Forensic trace DNA recovery and amplification from metal and metal-coated surfaces

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Thesis Abstract

Metals are problematic substrates of interest in frontline forensic practice due to difficulties in obtaining probative DNA evidence from common metal objects and surfaces that are routinely submitted for trace DNA analysis, such as cartridges, bullets, and casings. The low success of trace DNA recovery from metal substrates has been linked to their physicochemical nature, which can degrade DNA following deposition or act as inhibitory contaminants that interfere with PCR amplification. However, the mechanisms behind metal-DNA interactions and how this impacts the efficiency of trace DNA recovery and downstream processes are poorly understood.

The research described in this thesis examined trace DNA samples recovered from metal and metal-coated substrates in relation to typical forensic workflows from sample collection through to short tandem repeat profiling. The studies aimed to identify and characterise the negative effect of metal ions on DNA integrity, the collection and/or extraction of trace DNA samples, the co-purification of inhibitory factors with DNA, the interference of metal ions with quantitation, and how these ultimately impact DNA profiling.

Seven data chapters illustrate the importance of sampling techniques for the successful recovery of trace DNA from metal substrates. The Isohelix™ swabbing system was shown to be a more effective sampling tool than a Rayon swab. Depending on the chemistry of the qPCR assay, the DNA template input, and the type and quantity of metal ions in the PCR reaction, I observed non-patterned, complex interactions with unexpected DNA quantification results. Additionally, metal ions in qPCR caused direct inhibition or secondary interference of qPCR dye chemistry, leading to under and over-estimation of DNA concentration. I also show that metal-mediated inhibition/degradation of cellular DNA is matrix-dependent, paramagnetic DNA extraction may not be optimum for samples

contaminated with ferrous metals, and co-purified metal inhibitors can lead to an imbalance in STR profiles. When exposed to sunlight, self-cleaning metal-coated substrates, such as those coated with titanium dioxide, promote the photocatalytic destruction of trace DNA. Overall, this research highlights the importance of investigating novel trace DNA sampling and quantitation strategies, as well as more sensitive and robust amplification methods, while working with metal substrates.

Thesis Declaration

I, Dan Nana Osei Amponsah Mensah Bonsu, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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04 November 2022

Dan Nana Osei Amponsah Mensah Bonsu Date

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Chapter 1

General Introduction

In both their elemental and alloyed states, metals contribute significantly to the functioning of modern society. Metals have a wide range of applications from jewellery, machinery, tools, construction, electronics, cable networks, and transportation. Due to their widespread presence in the environment, metals are frequently encountered at crime scenes as structural components, personal accessories, coatings of another material (such as titanium dioxide coatings on floor tiles), or weapons used in the commission of the crime [1]. Recent years have seen an upsurge in crimes involving knives [2], firearms [3–5], volume and terrorism, as well as the theft of metal goods. Most likely, the increase in metal theft can be attributed to the COVID-19 pandemic [6] and the exorbitant price hikes for metals on the commodity market (36% higher in 2021 than the previous year) [7] . Theft of metals is a lucrative business for criminals [8,9]. For example, the nearly 7000 cases reported per month, compelled the enactment of the Scrap Metal Dealers Act [10] by UK government in response to the estimated annual economic loss of £770 million [11–13]. Similar legislation has been passed by the state [14,15] and federal [16] governments of Australia to combat metal theft. Surprisingly, the costs associated with the aftermath of metal thefts, such as protracted power outages [17], disruptions to rail traffic [13,18–20], school closures [21,22] and damage of prestigious statues and war memorials [23–25] often outweigh the value of the metal that was stolen. For example, in Droitwich Spa, United Kingdom, a copper pipe was stolen from a high school building and the associated costs were £250,000 [26]. However, the pipe's black market worth was merely £15 [26].

Recovery of trace biological evidence from metal substrates is becoming increasingly important due to the correlation between metals and weapons used in violent crimes and the rising occurrence of metal theft. At the scenes of hate crimes, homicides, and illegal wildlife poaching, knives and spent bullet casings are among the pieces of evidence that are found the most

frequently [27,28]. Copper wires that are discovered in improvised explosive devices (IEDs) that are utilised in terrorist attacks may also serve as a source of touch DNA. As an illustration, IEDs are frequently "reinforced" with materials like nails or metal bits to inflict maximal shrapnel damage, hence increasing the number of fatalities after detonation [29]. Metallic surfaces are said to be substrates that obstinately “refuse to reveal their secrets" [30,31], as such, there is a minimal likelihood of successfully recovering fingerprints and particularly trace/touch DNA from metal surfaces, according to the research [1].

Trace samples constitute at least 40 to 50% of biological samples evaluated at Australian forensic laboratories such as Forensic Science South Australia (FSSA) [32]. Trace DNA deposited at a scene typically originate from a person touching or wearing an object (touch or contact DNA). An intruder might, for instance, leave fingerprints on a metal window frame or door handle after a break-in. Likewise, when a gun is handled with bare hands, touch DNA may be left on the slide, trigger, or butt. In order to conceal their trail, criminals frequently wipe down their firearms, but are less likely to wipe the ammunition. Consequently, a suspect can be connected to a weapon or crime scene through successful profiling of touch DNA acquired from such fired or unfired ammunition [33,34].

The vast majority of trace samples contain very little DNA, making DNA profiling from them a difficult task (10-30% success rate) [35]. The chemistry of metal surfaces, such as hydration, oxidation, and weathering (ageing) over time through environmental exposure, present significant challenges for touch DNA recovery and amplification. The foregoing liberates ions on the metal surface, which facilitates strong metal-DNA interactions, making it difficult to release and recover bound DNA from the substrate [36]. Analysis of trace DNA on metal surfaces is also complicated by the inefficiencies of current sample recovery methods [1].

1.1 Metals, physicochemical properties, and DNA interaction

1.1.1 Environmental impact

Metal substrates, especially in outdoor locations, undergo deterioration induced by a complex interplay of various environmental factors, including contaminants and pollutants [37]. Humidity, temperature, and moisture can affect the degree of persistence and recovery of trace biomaterial deposited via contact, including touch DNA, from metal substrates. Relative humidity (RH), for instance, plays a significant role in open-air metal corrosion [38]. A thick electrolyte film that facilitates corrosion forms when a threshold RH of at least 60% is attained (at 20 ^{o}C) due to the reaction of moisture-saturated air, oxygen and metal's surface electrons. Additionally, in a polluted environment, a rise in ambient temperature can hasten metal deterioration by increasing chemical reactions on the surface. A prolonged period of metal surface wetness, together with an increased deposition rate of environmental contaminants/pollutants, further reduces the prospect of trace biomaterial recovery [26]. As an example, a firearm suspected of being used in a homicide in New South Wales was thrown in a storm drain. It was retrieved nine days later, after a period of heavy downpours. Apart from the protected inside surface of the plastic grip, the rusted metallic parts of the pistol yielded no touch DNA profile [1]. The pH of water from sources such as floods, rain, melting snow and condensation can be influenced by contaminants, including solid particles, microbes and dissolved substances like salts, chlorides, and carbon dioxide, which may exacerbate metal corrosion. Corrosion frees up metal ions contributing to the degradation of any persisting biomaterial, hence reducing the chance of recovery and DNA profiling of trace biological evidence. Therefore, the impact of specific environmental and climatic conditions is relevant to understanding trace DNA persistence and recovery from metal substrates.

1.1.2 Surface characteristics

The surface properties of a substrate influence the persistence and recovery of trace DNA. A foreign object may, for instance, generate abrasions, wear, and fatigue that alter the surface properties of a metal substrate. These modifications affect the persistence of biomaterials on such surfaces. Therefore, collecting trace/touch DNA from worn and rough-textured surfaces may be more difficult than from smooth (polished) surfaces [1]. A likely intricate metal-DNA interaction influenced by factors such as the composition of the metal surface or alloy, the texture and oxidation state of the surface mediates the binding and persistence of DNA on metal surfaces. While empirical data in forensic research on touch DNA recovery from metal surfaces is currently limited, an extensive study by the European Network of Forensic Science Institutes (ENFSI) provides invaluable insights into fingerprints' persistence and recovery from metal substrates [39]. This collaborative research involving many ENFSI member countries revealed low recovery rates of fingerprints on weathered metal surfaces due to the interaction of the corroded metal with especially the water-insoluble constituents of the fingerprints. Similar interactions are at play regarding DNA on metal surfaces and have been noted to result in low recovery rates, often in the range of 0 to 26% [40]. For instance, trace DNA on copper-containing substrates such as cartridges, bullets and casings (CBCs) are prone to degradation from direct contact with the metal surface [41]. Therefore, considering the surface type and condition is fundamental to understanding metal-trace biomaterial interactions and the impact on the persistence and effective recovery of DNA for forensic analysis.

1.1.3 Metal ions

Metal ions interact with DNA at different stages of the forensic analytical process, from extraction to PCR amplification, with a resultant adverse impact on DNA profiling [42–46]. The effect of this interference is contingent on the metal type and ion concentration involved. For instance, by inhibiting the DNA polymerases utilised in polymerase chain reactions (PCR), copurified metal ions may contribute to a low yield of PCR products. Metals have a wide spectrum of ionisation and electron affinities, allowing them to react with negatively charged molecules such as DNA. This affinity is mediated by the negative charge on DNA, with the phosphate backbone interacting with the metal cation [1]. The sequence-specific, strong DNA binding of lead (Pb) [47] and the at least three crosslinks that aluminium forms with DNA [48] are notable examples of metal ion - DNA interactions. Nickel (Ni) binds to DNA in a sequence and pH-specific manner [48], whereas copper preferentially binds to DNA bases [49].

Nevertheless, the exact mechanism underlying the influence of metal ions on DNA recovery, extraction, and amplification is poorly known at present. At crime scenes, the types of samples that may be contaminated with metal ions include swabs taken from metal substrates such as CBCs, weapons (e.g., guns and knives), metal wires and surfaces [50]. When obtaining DNA samples from a variety of metal substrates, it is not practically possible to exclude the possibility of contamination with metal ions. While the standard DNA extraction processes remove most of the inhibitory metal ions, the possibility of co-purification with the genetic material is well documented [51–53] and can negatively impact DNA analysis [54].

1.1.4 Metal-coated surfaces

Metals are also encountered in daily life and at crime scenes as coatings applied to metallic or non-metallic substrates. Coatings are typically applied to surfaces as a protective layer to reduce deterioration and/or for their aesthetic effects. For instance, molten zinc coating over steel (referred to as galvanised steel) provides a robust, tough, abrasion-resistant layer and cathodic protection to any small, damaged regions of the exposed steel substrate [55]. In addition, zinc-aluminium alloy coatings are utilised to give long-term corrosion protection to steel structures (e.g., rail tracks, steel bridge decks) exposed to harsh environments [56,57]. Due to their unique physicochemical properties and current (and potential) applications, the metal oxides of titanium and zinc appear to be the most relevant coatings (of non-metallic and metallic objects) of forensic research interest, particularly in the SARS-CoV-2 pandemic.

Titanium dioxide (titania, TiO_2) is a non-hazardous and inert material with numerous applications. Nanostructured coatings of titanium dioxide (TiO2) have, for instance, been utilised in products such as ceramic tiles [58], anti-fogging mirrors [59], pollutant-abating paints [60,61], concrete and asphalt [62–65]. Titania-coated surfaces possess the ability to eliminate dirt and microbial contaminations without the need for direct human intervention [66]. Through ultraviolet (UV) photocatalysis, titania efficiently absorbs UV light, the energy of which destroys organic molecules at their surfaces by the induction of oxidative stress [67]. The preceding process has been described as self-cleaning [68]. Titanium-coated glass has found essential indoor, and outdoor applications [60,67] and its use is expected to have an extended global reach [69]. The antimicrobial activity of the self-cleaning process can eliminate viruses that settle on such surfaces to curtail subsequent transmission, such as during the COVID-19 pandemic [70,71].

Similarly, zinc oxide (ZnO) possesses higher photocatalytic efficiency and is a subject of recent interest [72]. A UV-irradiated ZnO-coated substrate shows a phototoxic effect that has been proven to promote the generation of biologically essential reactive oxygen species (ROS) like superoxide ions (O^{2-}) and hydrogen peroxide (H_2O_2) [73]. The ROS generated are capable of intracellular penetration and consequently inhibiting or killing microbes [74] including influenza and coronavirus strains [75]. The antimicrobial activity of zinc ions and titania are now being harnessed for SARS-CoV-2 pandemic control by being embedded in fabrics to manufacture PPE, including facemasks [75–78]. Locard's principle, a fundamental tenet of forensic science, holds that every contact leaves a trace [79]. Self-cleaning and antimicrobial metal-coated surfaces have, however, not been studied in the context of this principle. An understanding of the impact of such substrates on the transfer, persistence, recovery, and amplification of contact DNA is thus vital to forensic science research.

