RFU ± SD)</th>
<th>Profile balance (CoV ± SD)</th>
<th>STR alleles detected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (neat saliva)</td>
<td>5383 ± 3119</td>
<td>0.454 ± 0.038</td>
<td>100</td>
</tr>
<tr>
<td>Brass</td>
<td>2938 ± 1679</td>
<td>0.538 ± 0.074</td>
<td>100</td>
</tr>
<tr>
<td>Cr</td>
<td>3347 ± 2366</td>
<td>0.554 ± 0.065</td>
<td>100</td>
</tr>
<tr>
<td>Cu</td>
<td>4100 ± 2445</td>
<td>0.541 ± 0.047</td>
<td>100</td>
</tr>
<tr>
<td>Fe(II)</td>
<td>2106 ± 1194</td>
<td>0.568 ± 0.083</td>
<td>100</td>
</tr>
<tr>
<td>Sn</td>
<td>2028 ± 1407</td>
<td>0.608 ± 0.170</td>
<td>100</td>
</tr>
<tr>
<td>Zn</td>
<td>3130 ± 2175</td>
<td>0.524 ± 0.137</td>
<td>100</td>
</tr>
</tbody>
</table>

Abbreviations: CoV, coefficient of variation; RFU, relative fluorescence unit; SD, standard deviation.

Figure 5. Comparison of GlobalFiler profile data for neat saliva samples treated with 5 mM metal (n = 3 for each sample) and extracted using DNA IQ. (A) Total profile relative fluorescence unit (RFU) and (B) profile balance (coefficient of variation of profile RFU).

Figure 5). The lowest average peak heights were obtained for Sn (2028 RFU) and Fe(II) (2106 RFU) spiked samples (Table 3, Figure 5).

Profile imbalance was the lowest in the control samples and highest in Fe(II) and Sn-treated samples (Table 3, Figure 5). Despite the lower average peak height RFU and relatively poor profile balance, all metal–ion-treated samples returned full STR profiles that were 100% concordant with the untreated control sample.

4 | DISCUSSION

4.1 | Impacts of metal ions on qPCR estimation of DNA concentration

Quantitative PCR assays have become the tool of choice for the rapid and sensitive quantitation of DNA in forensic DNA testing laboratories. However, the technique is prone to adverse impacts by contaminants often co-purified with DNA [46]. Metals have been demonstrated to exhibit rather complex effects during the qPCR process depending on the physicochemical properties of co-extracted inhibitor and the type of qPCR assay employed for DNA quantitation [7, 13, 47]. Therefore, this study examined the inhibition activity of metal ions on the Quantifiler Trio DNA Quantification kit and a custom assay quantification of different sample types.

The results show that of the metals tested, brass, Ni, Sn, Cr, Pb and Zn cause higher PCR inhibition (lower IC_{50} Table 1 and Table 2). Interestingly, these metals make up most of the so-called ‘common workhouse’ routinely used in the manufacture of firearms and ammunition; other weapons like razors, knives, screwdrivers and so forth, used in the commission of crime [8] and commonly submitted to forensic science laboratories for DNA evidence recovery [48]. In their evaluation of the influence of metal ions on the real-time quantitation of DNA, Kuffel et al. [7] obtained an IC_{50} of 2.79 mM for Al, whereas Combs et al. [13] identified the same metal as the most effective inhibitor, with 50% inhibition achieved at a very low concentration of 0.1 mM. The observed variance in the
inhibitory potency of the same metal was attributed to the differences in the qPCR assay [7].