1.1.5 DNA recovery from metal substrates

Trace samples potentially containing DNA are among the most difficult specimens to process. These samples are frequently limited in quantity, may be environmentally exposed, or located on substrates like metals that contain PCR-inhibitory substances, which may lead to the generation of blank or incomplete profiles [80]. The quality and quantity of DNA extracted from a forensic sample are directly correlated to the success of downstream analysis; thus, extensive cleaning procedures are often used to rid samples of inhibitors, despite the increased risk of DNA loss [81]. Currently, there is no consensus among practitioners and forensic laboratories regarding the most effective method for retrieving DNA from metals to aid in investigations. Despite this, standard efforts to develop methods on this topic have centred on five basic techniques: swabbing, tape lifting, soaking, vacuum filtering (also known as the Bardole method), and direct

PCR [1]. Except for direct PCR, a standard DNA extraction process is performed after sampling and before amplification. However, there are certain downsides to the recovery procedures. For instance, the deterioration of the critical grooves on CBCs after substrate soaking hinders DNA recovery, as does the increased leaching of metal ions into solution that causes nucleic acid degradation [82].

Similarly, direct PCR of swabbed trace samples has seen limited operational adoption in forensic laboratories due to the risk of contaminants, either extraneous or inherent to the swab, going straight into the amplification reaction without the sample clean-up. In many cases, the samples are so small that they cannot be tested more than once for a useful DNA profile, hence the need for optimal recovery methods. Swabbing, however, is the preferred method for recovering trace DNA from metal substrates since it is less expensive, simpler to use, and compatible with a variety of robotic extraction instruments [1,83].

1.2 Scope of thesis and data chapter summaries

This thesis aims to synthesise existing information and generate new data on the effects of different metals on the persistence, recovery, and amplification of DNA from metal surfaces. This knowledge will allow recommendations for developing novel solutions for forensic analysis of these challenging evidential samples. The outcome of this research will provide law enforcement and forensic practitioners with practical insight to enable the triage of metal exhibits and to improve the quality and quantity of DNA evidence recovered from such materials. This will facilitate improved analytical throughput for the recovery and DNA profiling of trace biological evidence recovered from problematic metal surfaces during forensic examinations.

The first study (Chapter 2) is a comprehensive review that brings together a range of scientific literature sources to examine the effect of metal surfaces on DNA. This study examines the rudiments of metal-DNA interactions and touch DNA persistence on forensically significant metal surfaces, as well as their impact on collecting enough DNA for successful forensic profiling. It addresses the advantages and limitations of present technologies, the impact of metal surface properties, and methods for improving touch DNA recovery and amplification from metal surfaces.

The second study (Chapter 3) investigates DNA recovery from various metal surfaces using different swab types and wetting solutions. This ‘proof-of-concept’ study, utilising purified DNA, demonstrates that the Isohelix™ swab moistened with isopropyl alcohol is more efficient than Rayon swabs wetted with sterile water for recovering and amplifying DNA from metal surfaces. It underscores the need for further testing with “real-world” trace biological samples to ascertain the actual performance of the swabs in a potential casework scenario.

Chapter 4 verifies the findings of Chapter 3 by using the same swabbing methods on frequently touched metal surfaces in a building, thus simulating a real-world scenario. The results match those of the 'proof-of-concept,' and demonstrates that extra cleaning measures implemented during a pandemic are likely to affect the persistence and recovery of touch DNA from metal substrates.

As Chapters 3 and 4 examine trace DNA sample recovery from metal substrates, Chapter 5 takes a closer look at the impact of selected metal ions on DNA extraction, real-time PCR quantitation and STR profiling efficiencies. This study demonstrates that the sample type (cellular or acellular), type of metal, chemistry of the qPCR assay and amount of template are important factors to the extent of metal ion-mediated inhibition and degradation of DNA.

Chapter 6 reports on a unique discovery that the presence of tin (Sn) ions causes the quenching of the fluorescence of the passive reference dye in the Quantifiler™ Trio DNA quantification Kit, resulting in a template overestimation of at least 30,000-fold.

Chapter 7 provides a comprehensive analysis of whether copper, zinc, or their brass alloy is responsible for the detrimental effects on DNA integrity observed in trace samples collected from CBCs. This study reveals that the higher inhibition/degradation potency of brass is connected to a synergistic interaction between the two metals, which is primarily driven by zinc.

The work discussed in Chapter 8 investigates how photocatalysis affects the persistence of trace DNA on self-cleaning glasses. This study is the first to demonstrate substantial deterioration of trace biomaterial on titania coated substrates after exposure to sunlight, and it also looks at the implications for crime scene analysis.

The final study in chapter 9 examines data from original research articles published in six of the top forensic science journals over a ten-year period. This reveals how frequently ethical approval and informed consent are mentioned in manuscripts involving humans or animals subjects. During the COVID-19 pandemic lockdowns, when I could not access labs to continue working on my experiments, I conducted this study as a stopgap. This study highlights the need for forensic science research to improve the surprisingly low level of ethics reporting.

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Chapter 2

Forensic touch DNA recovery from metal surfaces – A review

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Chapter 3

Evaluation of the efficiency of Isohelix™ and Rayon swabs for recovery of DNA from metal surfaces

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Chapter 4

Comparison of Isohelix™ and Rayon swabbing systems for touch DNA recovery from metal surfaces

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Chapter 5

Metal ions disrupt paramagnetic extraction, quantitative PCR and STR profiling, but their degradation of cellular DNA is matrix-dependent.

Bonsu DOM, Higgins D, Austin JJ.

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Chapter 5

Metal ions disrupt paramagnetic extraction, quantitative PCR and STR profiling, but their degradation of cellular DNA is matrix-dependent.

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 PCR amplification or both. This study evaluated the impact of metal ions on DNA extraction, quantitation, and STR profiling using cell-free and cellular (saliva) DNA. Of the eleven metals assessed, brass exhibited the strongest PCR inhibitory effects, for both custom and Quantifiler™ Trio quantitation assays. Metal ion inhibition varied across the two qPCR assays and the amount of DNA template used. The Quantifiler™ Trio internal PCR control 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 dilution of the sample, suggesting that the saliva matrix provides protection from metal ion effects.

Keywords

Forensic Science, DNA analysis, quantitative PCR, Metal ions, Quantifiler Trio

5.1 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 PCR (qPCR) and 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 inhibition of the polymerase, and interaction with target DNA, internal positive control (IPC) or the passive reference dye can cause over- or under-estimation of DNA concentration [7,12] leading to sub-optimal DNA input into subsequent STR profiling reactions.

The impact of metals on DNA quantitation has been evaluated using custom and commercially-available qPCR assays. 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) [13], InnoQuant® HY (InnoGenomics Technologies) [14], PowerQuant® System (Promega Corporation) [15] and Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific) [16] 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™ [13–15,17]. The passive reference dye is required for normalisation between sample wells since its fluorescence is unaffected by the amplification cycles [18]. 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 [19]. 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 [20]. 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 [21,22]. The quality data enables the prediction of STR typing success and provides a streamlined and efficient forensic analysis workflow [22]. 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 [16,21,22]. The test sample is amplified, and a degradation index (DI) is calculated from the resulting SA and LA target DNA concentrations [16,23,24]. 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 [25]. 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., [7,12]) 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 [10,26] and DNA profiling [22]. 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.

5.2 Materials and Methods

5.2.1 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 [27,28]. Tin is regularly employed in food packaging and beverage containers [29], and copper is a significant component of most ammunition and improvised explosive devices encountered at crime scenes [8,28,30]. 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 [31]. Lead is one of the main elements of gunshot residue (GSR) frequently found on surfaces of discharged firearms and/or cartridges, bullets, and casings (CBCs) [32] potentially harbouring touch DNA. Hard tissues (teeth and bones) are sources of calcium, while aluminium and brass are frequently used for door/window frames and knobs and household items, among others [33]. 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. Detailed examination of the impacts of brass, copper and zinc, and tin are described in Chapters 7 and 6, respectively. They are included here to enable comparisons of inhibition and DNA damage across a broad range of metals.

5.2.2 Metal ions

Stock solutions (50 mM) of ten 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 sulfate hydrate (≥99.99% trace metal basis)(Al), calcium chloride (anhydrous, powder ≥97%) (Ca), chromium (III) chloride hexahydrate (purum p.a ≥98.0% (RT)) (Cr), copper (II) sulfate (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) sulfate hydrate (99.999% trace metals basis) (Fe (II)), nickel (II) sulfate 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.

5.2.3 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 ng 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 ng and 0.2 ng of template DNA were used as these are the optimal input amount for GlobalFiler STR profiling as validated by FSSA 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 were directly added to the PCR reaction mixture using an automated Tecan™ Liquid Handling Platform (LHP) (Tecan Group Ltd., Männedorf, Switzerland), and the selected concentrations were based on previous research [7,12]. For example, a previous study reported inhibition (IC_{50}) values between 0.26 to 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 case-work samples following DNA extraction [34].

5.2.4 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 extracted using the DNA IQ™ system (Promega, Madison, WA, USA) on a Hamilton AutoLys liquid handling platform 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 x 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.

5.2.5 Matrix effect on metal ion DNA damage and PCR inhibition

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 IC_{50} (brass, chromium, copper, iron (II), tin and zinc). 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 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 small autosomal (SA) target yield, degradation index (DI) and the cycle threshold (C_T) of the SA and IPC data. STR profiling of the extracts was performed using the GlobalFiler™ PCR amplification kit as previously described [33].

5.2.6 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.

5.2.6.1 Custom assay

The custom assay (in-house) qPCR assay using SYBR green chemistry targeting a small (67 bp) (Forward: GGGCAGTGTTCCAACCTGAGGAAA ACT; reverse: GAGACACAGGGTGGTTA) human-specific nuclear DNA amplicon was performed as previously described [35] on a QuantStudio™ 6 Flex Real-Time PCR System (Thermofisher Scientific). The 10 µL reaction volumes consisted of 1X Brilliant III Ultra-Fast SYBR Green Low ROX qPCR Master Mix (Agilent Technologies, USA), 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 ºC for 20 s, and 72 ºC for 15 s. DNA concentration was determined using the comparative C_T 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 [35]. 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.

5.2.6.2 Quantifiler Trio™ assay

Quantification with the commercial kit employed Quantifiler Trio™ DNA Quantification Kit (Thermo Fisher Scientific, Waltham, MA, USA) on an Applied Biosystems 7500 Real-Time PCR System with the HID Real-time PCR Analysis software v1.2 (ThermoFisher 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 and 1 µL of DNA template. 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.

5.2.6.3 DNA Profiling

Short tandem repeat (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).

5.2.7 Data analysis

DNA quantity was estimated using the small autosomal (SA) DNA yield. DNA quality was assessed using the degradation index (DI), calculated as the ratio of the small to the large autosomal target concentrations. A degradation value of < 1 indicates no degradation, 1–10 indicates slight to moderate degradation, and > 10 indicates severe degradation. Samples where no large autosomal target was amplified were considered severely degraded. PCR inhibition was assessed using the internal PCR control (IPC) cycle threshold (C_T) values, with values above 30 indicating inhibition.

The level of PCR inhibition by metal ions was determined using the small autosomal (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 (IC_{50}) 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, San Diego, California, USA). 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 [36]. 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 $p < 0.05$.

5.2.8 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 (NHMRC) National Statement of Ethical Conduct in Human Research [37]. In addition, written informed consent was obtained from the donor of the saliva samples.

5.3 Results

5.3.1 Impacts of metal ions on qPCR estimation of DNA concentration

5.3.1.1 Custom Assay

All ten 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 IC_{50} results show that brass, nickel, chromium, lead and zinc, were the strongest PCR inhibitors (IC_{50} 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. DNA input amount influenced metal ion inhibitory effects. For all metals, the IC_{50} was 1.6-23 times higher for 0.5 ng, compared to 0.2 ng of input DNA, suggesting synergistic effects between the amount of DNA in the reaction and the level of metal ion inhibition.

Table 1: IC_{50} values for qPCR inhibition for two DNA template input amounts (0.5 ng and 0.2 ng) for an in-house assay utilising SYBR green chemistry and ROX as the passive reference.

Metal	IC_{50} (0.5 ng) ± SD (mM)	IC_{50} (0.2 ng) ± SD (mM)
Brass	0.050 ± 0.042	0.029 ± 0.015
Ni	0.058 ± 0.018	0.036 ± 0.025
Cr	0.064 ± 0.020	0.038 ± 0.022
Pb	0.064 ± 0.018	0.029 ± 0.030
Zn	0.099 ± 0.045	0.041 ± 0.020
Al	0.124 ± 0.031	0.030 ± 0.028
Fe (III)	0.437 ± 0.037	0.144 ± 0.024
Cu	0.497 ± 0.044	0.099 ± 0.013
Ca	1.302 ± 0.064	0.055 ± 0.020
Sn	1.541 ± 0.030	0.914 ± 0.021
Fe (II)	1.671 ± 0.037	0.631 ± 0.021

SD = standard deviation

5.3.1.2 Quantifiler™ Trio assay

The ten 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 (Fig. 1). The IC_{50} results show that brass, chromium, zinc, and aluminium were stronger PCR inhibitors (IC_{50} values less than or equal to 0.1 mM, Table 2), while calcium, copper and nickel were the least inhibitory. As with the custom assay, the inhibitory effect of metal ions (as measured by the IC_{50}) was influenced by DNA input amount - IC_{50} 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_{50}.

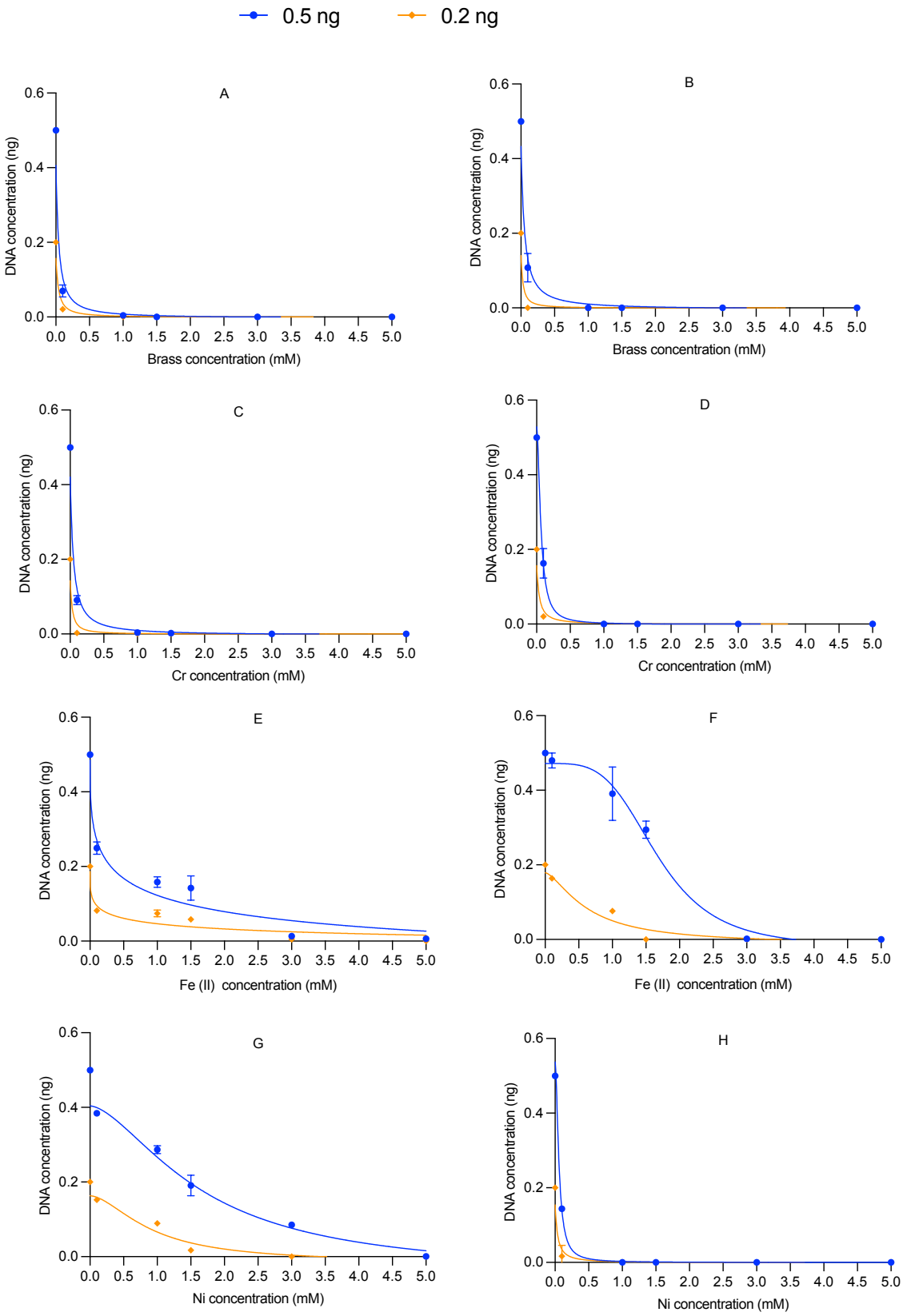
Table 2: IC_{50} values for qPCR inhibition for two DNA template input amounts (0.5 ng and 0.2 ng) for Quantifiler™ Trio assay utilising TaqMan chemistry and Mustang Purple as passive reference.

Metal	IC_{50} (0.5 ng) ± SD (mM)	IC_{50} (0.2 ng) ± SD (mM)
Brass	0.042 ± 0.017	0.028 ± 0.021
Sn	***	***
Cr	0.046 ± 0.020	0.020 ± 0.013
Zn	0.062 ± 0.03	0.027 ± 0.012
Al	0.101 ± 0.020	0.053 ± 0.011
Pb	0.388 ± 0.038	0.205 ± 0.007
Fe (II)	0.498 ± 0.044	0.313 ± 0.023
Fe (III)	0.794 ± 0.061	0.219 ± 0.015
Ni	1.660 ± 0.048	0.911 ± 0.023
Cu	1.807 ± 0.073	0.280 ± 0.014
Ca	2.584 ± 0.035	1.879 ± 0.010

SD = standard deviation *** could not be estimated

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 enhanced inhibition potential than brass at 0.2 ng template (IC_{50}: 0.02 ± 0.01 mM vs 0.03 ± 0.02 mM, respectively), while 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 (Fig. 1, Table 1 and Table 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.



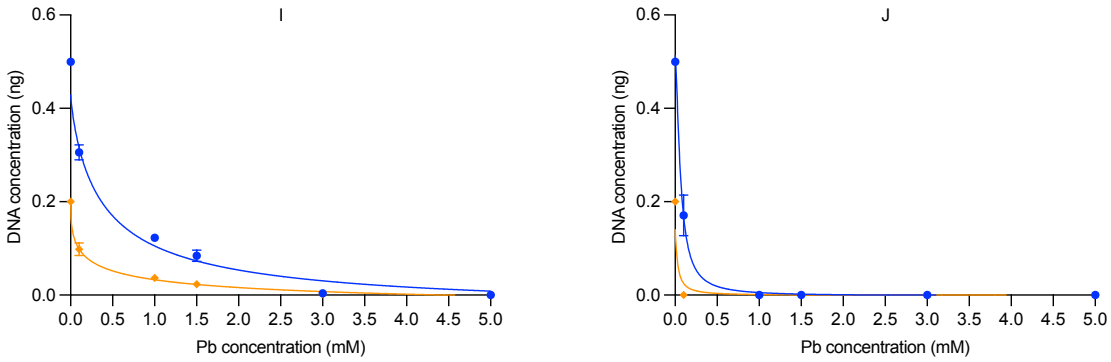


Fig 1. Representative inhibition (IC_{50}) data of Quantifiler™ Trio assay (left panel) versus custom assay (right panel) by brass (A vs B), Cr (C vs D), Fe (II) (E vs F), Ni (G vs H) and Pb (I vs J) with 0.5 ng and 0.2 ng DNA input.

5.3.1.3 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 (Fig. 2). Aluminium had no impact on the IPC C_T which stayed within the typical Quantifiler™ Trio threshold of 20 – 30 (Fig. 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 5mM (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 adverse downstream effects. Fe (II) had almost no impact on the IPC C_T, whilst 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 C_T (20), irrespective of the amount of template DNA (Fig. 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)), 3mM (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.

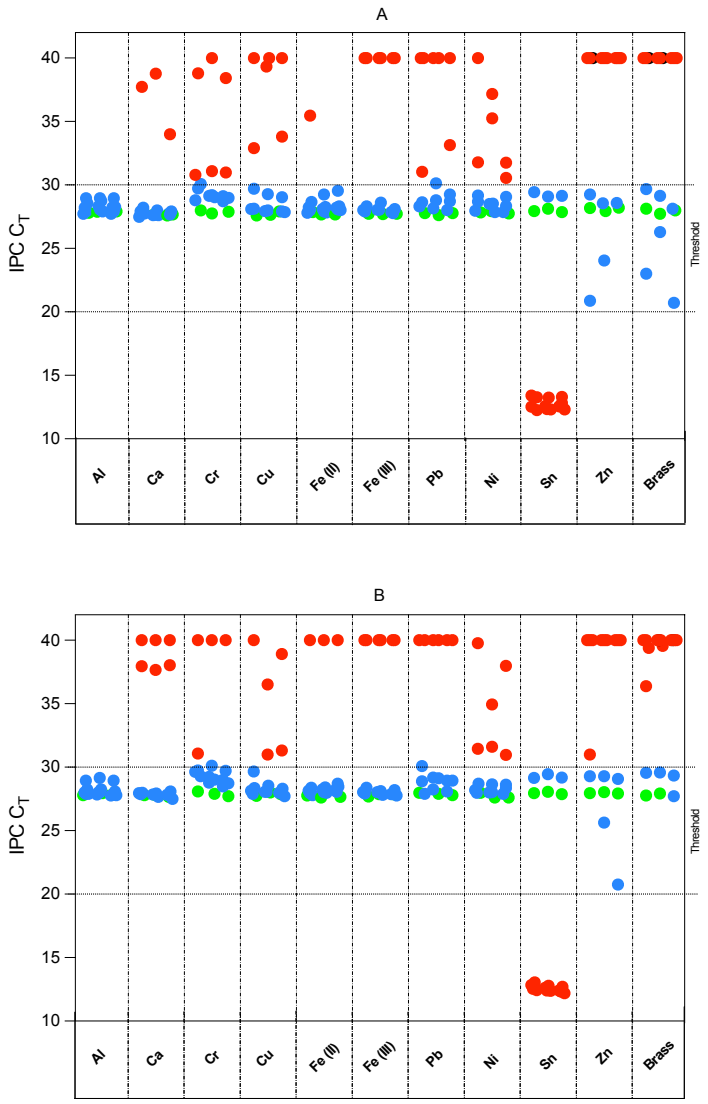


Fig 2. Quantifiler Trio of IPC C_T results for different DNA input amounts (A: 0.5 ng, B: 0.2 ng) for 10 metal ions and brass. Three quantification runs per treatment. Green dots: control DNA sample with no metal ions added; blue and red dots: DNA sample with 0.1, 1, 1.5, 3 or 5 mM metal ion. The expected IPC C_T threshold for Quantifiler™ Trio of 20 - 30 is indicated. Red dots indicate IPC C_T values significantly higher (> 30) or lower (< 20) than the threshold.

5.3.2 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.

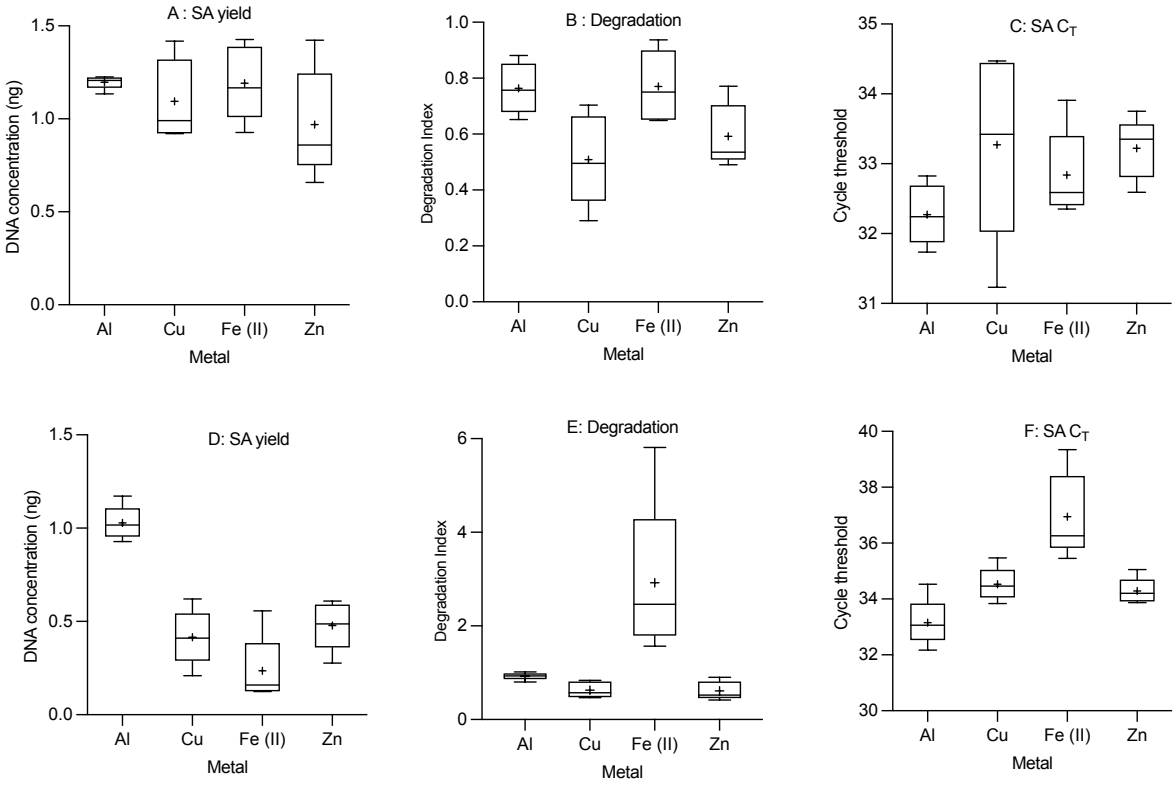


Fig 3. Autosomal DNA (SA) yield, degradation index and SA cycle threshold (SA C_T) of DNA samples (3 µL of 0.5 ng/µL) treated with 3 µL of 1 mM (top panel, A, B and C) and 5 mM (bottom panel, D, E and F) of Al, Cu, Fe (II) and Zn metals, (n = 5). Mean values represented by '+'.