Accurate DNA quantitation is vital in the triage of casework samples and offers data to mitigate potential problems before profiling. The outcome of the current study shows that metal ions’ impact on the qPCR process may be multi-faceted. For instance, IC$_{50}$ values were higher (low inhibition) for an increased quantity of template DNA for all metals for both assay types investigated. This means that the amount of DNA in the PCR affects the extent of inhibition. Higher amounts of template in the reaction mix offers an enhanced surface area for the PCR to proceed, albeit sub-optimally and with decreased efficiency. This may account for the relatively high IC$_{50}$ values reported by Kuffel et al. [7], who used 5 ng template DNA, well above the recommended 0.4–2 ng [49] in contrast to the 0.1 ng of DNA input per reaction used by Combs et al. [13]. Moreover, the observed effect could be due to metal interaction with other assay components. As noted in Tables 1 and 2, Fe(II) shows heightened qPCR inhibition (IC$_{50}$: 0.31–0.50 mM) with the Quant Trio but not custom (IC$_{50}$: 0.63–1.67) assay (Figure 1E vs. F). The custom assay includes rabbit serum albumin, which is known to chelate metal contaminants [50], hence requiring more Fe(II) ions to cause inhibition.

4.2 IPC $C_T$ variability and impact on DNA quantification

Internal PCR control facilitates inhibitor detection if the inhibitor blocks essential reagents, inactivates or interferes with the polymerase’s processivity, and in some cases, binds to the DNA template [13]. Therefore, a sufficient amount of potential inhibitors in a reaction that affects the IPC template and sample template confounds the quantitative assessments of the DNA quantity [51]. Metal contaminants in forensically relevant samples have been documented as potent inhibitors in PCR-based STR assays [8] and a recent study has highlighted a quenching effect of various metal ions on fluorescence [52]. The foregoing effect may be implicated in the interference observed for samples treated with Sn ions where IPC $C_T$ values were at least 12 $C_T$ units lower with increasing Sn concentration compared to the average of controls (Figure 2). The data showed that excepting Zn, Sn and brass, a relatively high amount of metal inhibitors was required to trigger the IPC inhibition detection system of Quantifiler Trio, despite apparent evidence of inhibition in the autosomal DNA by target, as noted for Al (Figure 2). Therefore, the IPC $C_T$ alone may not be a good indicator of the presence of inhibitors in a sample with potential metal ion contamination.

4.3 Impact of metal ions on DNA extraction and qPCR

Sample purification and quantification are crucial to forensic DNA recovery from metals. However, the ability of the paramagnetic bead extraction kits to effectively remove specific metal ions, which are often magnetisable, has not been investigated. Purification of samples spiked with metal ions resolved, in most instances, the inhibitory effect observed when DNA was spiked directly into qPCR reactions. However, this was dependent on the type and amount of metal inhibitor (Figure 3). For example, when enough contaminants (5 mM) were present in the sample, less than one third of the DNA was recovered (Figure 3D). This was notable in Fe(II)-treated samples and corresponded with increased degradation (Figure 3E). A plausible reason may be that being paramagnetic, Fe(II) potentially saturates the binding sites of the DNA-IQ magnetic beads, limiting the resin-DNA binding necessary for sample purification. Possible loss of non-resin-bound DNA in the extraction medium may ensue via the repeated washing steps (decreased recovery) with an enhanced prospect of co-extraction of inhibitors that may facilitate degradation and/or adversely impact subsequent PCR reactions. This outcome suggests that paramagnetic bead-based extraction technique utilised in this study may probably not be the ideal sample purification method for samples potentially contaminated with ferrous ions. Further investigation of other alternatives, such as silica membrane columns [53] or use of chelating additives [10] with the magnetic beads techniques is recommended.