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) (Fig 3A). 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 (Fig 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 (Fig 3E), while all others for both metal concentrations

were within the range (DI ≤ 1) of undegraded DNA (Fig 3B and Fig 3E). In all instances, irrespective of metal ion concentration, IPC C_T was within the expected range (~ 27 - 28).

5.3.3 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 (Fig. 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 dilution). The average DI was ≤ 1, and no significant differences in the values between the three saliva samples (Fig. 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, while the IPC data for all metal-treated samples were not different from the non-treated sample (Supplementary Table 1), the SA C_T (Fig 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 Fig. 4A).

For neat saliva samples treated with metal ions, the DNA yield (range 1.0 ng to 1.2 ng) was not significantly different to the untreated control sample for all metals tested except for Fe (II) which averaged 0.8 ng (Fig. 4A). The average DI value was below 2.5 for metal ions (Fig. 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 (Fig. 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 (Fig. 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 long autosomal (LA) target (Supplementary Table 1). 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 Fig. 4B) but none in Cr-treated samples (asterisked space in Fig 4B) indicating either severe DNA degradation or PCR inhibition [24].

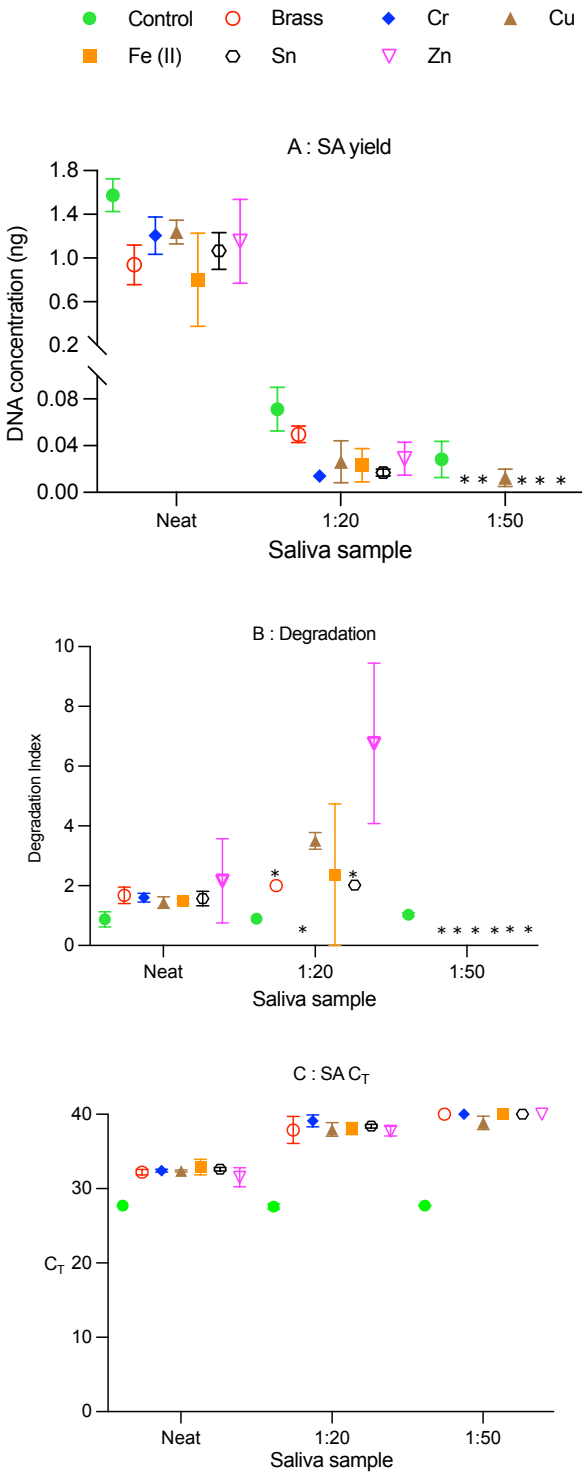


Fig 4. Effect of sample matrix on (A) autosomal DNA yield, (B) degradation and (A) SA C_T of neat, 1:20 and 1:50 saliva samples treated with brass, Cr, Cu, Fe (II), Sn and Zn.

Apart from two replicates of Cu-spiked samples that gave a detectable SA target signal (Supplementary Table 1) but failed to give a signal for the large autosomal (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 Fig 4A).

5.3.3.1 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.

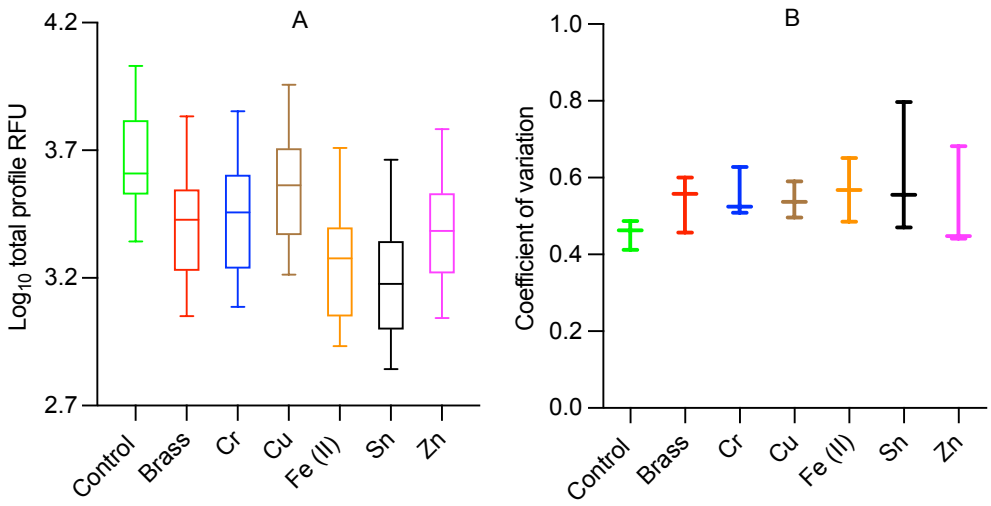


Fig 5. Comparison of GlobalFiler profile data for neat saliva samples treated with 5 mM metal (n = 3) and extracted using DNA IQ. (A) Total profile RFU and (B) profile balance (coefficient of variation of profile RFU).

For neat saliva samples the average STR peak height was highest for the non-treated saliva samples (5383.95 ± 3119.92) compared to saliva spiked with metal-ions, and significantly different from all but Cu-treated samples (4100.08 ± 2445.43, p = 0.3240, Table 3, Fig. 5). The lowest average RFU was obtained for Sn (2028.30 ± 1407.48) and Fe (II) (2106.23 ±

1194.37) spiked samples (Table 3, Fig. 5). Profile imbalance was lowest in the control samples and highest in Fe (II) and Sn treated samples (Table 3, Fig. 5). Despite the lower average peak height RFU and worse profile balance, all metal-ion treated samples returned full STR profiles that were 100% concordant with the untreated control sample.

Table 3. Summary of quantitative STR data for metal-treated neat saliva samples

Sample	Peak Height (RFU) ± SD	Profile Balance (CoV ± SD)	STR Alleles Detected
Control (neat saliva)	5383 ± 3119	0.454 ± 0.038	100%
Brass	2938 ± 1679	0.538 ± 0.074	100%
Cr	3347 ± 2366	0.554 ± 0.065	100%
Cu	4100 ± 2445	0.541 ± 0.047	100%
Fe (II)	2106 ± 1194	0.568 ± 0.083	100%
Sn	2028 ± 1407	0.608 ± 0.170	100%
Zn	3130 ± 2175	0.524 ± 0.137	100%

5.4 Discussion

5.4.1 Impacts of metal ions on qPCR estimation of DNA concentration

Real-time quantitative PCR (qPCR) 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 [38]. Metals have been demonstrated to exhibit a 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 [7,12,39]. 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 causes 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, etc., used in the commission of crime [8] and commonly submitted to forensic science laboratories for DNA evidence recovery [40]. 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. [12] 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 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 to 2 ng [41] in contrast to the 0.1 ng of DNA input per reaction used by Combs et al. [12]. 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 (Fig 1 E vs F). The custom assay includes Bovine Serum Albumin (BSA), which is known to chelate metal contaminants [42], hence requiring more Fe (II) ions to cause inhibition.

5.4.2 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 [12]. 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 [43]. 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 [44]. The foregoing effect may be implicated in interference observed for samples treated with Sn ions where IPC C_T values were at least lowered with increasing Sn concentration compared to the average of controls (Fig 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 (Fig 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.

5.4.3 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 (Fig 3). For example, when enough contaminants (5 mM) were present in the sample, less than one-third of the DNA was recovered (Fig 3D). This was notable in Fe (II)-treated samples and corresponded with increased degradation (Fig 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. Consequently, more DNA goes to waste via the repeated binding/washing steps (decreased recovery) with an enhanced prospect of co-extraction of inhibitors that may facilitate degradation and/or adversely impact subsequent PCR reactions. Thus, paramagnetic bead-based extraction techniques may not be the ideal sample purification method for samples potentially contaminated with ferrous ions.

In a study by Akhidime et al. [45], the concentration of metal ions that leached off different metal surfaces, including Cu, Fe and Zn, was assessed. 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 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 consistently high SA C_T data (Fig 3C and Fig 3F) 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 affected. The kit, however, provides data on the DI, which is probably more helpful than the IPC C_T in identifying inhibition as there is preferential amplification of the SA target than the LA target with inhibited samples, as observed for Fe (II)-treated samples.

5.4.4 Matrix effect on metal ion inhibition and STR genotyping

The impact of the sample matrix is critical to the success of the analytical process [46]. As such evaluating inhibitor interactions with the matrix in the analysis of forensically relevant biological samples provides insight into method limitations, troubleshooting strategies and/or novel approaches to detect and mitigate contaminants co-purified with the samples. In general, the success of DNA recovery decreased with increasing saliva dilution for all metals tested, compared to the untreated saliva samples (Fig 4). For instance, DNA recovery was higher in the neat saliva solution despite the presence of an inhibitor (Fig 4A). This is because in its encapsulated form in nucleated cells, the DNA benefits from a protective barrier by being enclosed by the cell and nuclear structure and tightly bound to the histones [47,48]. Metals typically chelate cellular proteins [49] and phospholipid bilayers [50,51], ensuring nucleic acid from the lysed buccal cells preferentially binds to the magnetic resin while other constituents of saliva matrix interact with the interferent.

The dilution effect reduces the matrix in the sample, enhancing metal ions interaction and potential co-elution with the DNA. As previously noticed, extraction may allow any co-sampled metal ions to interact with the DNA during the extraction process, whereas direct PCR avoided this partially [52]. Thus, while the DNA extraction process removes more impurities, the DNA response is less in the diluted samples due to the reduced pre-extraction matrix, and the effect (increased inhibition and degradation) is noticeable from the higher DI (Fig 4B) and SA C_T (Fig 4C) values. Further, the IPC C_T remained normal in all instances of non-amplification of the LA targets (Fig. 4A). These results suggest that while the inhibition potential of metals on cellular DNA is matrix dependent, interferent levels that cause detectable inhibition/degradation may not necessarily be optimal to effect an inhibitory IPC C_T response. As such, when dealing with samples recovered from metal substrates, the

determination of a 'true negative' should not be solely based on the failure of the IPC to indicate the presence of inhibitors. Amplification of all samples may thus be warranted regardless of the quantification results.

5.4.5 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. 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. Regardless, samples with intact matrix showed higher variability in profile balance, with Sn and Fe (II) treated samples exhibiting the worst interlocus balance (Fig. 5B). These findings 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.

5.5 Conclusions

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. Whereas 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 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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Supplementary Table 1: Saliva samples spiked with metal ions

	Neat saliva + metal					
	SA (ng/µL)	**LA (ng/µL)**	**CT_{SA}**	**IPC C_T**	**DI**	**SA (ng)**
	0.0122	0.0083	32.61	27.83	1.47	0.73
Brass	0.0171	0.0086	31.89	27.40	2.00	1.02
	0.0177	0.0112	32.16	27.56	1.57	1.06
Mean	**0.0156**	**0.0094**	**32.22**	**27.60**	**1.68**	**0.94**
SD	**0.0030**	**0.0016**	**0.36**	**0.21**	**0.28**	**0.18**
	0.0185	0.0104	32.53	27.59	1.77	1.11
Cr	0.0234	0.0154	32.20	27.59	1.52	1.40
	0.0184	0.0121	32.54	27.56	1.52	1.10
Mean	**0.0201**	**0.0127**	**32.42**	**27.58**	**1.60**	**1.21**
SD	**0.0028**	**0.0025**	**0.20**	**0.02**	**0.15**	**0.17**
	0.0191	0.0135	32.49	27.47	1.41	1.15
Cu	0.0226	0.0138	32.24	27.59	1.64	1.36
	0.0202	0.0160	32.41	27.51	1.26	1.21
Mean	**0.0206**	**0.0144**	**32.38**	**27.52**	**1.44**	**1.24**
SD	**0.0018**	**0.0014**	**0.12**	**0.06**	**0.19**	**0.11**
	0.0204	0.0146	32.39	27.92	1.40	1.23
Fe (II)	0.0062	0.0040	34.11	28.36	1.56	0.37
	0.0134	0.0091	32.19	27.63	1.47	0.80
Mean	**0.0134**	**0.0092**	**32.90**	**27.97**	**1.48**	**0.80**
SD	**0.0071**	**0.0053**	**1.05**	**0.37**	**0.08**	**0.43**
	0.0149	0.0096	32.85	27.52	1.55	0.89
Sn	0.0179	0.0134	32.58	27.79	1.33	1.07
	0.0205	0.0113	32.39	27.71	1.82	1.23
Mean	**0.0178**	**0.0114**	**32.61**	**27.67**	**1.57**	**1.07**
SD	**0.0028**	**0.0019**	**0.23**	**0.14**	**0.24**	**0.17**
	0.0254	0.0182	32.08	27.51	1.39	1.52
Zn	0.0126	0.0033	30.06	26.01	3.79	0.76
	0.0197	0.0150	32.45	27.47	1.31	1.18
Mean	**0.0192**	**0.0122**	**31.53**	**26.99**	**2.16**	**1.15**
SD	**0.0064**	**0.0079**	**1.28**	**0.85**	**1.41**	**0.38**
	0.0258	0.0240	27.24	27.86	1.08	1.55
Control	0.0289	0.0493	27.43	27.65	0.59	1.73
	0.0240	0.0249	27.06	27.60	0.96	1.44
Mean	**0.0262**	**0.0327**	**27.24**	**27.70**	**0.88**	**1.57**
SD	**0.0025**	**0.0144**	**0.18**	**0.14**	**0.26**	**0.15**

	1 in 20 saliva dilution + metal					
	SA (ng/µL)	**LA (ng/µL)**	**CT_{SA}**	**IPC C_T**	**DI**	**SA (ng)**
Brass	UNDET	UNDET	UNDET	27.73		UNDET
	0.0009	UNDET	36.70	27.68		0.055
	0.0007	0.0004	36.99	27.70	2.00	0.045
Mean	**0.0008**		**36.85**	**27.70**		**0.050**
SD	**0.0001**		**0.20**	**0.03**		**0.007**
Cr	UNDET	UNDET	UNDET	27.53		UNDET
	0.0002	UNDET	38.85	27.40		0.012
	0.0003	UNDET	38.49	27.52		0.016
Mean	**0.0002**		**38.67**	**27.48**		**0.014**
SD	**0.0000**		**0.25**	**0.08**		**0.002**
Cu	0.0008	0.0002	36.92	27.65	3.20	0.047
	0.0003	0.0001	38.31	27.55	3.54	0.018
	0.0002	0.0001	38.66	27.74	3.76	0.014
Mean	**0.0004**	**0.0001**	**37.96**	**27.65**	**3.50**	**0.026**
SD	**0.0003**	**0.0001**	**0.92**	**0.09**	**0.28**	**0.018**
Fe (II)	0.0007	0.0001	37.16	27.54	5.10	0.040
	0.0003	0.0003	38.54	27.65	0.82	0.015
	0.0003	0.0002	38.56	27.57	1.11	0.015
Mean	**0.0004**	**0.0002**	**38.09**	**27.59**	**2.35**	**0.023**
SD	**0.0002**	**0.0001**	**0.80**	**0.05**	**2.39**	**0.014**
Sn	0.0002	UNDET	38.63	27.47		0.014
	0.0003	0.0001	38.44	27.62	2.02	0.016
	0.0003	UNDET	38.14	27.38		0.020
Mean	**0.0003**		**38.40**	**27.49**		**0.017**
SD	**0.0000**		**0.25**	**0.12**		**0.003**
Zn	0.0008	0.0001	36.98	27.41	9.53	0.045
	0.0003	0.0001	38.08	27.66	6.59	0.021
	0.0003	0.0001	38.12	27.76	4.16	0.020
Mean	**0.0005**	**0.0001**	**37.73**	**27.61**	**6.76**	**0.029**
SD	**0.0002**	**0.00002**	**0.65**	**0.18**	**2.68**	**0.014**
Control	0.0010	0.0010	27.65	27.80	1.03	0.060
	0.0015	0.0018	27.71	27.19	0.85	0.093
	0.0010	0.0013	27.86	27.78	0.79	0.061
Mean	**0.0012**	**0.0014**	**27.74**	**27.59**	**0.89**	**0.071**
SD	**0.0003**	**0.0004**	**0.10**	**0.35**	**0.13**	**0.019**

	1 in 50 saliva dilution + metal					
	SA (ng/µL)	**LA (ng/µL)**	**CT_{SA}**	**IPC C_T**	**DI**	**SA (ng)**
Brass	UNDET	UNDET	UNDET	27.67		
	UNDET	UNDET	UNDET	27.81		
	UNDET	UNDET	UNDET	27.72		
Mean						
SD						
Cr	UNDET	UNDET	UNDET	27.52		
	UNDET	UNDET	UNDET	27.72		
	UNDET	UNDET	UNDET	27.56		
Mean						
SD						
Cu	0.0003	UNDET	38.2029	27.65		0.018
	UNDET	UNDET	UNDET	UNDET		
	0.0001	UNDET	39.4715	27.81		0.007
Mean	**0.0002**		**38.8372**	**27.73**		**0.012**
SD	**0.0001**		**0.8970**	**0.11**		**0.007**
Fe (II)	UNDET	UNDET	UNDET	27.59		
	UNDET	UNDET	UNDET	27.78		
	UNDET	UNDET	UNDET	27.65		
Mean						
SD						
Sn	UNDET	UNDET	UNDET	27.54		
	UNDET	UNDET	UNDET	27.52		
	UNDET	UNDET	UNDET	27.47		
Mean						
SD						
Zn	UNDET	UNDET	UNDET	27.54		
	UNDET	UNDET	UNDET	27.60		
	UNDET	UNDET	UNDET	27.76		
Mean						
SD						
Control	0.0007	0.0008	26.74	27.78	0.99	0.04
	0.0004	0.0004	27.34	27.69	1.00	0.03
	0.0002	0.0002	27.43	27.71	1.11	0.01
Mean	**0.0005**	**0.0005**	**27.17**	**27.73**	**1.03**	**0.03**
SD	**0.0003**	**0.0003**	**0.37**	**0.05**	**0.06**	**0.02**

Chapter 6

Real-time qPCR overestimation of DNA in samples contaminated with tin.

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Contribution to the Paper	Conceived the study, designed experiments, collected, analysed and interpreted data, drafted manuscript and produced figures.			
Overall percentage (%)	70%			
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.			
Signature			Date	1 November 2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 6

Real-time qPCR overestimation of DNA in samples contaminated with tin

Abstract

Metals are problematic substrates in forensic DNA analysis, mainly because metal ions in crime-scene DNA extracts can degrade DNA or inhibit qPCR-based DNA quantitation leading to low STR profiling success. As part of a broader study of metal-ion interaction with human DNA, we identified a contradictory example where tin ions led to at least a 38,000-fold overestimation of DNA concentration when utilising the Quantifiler Trio™ DNA Quantification Kit. We did not observe this effect when DNA was quantified using an in-house qPCR assay based on SYBR green and utilising ROX™ as the passive reference, nor when DNA was extracted and purified prior to Quantifiler Trio™ quantitation. We show that Sn suppresses the passive reference dye (Mustang Purple, MP) fluorescence at concentrations above 0.1 mM when utilising the Quantifiler Trio™ DNA Quantification Kit leading to massive overestimation of DNA concentrations. Our results show that metal contaminants can disrupt qPCR-based DNA quantitation in unexpected ways and may be assay dependent. Forensic workflows that involve direct PCR or lysis only extraction should recognise the risk of inaccurate DNA quantitation in DNA samples that are collected from metal objects containing tin.

Keywords

Tin, Fluorescence quenching, Passive reference dye, PCR Inhibition, Quantifiler Trio™, qPCR

6.1 Introduction

DNA testing workflows in operational forensic laboratories include a DNA quantification step, following DNA extraction, to ensure optimal DNA input into downstream analyses. Typically, quantitative real-time PCR (qPCR) is used to estimate the amount of DNA present based on a short autosomal amplicon (about 80 bp) and a larger autosomal amplicon (typically 200 - 300bp), the latter used mainly as an indicator of DNA degradation. In addition, modern qPCR kits include an internal PCR control (IPC) to detect the presence of PCR inhibitors [1]. Together, the resultant data provides insight into DNA quality and quantity and how much DNA extract to forward to STR profiling [2]. Hence, accurate quantitation is vital in forensic DNA testing. However, qPCR analysis may be impacted by the presence of contaminants in samples that causes amplification or detection inhibition [1,3,4].

The impact of contaminants such as humic acid [3,5], fulvic and tannic acid [6–8], haematin [9], residual dithiothreitol (DTT) [10,11] and other environmental contaminants [12] on qPCR and STR typing are well-characterised. Metal ions are common contaminants in samples collected from metal objects [13,14]. The effects of metal ions in crime-scene DNA extracts on STR profiling success, and qPCR-based DNA quantitation are not fully understood. For example, DNA extracted from a fired cartridge case sample, and analysed with the Quantifiler™ Trio (Thermofisher) DNA Quantitation kit, yielded an undetectable DNA result with no evidence of inhibition by the internal PCR control [15]. Typically, such a result would indicate that the sample lacked amplifiable DNA [16] but a nearly complete DNA profile was generated in this instance. In contrast, Combs et al. [17] found that 1.5 mM copper (Cu) caused about a 100-fold apparent increase in DNA concentration using the Quantifiler™ Human DNA Quantification Kit (Thermofisher Scientific). Kuffel et al. [18] did not observe an overestimation of DNA concentration in the presence of copper

contamination when utilising an in-house assay. These outcomes suggest that metal ion interference in qPCR may be assay-specific and may occur via a complex interaction with specific kit components.

The presence of metal ions has been shown to alter fluorescence emission, presumably by absorbing the energy generated during electron transfer, leading to fluorescence quenching [19]. This suggests that metal ions can interfere with the qPCR technique, which measures DNA amplification in real time using the fluorescence of DNA binding dyes.
During an 'inhibition study' to evaluate the impact of different metal ions on DNA quantitation and STR profiling success, we observed that samples treated with tin (Sn) showed overestimated DNA concentrations. We report here the specific impact of Sn on DNA quantitation, provide a mechanism for this unexpected result, and suggest methods to resolve this issue.

6.2 Methods

Tin ion solution (50 mM) (Tin (II) chloride, ≥99.99% trace metal basis, Sigma-Aldrich) was prepared in DNA-free water and then diluted to working stocks of 10 mM using DNA-free water. The impact of the metal ion was tested at five concentrations (final concentration of 0.1, 1, 1.5, 3 and 5 mM in the PCR) on two DNA input amounts (0.5 ng and 0.2 ng of Human Male Genomic DNA, Promega, cat#: G1471). We included control samples, at both DNA input amounts, with no added Sn. Samples were quantified using the Quantifiler Trio™ DNA Quantification Kit (Thermo Fisher Scientific, Waltham, MA, USA) and an in-house assay. DNA quantitation with Quantifiler Trio™ was performed 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.

The kit uses a TaqMan® assay and contains four target-specific primer pairs for an 80 bp small (SA) and 214 bp large autosomal (LA) human targets, 75 bp Y chromosome (male) target, and a 130 bp (synthetic) internal PCR control (IPC) [1,2,20]. The TaqMan® probes for SA, LA, Y and the IPC are labelled with the VIC®, ABY™, FAM™, and JUN® dyes, respectively. The assay also includes Mustang Purple (MP) as the passive reference dye. Being inert, the fluorescence of the passive reference dye is not affected by the amplification cycles and is used to normalise target signals to reduce well-to-well variation [20–22].
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 Sn. The Sn ions were directly added to the PCR reaction mixture using an automated Tecan™ Liquid Handling Platform (LHP) (Tecan Group Ltd., Männedorf, Switzerland).