In a study by Akhidime et al. [54], the concentration of metal ions that leached off different metal surfaces, including Cu, Fe and Zn, into a bacterial growth broth after 24 h incubation with constant agitation and at elevated temperatures was assessed for antimicrobial activity. At least 56 ppm (0.88 mM), 10 ppm (0.15 mM) and 0.4 ppm (0.007 mM) of Cu, Zn and Fe ions, respectively, was determined to leach from substrates coated with these metals. These concentrations, while unlikely to obtained for instance, during swabbing, relate to the 1 mM of metal ions spiked into samples in this study and could ordinarily be removed by sample purification, decreasing the potential for inhibition. However, an assessment of the consistenctly high SA $C_T$ data (Figure 3C,F) provides a curious perspective. Thus, with inhibitors of metal origin, normal IPC $C_T$ values should not be interpreted as meaning there is no inhibition when the Quantifiler Trio kit is used. The effect may be seen in the target $C_T$ values and downstream profiling, although the IPC $C_T$ can remain normal. It appears that there must be extreme levels of inhibition for the Quantifiler Trio IPC $C_T$ to be
types of biomaterial are required to assess the specific relationship between biological matrices and the extent of metal inhibition and/or DNA degradation.

4.5  |  STR genotyping

Metal-treated saliva DNA profiles were examined and contrasted to non-treated control samples to assess the impact of metal ions on the quality and informativeness of STR profiles. The inability to generate profiles for all the treated 1:20 saliva extracts suggests that the decreased sample matrix caused substantial metal-induced DNA inhibition/degradation, an observation consistent with the increased DI and the non-amplification of SA and/or LA targets for these samples. In contrast, complete and informative STR profiles were detected for the treated clean saliva samples without any dropouts, regardless of metal type, and were congruent with the respective quantitation and DI findings. Although the relatively high standard deviation of peak intensities of metal-spiked samples is partly related to the sample size, it may possibly reflect a differential impact of metal inhibitors on different loci/alleles and provides a baseline for further evaluation of same, utilising more samples. Regardless, samples with intact matrix showed higher variability in profile balance, with Sn and Fe(II)-treated samples exhibiting poorer interlocus balance (Figure 5B). These findings, in concert with the lack of successful amplification of metal-treated diluted saliva matrix, indicate that the sample matrix is crucial in reducing the impact of metals on forensic DNA analysis and that metal effects may persist during sample purification, resulting in less balanced STR profiles in the best case scenario.

5  |  CONCLUDING REMARKS

The intricate interactions between metals and DNA continue to pose various problems for DNA analysis. Therefore, it is essential to evaluate the effects of metals at various phases of the forensic DNA analysis workflow. In this study, we demonstrated that the level of metal inhibition on DNA quantification is matrix-dependent and correlated with the qPCR assay type and template quantity. Brass was shown to have the strongest tendency to interfere with amplification, with an IC$_{50}$ ranging from 0.03 to 0.05 mM, across both assays evaluated. Our results highlight the need for cautious interpretation of normal IPC $C_T$ results as suggestive of no inhibition when dealing with metal contaminants that make it through the extraction process. Higher levels of metal ions were necessary for the Quantifiler Trio IPC $C_T$ to be affected, so although the
inhibitory effect of metals can be observed on the target $C_T$ values and downstream profiling, the IPC $C_T$ can stay normal. Although typical magnetic bead-based extraction eliminates most metal impurities, inhibitors such as Fe(II) lower the extraction efficiency, perhaps by preferentially saturating the DNA binding sites of the magnetic beads, as suggested by this study. Lastly, metal-contaminated samples are more susceptible to STR profile imbalance. This work establishes a foundation for future research, with larger sample sizes and types, into the effects of metals and constraints for DNA purification and any additional cleaning procedures. Specifically, to answer questions regarding the metal ion concentration at which purification/cleanup becomes inefficient to the extent that carryover is observed at qPCR; and whether some purification/cleanup processes are more effective than others at removing metal inhibitors.

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**CONFLICT OF INTEREST STATEMENT**

The authors have declared no conflict of interest.

**DATA AVAILABILITY STATEMENT**

The data that supports the findings of this study are available in the supplementary material of this article.

**ORCID**

Dan Nana Osei Bonsu https://orcid.org/0000-0002-4671-0521

**REFERENCES**


SUPPORTING INFORMATION
Additional supporting information can be found online in the Supporting Information section at the end of this article.

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