The in-house qPCR assay was based on SYBR green chemistry and targeted a small (67 bp) (Forward: GGGCAGTGTTCCAACCTGAGGAAAACT; reverse: GAGACACAGGGTGGTTA) human-specific nuclear DNA amplicon with ROX™ dye as the passive reference. qPCRs were performed on a QuantStudio™ 6 Flex Real-Time PCR System (Thermofisher Scientific) as described previously [23]. The 10 µL reaction volume comprised 8 µL of master mix, 1 µL template DNA and 1 µL of Sn. Thermal cycling conditions were a 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 C_T method by comparing unknown samples to a standard curve using the QuantStudio™ 6 Flex Real-Time PCR Software v1.3 [23].

To assess the effect of sample clean-up on the quantitation of Sn-tainted samples, 3 µL of 0.5 ng/ µL single source Human Male Genomic DNA (Promega, cat#: G1471) was mixed with 3 µL of 5 mM Sn. The resulting DNA-metal (n = 5) samples were extracted using the DNA

IQ™ system (Promega, Madison, WA, USA) on a Hamilton AutoLys LHP with a final elution volume of 60 µL per the manufacturer's protocol [24] with a final elution volume of 60 µL. The extraction batch included two reagent blanks and one positive control (2 µL of whole human blood spotted on a 5 mm x 5 mm square FTA card (Whatman, GE Healthcare) according to the standard operating procedure of Forensic Science SA (FSSA). Quantifiler™ Trio sample quantitation was performed as described above.

6.3 Results and Discussion

6.3.1 Impacts of tin on Quantifiler™ Trio DNA quantitation

The Internal PCR control cycle threshold (IPC C_T) facilitates inhibitor detection if the inhibitor blocks essential reagents, inactivates or interferes with the polymerase's processivity, or in some instances, binds to the DNA template [17]. The IPC C_T value for non-treated control samples was 27.9 and showed no difference for the two different template input amounts. In contrast, samples spiked with Sn showed two distinct types of IPC C_T response: 'normal' and 'unusual' (Fig. 1). The 'normal' samples - with 0.1 mM Sn, had IPC C_T values similar to the controls (approximately 28). On the other hand, 'unusual' samples with Sn concentrations above 1 mM, returned IPC C_T values of 12-13, at least 12 C_T units lower than the average of controls and below the lower limit of Quant Trio IPC C_T, range (20 – 30) irrespective of the amount of template DNA (Fig 1).

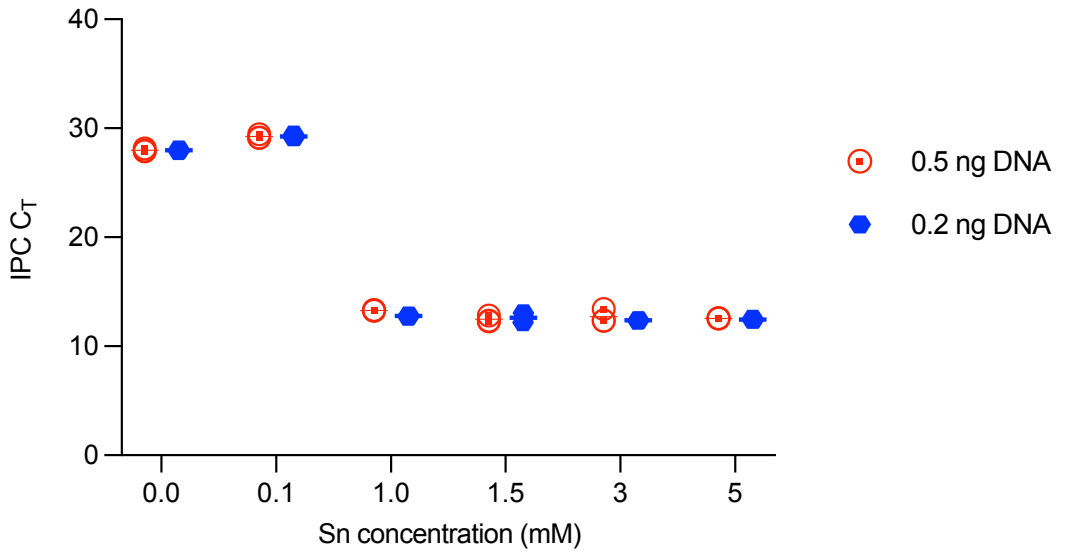


Fig 1. Quantifiler Trio IPC C_T results for 0.5 ng and 0.2 ng of human genomic DNA treated with 0, 0.1, 1, 1.5, 3 and 5 mM Sn. Red dots indicate IPC C_T values for 0.5 ng template DNA input, and Blue represents 0.2 ng template DNA input. The typical IPC C_T threshold for Quantifiler™ Trio is 20 – 30.

Metal contaminants in forensic samples have been documented as potent inhibitors in PCR-based assays [13]. Also, a recent study has highlighted the quenching effect of Sn on fluorescence [25]. This may explain the interference observed for Sn-spiked samples where IPC C_T values were substantially lower than controls. We also observed a similar pattern of Sn impact on DNA concentration. At 0.1 mM Sn, the estimated DNA concentration was reduced by more than 75% (Fig 2), indicating PCR inhibition or degradation of the DNA template. However, at Sn concentrations of 1-5 mM, input DNA concentration was overestimated by at least 38,000-fold (Fig 2, Supplementary Table 1), corresponding to the lowered IPC C_T data (Fig 1).

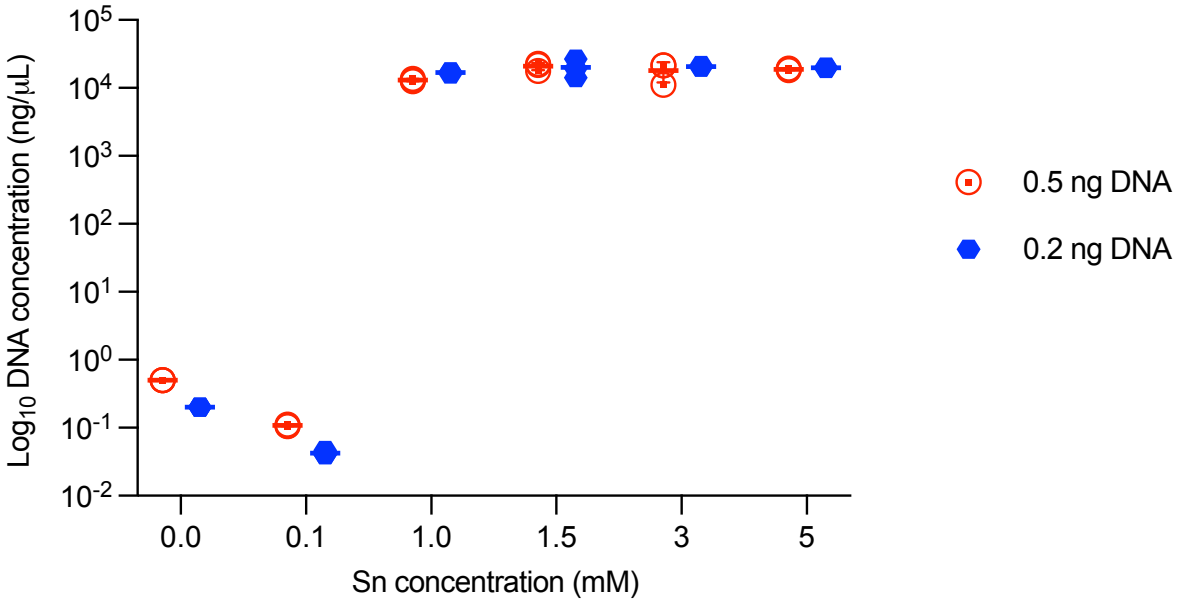


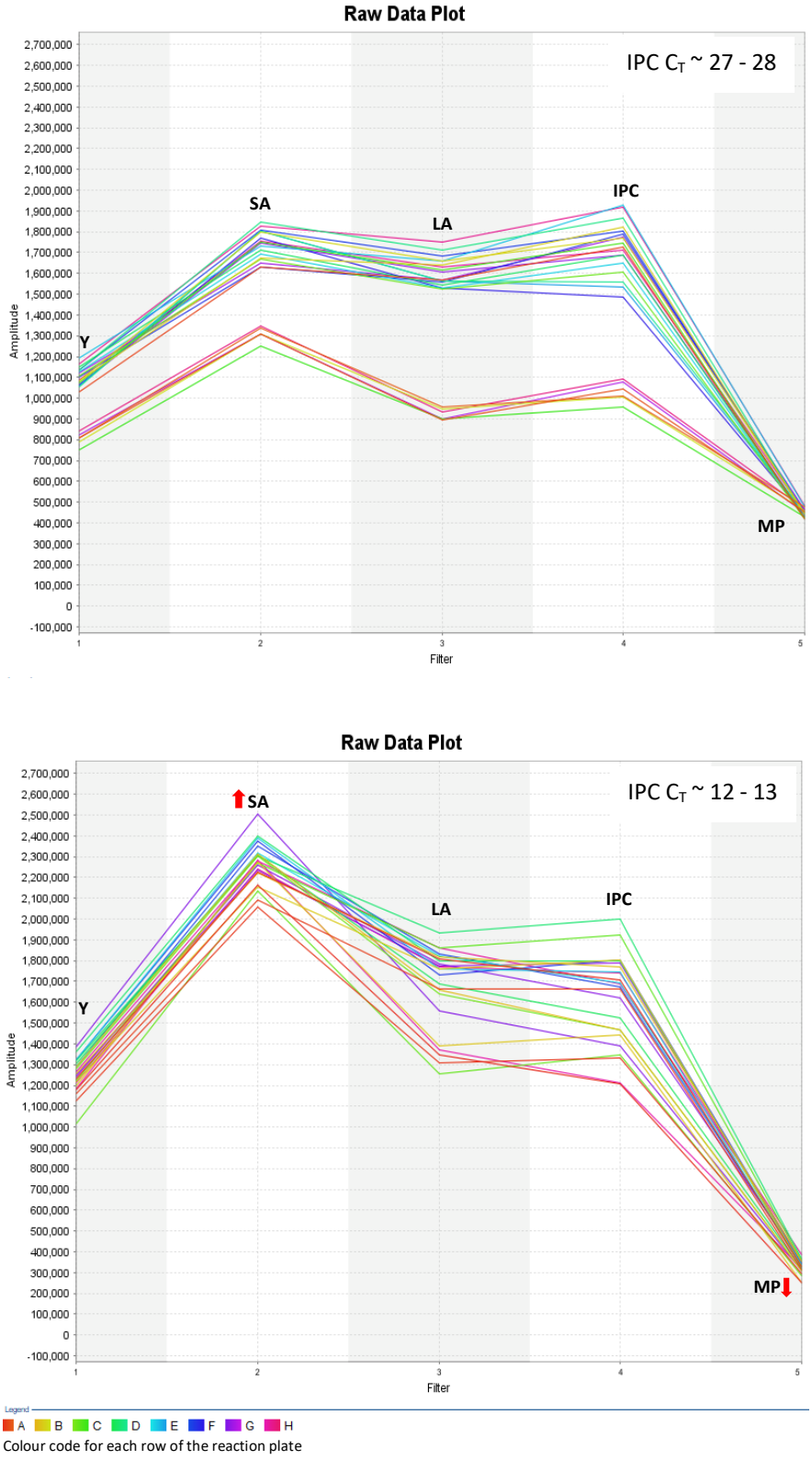
Fig 2. Quantifiler Trio estimated DNA concentration results for 0.5 ng and 0.2 ng of human genomic DNA treated with 0, 0.1, 1, 1.5, 3 and 5 mM Sn.

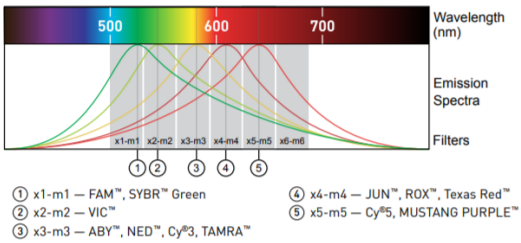
6.3.2 Causes of DNA overestimation in tin contaminated samples

To determine the likely effect of Sn ions, a thorough analysis of the order of data collection and processing through the HID Real-time PCR Analysis software v1.2 (Thermo Fisher Scientific) (the software) to produce the final quantitation, across the 29 cycles of amplification was conducted by reviewing the raw data (Fig. 3), multicomponent (Fig. 4) and amplification (Fig. 5) plots of the standard (IPC C_T ~ 27 - 28) and abnormal (IPC C_T ~ 12-13) samples.

The detector system of the Applied Biosystems (AB) 7500 Real-Time PCR System has optical filters with wavelengths adapted to the spectra-signal of each dye following excitation during the PCR (Fig. 3). After each run, the software receives raw spectra-signal data for each reading and determines the contribution of each fluorescent dye. Fig. 3 shows the raw fluorescence (not normalised) for each optical filter for the selected wells during each cycle of the real-time PCR.

Generally, the unusual samples (IPC C_T ~ 12-13) show an increase in fluorescence at each filter, particularly the SA target (filter 2) and a notably lower fluorescence at Mustang Purple (MP, filter 5) compared to the normal (IPC C_T ~ 27 – 28) samples. The lowered MP fluorescence and elevated target fluorescence (mainly SA) are more apparent in the multicomponent plot (Fig. 4), which displays the fluorescence data for each SA, LA, and Y target, and IPC in the quantification assay plotted against cycle number.

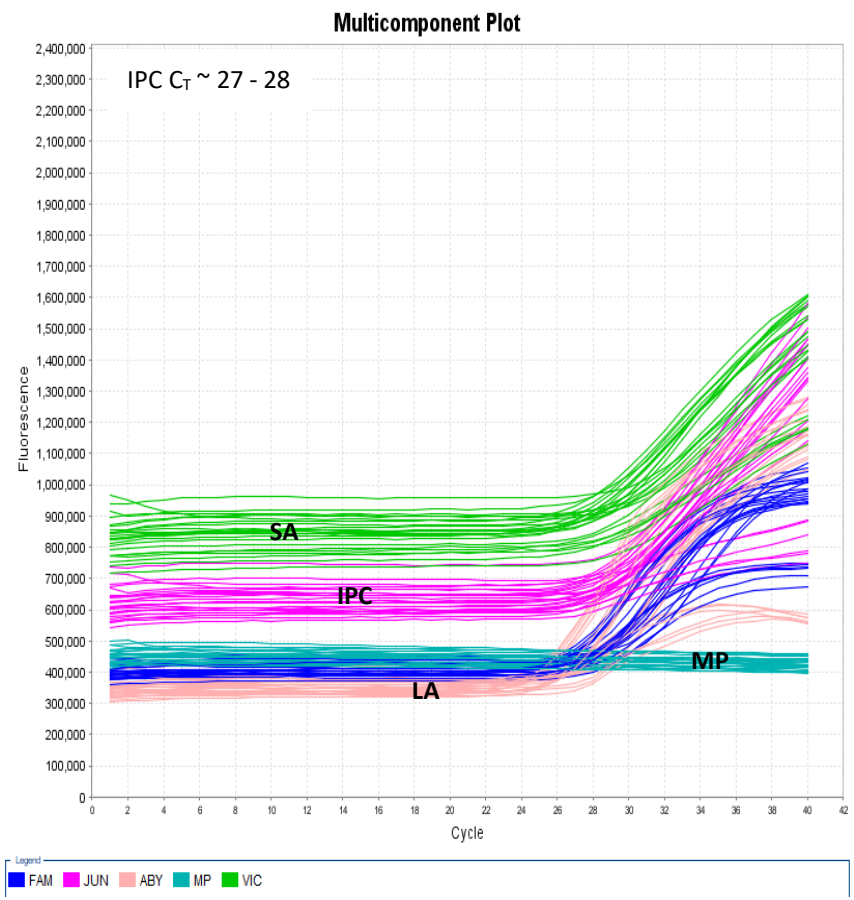


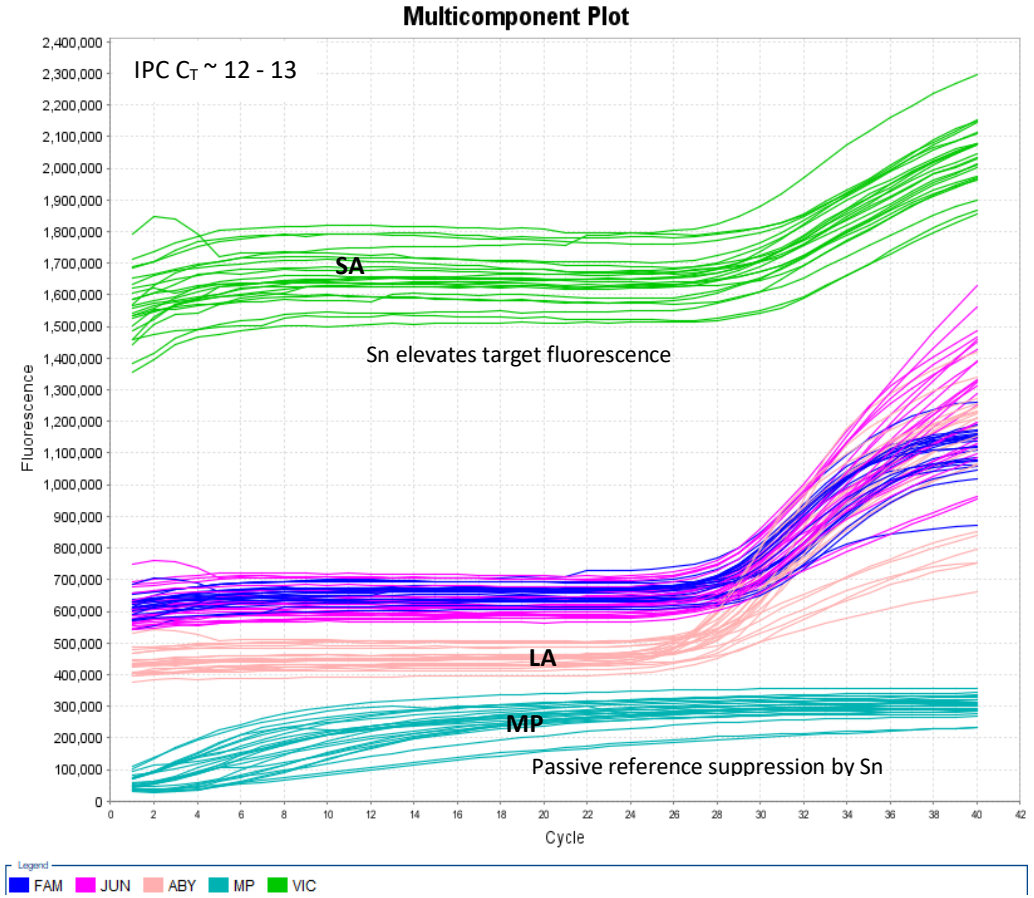


FAM = Y chromosome target (filter 1)
VIC = Small autosomal (SA) target (filter 2)
ABY = Large autosomal (LA) target (filter 3)
JUN = Internal PCR control (IPC) (filter 4)
MP (Mustang Purple) = Passive reference (filter 5)

Fig 3. Raw data plots of normal samples (IPC ~27-28) and the unusual samples (IPC ~12-13) showing the raw fluorescence (not normalized) for each optical filter for the selected wells for samples treated with Sn. The unusual samples show a general increase in target fluorescence, most noticeable at SA (filter 2) and a passive reference (Mustang Purple) fluorescence suppression (filter 5).

In the multicomponent plot, normal samples typically exhibit a flat line (i.e., constant background fluorescence) for at least the first 15 – 20 cycles, before exponential growth of the PCR product can be detected, as is the case for the IPC C_T ~ 27 – 28 samples (Fig 4). However, the plot for the 'unusual' samples shows short dips and increased fluorescence readings (also called fluorescence noise) for the targets for the initial 20 cycles due to the presence of Sn ions. For example, the SA signal at cycle 3 was approximately 900,000 relative fluorescence units (RFU) for the normal samples versus 1,800,000 RFU for the unusual samples, a two-fold increase in fluorescence due to the presence of Sn (Fig. 4). Notably, a drop in the MP signal close to zero RFU was observed between cycles 1 and 10 (Fig. 4) compared to the 'normal' samples. During the PCR process, the fluorescence signal (or normalised reporter, R_n) increases as the amount of specific amplified product increases. The DNA quantity estimates of samples are determined by comparing their respective cycle threshold (C_T) to an external calibration curve constructed from a dilution of reference standards [26]. The C_T is the cycle number at which there is a noticeable difference between the fluorescence signals of a sample and the passive reference [22]. Hence, any adverse impact on the MP signal affects the C_T value calculated for the DNA targets.





FAM = Y chromosome target (filter 1)
VIC = Small autosomal (SA) target (filter 2)
ABY = Large autosomal (LA) target (filter 3)
JUN = Internal PCR control (IPC) (filter 4)
MP (Mustang Purple) = Passive reference (filter 5)

Fig 4. Multicomponent Plots of fluorescence data of SA, LA, Y, IPC and MP plotted against cycle number. Normal samples (IPC C_T ~27 – 28) show the typically expected flat line for at least the first 15–20 cycles, before the exponential phase, compared to the low MP fluorescence (quenching) and elevated target fluorescence (mainly the SA target) seen in the unusual samples (IPC C_T ~12 – 13).

The software determines the C_T value using the R_n data collected from a predefined range of PCR cycles termed the baseline [20]. On the AB 7500 instrument, the default baseline occurs between cycles 3 and 15 (Fig 5). Consequently, a baseline-subtracted amplification plot of ΔR_n versus cycle number is generated, and the cycle where the ΔR_n value crosses the threshold set as the C_T is defined [16] (Fig 5).

The typical expectation from normal samples is a constant background fluorescence that grows when the exponential amplification occurs (Fig. 4). However, due to the effect of Sn ions, the normalised reporter signal's amplification plot (before baseline subtraction) exhibits a significant increase in the background and an irregularly shaped total fluorescence (arrowed, Fig. 5). The software is set to take cycles 3 - 15 for the baseline subtraction automatically. However, the Sn-induced fluorescence noise within these cycles leads to inaccurate baseline estimation. Consequently, the non-PCR background fluorescence is miscalculated, which causes fluorescence to remain abnormally high after normalisation (Fig. 5) and overestimates DNA quantity.

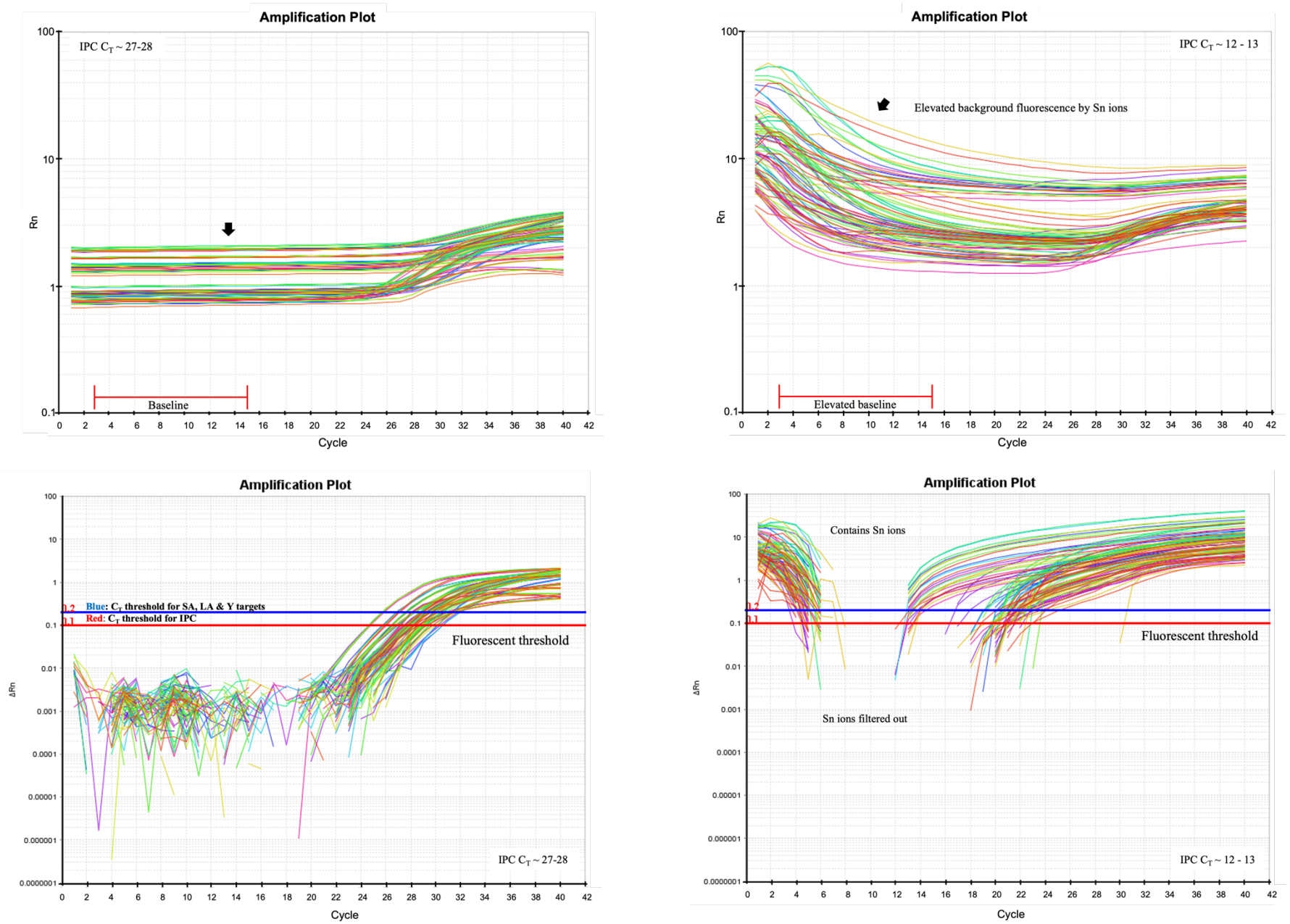


Fig 5. Amplification plots showing fluorescence signal (or normalised reporter, R_n) without baseline subtraction (top panel) and R_n with baseline subtracted (bottom panel) for normal samples (IPC ~27-28) and the unusual samples (IPC ~12-13) treated with tin ions. Automatic baseline = cycles 3 to 15.

6.3.3 Impacts of tin on in-house qPCR DNA quantitation

In contrast to the results obtained using Quantifiler Trio, the in-house qPCR assay that employs ROX as the passive reference dye showed no overestimation of Sn-treated DNA (Fig, 6). Instead, at least 50% inhibition was observed at Sn concentrations around 1.5 mM and 1 mM for the 0.5 ng and 0.2 ng DNA templates, respectively, with no DNA detectable above 3 mM Sn. This result suggests that Sn interacts differently with different qPCR chemistries, with Mustang Purple more susceptible to Sn-mediated fluorescence quenching than the ROX passive reference dye.

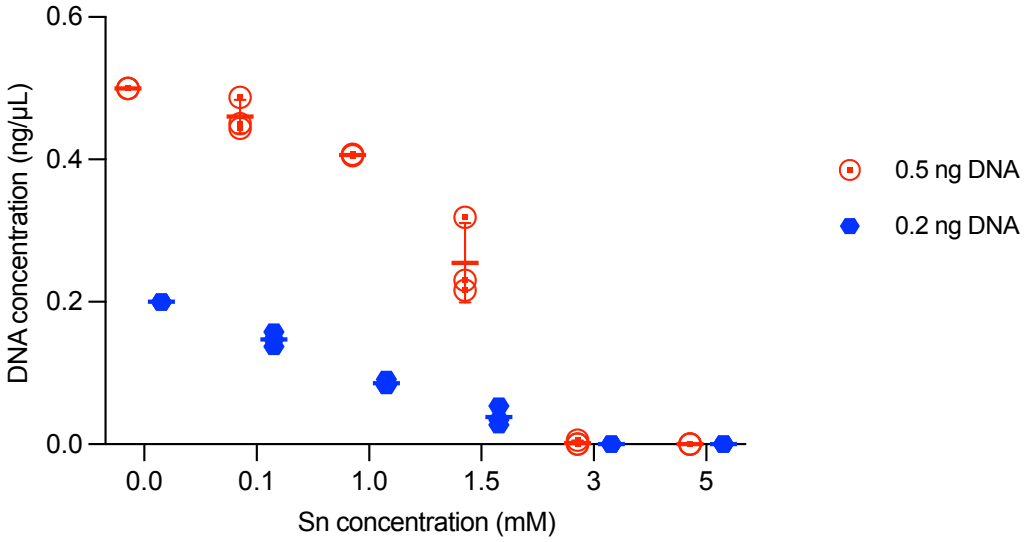


Fig 6. SYBR Green ROX estimated DNA concentration results for 0.5 ng and 0.2 ng of human genomic DNA treated with 0, 0.1, 1, 1.5, 3 and 5 mM Sn.

6.3.4 DNA extraction and purification removes the impact of tin

Similarly, no target overestimation or unusually lowered IPC C_T values were observed when Sn-spiked DNA samples were subjected to DNA extraction and purification prior to quantitation with the Quantifiler Trio™ kit (Supplementary Table 2). This suggests that efficient sample purification can resolve the adverse impact of Sn contamination and confirms that the presence of Sn was the cause of DNA excess estimation in the spiked samples analysed with Quantifiler Trio™ assay.

6.4 Conclusions

Overestimation of DNA induced by metal interaction with qPCR assay components has only been documented in one other study by Combs et al. [17], who examined copper (CU). Using the Quantifiler® Human DNA Quantification Kit (Thermofisher), the authors reported that samples treated with 18 mM Cu generated almost a 100-fold increase in quantity estimations compared to the actual template used, including in the negative template control (NTC). Interestingly, analysis of the component plots (raw data) for the Cu-treated DNA and NTC samples revealed a subtly increasing 6-FAM fluorescence concurrently with ROX passive reference signal suppression [17]. In contrast, Kuffel et al. [18] did not report any excess estimation for input DNA when utilising a custom (in-house) assay for the same metal. The enhanced fluorescence and resulting overestimation of DNA quantity suggest a complicated synergistic interaction between metal ions and qPCR reaction components.

It is also worth noting that residual dithiothreitol (DTT) in DNA extracts has been shown to prevent the detection of qPCR signals by quenching MP fluorescence, causing overestimation of DNA concentrations [10,27] when quantified with Quantifiler Trio™ [10] which is consistent with the findings in this study. However, unlike Sn, where the overestimation was directly proportional to the rise in Sn concentration (Fig 2), the DTT-mediated DNA quantity overestimation followed no regular pattern [10]. The specific mechanism underlying the Sn – Mustang Purple interactions, as observed with the Quantifiler Trio kit, is unknown, but the MP signal being diminished or MP losing fluorescence following interaction with Sn ions is probable. Therefore, a further study exploring the basis of Sn – MP interactions is recommended.

Tin is ubiquitous in everyday items such as food packaging and beverage containers [28], countertops, interior home décor, and jewellery that can be a source of touch DNA.

Therefore, a suitable DNA extraction and purification process may be necessary, especially with the advent of lysis-only techniques, to reduce or eliminate co-purification of tin that might lead to incorrect estimation of DNA concentration. We have shown that low levels of Sn contamination quench the Mustang Purple passive reference dye in the Quantifiler Trio kit, leading to inaccurate DNA quantitation. Also, qPCR assay chemistry utilising ROX as the passive reference dye, is more resistant to the Sn fluorescence quenching and offers an alternative for quantitation of samples with potentially co-extracted tin ions.

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Supplementary Table 1: Purified DNA samples spiked with different concentrations of Tin (Sn) ions

0.5 ng/µL DNA template							0.2 ng/µL DNA template					
[Metal ion]					IPC			LA			IPC	
(mM)	SA (ng/µL)	LA (ng/µL)	Y- (ng/µL)	CT_{SA}	C_T	DI	SA (ng/µL)	(ng/µL)	Y- (ng/µL)	CT_{SA}	C_T	DI
	0.46879	0.59559	0.53076	28.364	27.957	0.787	0.20159	0.25150	0.22696	29.524	27.967	0.859
0.00	0.52604	0.61926	0.55754	28.192	28.134	0.849	0.20022	0.26485	0.22263	29.637	28.071	0.756
	0.47889	0.55164	0.52357	28.332	27.877	0.868	0.20038	0.20207	0.27299	29.623	27.887	0.740
	0.10722	0.09317	0.10867	30.572	29.464	1.151	0.04038	0.03970	0.04472	32.033	29.164	1.017
0.10	0.10545	0.08199	0.11551	30.596	29.105	1.286	0.04027	0.04456	0.04582	32.037	29.445	0.904
	0.11089	0.08922	0.11208	30.521	29.158	1.243	0.04662	0.04529	0.04605	31.818	29.174	1.029
	13561.702	448.048	4024.714	12.994	13.301	30.268	15946.350	1227.886	7124.833	12.751	12.852	12.987
1.00	12921.680	775.026	5114.141	13.066	13.255	16.673	16982.637	1198.335	6962.269	12.657	12.779	14.172
	12552.279	664.599	4755.602	13.109	13.326	18.887	17368.650	1409.351	7683.973	12.623	12.700	12.324
	22820.289	2182.356	12122.096	12.215	12.290	10.457	14215.867	783.381	4884.220	12.923	13.065	18.147
1.50	21775.215	2165.464	11807.667	12.285	12.360	10.056	19271.543	1176.771	7549.903	12.468	12.623	16.377
	17887.174	804.982	5898.473	12.579	12.832	22.221	26715.820	1069.417	8424.616	11.979	12.188	24.982
	11129.077	855.305	4876.361	13.289	13.423	13.012	20013.152	2535.584	12245.849	12.411	12.428	7.893
3.00	21424.645	2497.653	12582.494	12.309	12.342	8.578	20679.111	2613.999	12614.480	12.362	12.375	7.911
	21457.389	2586.402	12954.389	12.307	12.335	8.296	21469.836	2538.275	12587.636	12.306	12.328	8.458
	19351.264	1694.988	9617.797	12.462	12.535	11.417	19172.869	1823.079	9602.043	12.475	12.539	10.517
5.00	18730.119	1535.652	8968.326	12.510	12.596	12.197	19877.852	2362.471	11544.518	12.421	12.424	8.414
	18354.420	2015.292	10305.438	12.541	12.565	9.108	20269.512	2252.314	11286.539	12.392	12.420	8.999

Supplementary Table 2: Purified DNA treated with 5 mM Tin ions

	3 µL of 5 mM Sn + 3 µL of 0.5 ng/µL of Pure DNA				
			IPC		
Replicate #	**SA (ng/µL)**	**LA (ng/µL)**	**Ct**	**DI**	**SA yield (ng)**
1	0.0164	0.0176	27.67	0.93	0.98
2	0.0175	0.0233	27.37	0.75	1.05
3	0.0203	0.0192	27.26	1.06	1.22
4	0.0154	0.0089	27.76	1.72	0.92
5	0.0189	0.0162	27.57	1.16	1.13
Mean	0.0177	0.0171	27.53	1.12	1.06
SD	0.0020	0.0052	0.21	0.37	0.12

Chapter 7

Zinc, not copper, is the major contributor to DNA degradation and PCR inhibition in DNA samples contaminated with brass.

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Principal Author

Name of Principal Author (Candidate)	Dan Osei Mensah Bonsu		
Contribution to the Paper	Conceived the study, designed experimensts, collected, analysed and interpreted data, drafted manuscript and produced figures.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	1 November 2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Chapter 7

Zinc, not copper, is the major contributor to DNA degradation and PCR inhibition in DNA samples contaminated with brass

Abstract

Limited success in DNA recovery and STR profiling from brass substrates, such as ammunition, has been ascribed to oxidative damage and/or inhibition caused by copper. However, brass is an alloy of copper and zinc and there is limited empirical data on the effect of zinc on DNA analysis. We assessed the direct inhibitory impact on DNA quantitation and STR profiling of five different concentrations of copper, zinc and brass on human genomic DNA. We then assessed DNA degradation by adding copper, zinc and brass to serial dilutions of human saliva, followed by DNA extraction, quantitation and STR profiling. Brass showed the strongest inhibitory impact, followed by zinc with copper 10-40 times less inhibitory. Brass and zinc also caused higher DNA degradation than copper. These findings contradict the Cu-induced DNA inhibition/degradation model and suggest that DNA degradation or PCR inhibition of samples collected from brass surfaces is predominantly driven by Zn, but with a synergistic interaction of both alloy components.

Keywords

DNA, Copper, Zinc, Brass, Inhibition, Degradation, QuantiFiler Trio

7.1 Introduction

The low success of forensic DNA profiling due to the contamination of crime scene samples with metal ions is a long-standing problem in forensic biology [1–3]. Notably, low rates of success in DNA recovery and STR profiling are well documented for trace DNA collected from brass substrates [4,5]. Brass is an alloy of copper (Cu) and zinc (Zn), traditionally used in the manufacture of many substrates of forensic interest, including firearms and ammunition (cartridges, bullets, and casings (CBCs)). The poor DNA recovery from brass has been linked to oxidative damage by Cu leading to fragmentation of DNA [6–8] or direct inhibition of STR profiling PCR reactions [2,9].

Previous research on the impacts of brass, and its constituent metals copper and zinc, on forensic DNA profiling have directly or indirectly assessed their roles in DNA damage and PCR inhibition. For example, Prasad *et al.* [7] reported significantly lower DNA recovery, higher DNA degradation, fewer STR alleles and a lower proportion of uploadable STR profiles from brass compared to nickel firearm cartridges spiked with human saliva, but no evidence of PCR inhibition from either source. They attributed the poor recovery and higher degradation of DNA from brass cartridges to copper induced DNA damage, rather than PCR inhibition. Kuffel *et al.* [2] directly tested the impact of various metal ions, including zinc and copper, on a custom qPCR assay. Using 5 ng of input DNA, they showed that copper and zinc could induce 50% PCR inhibition (IC_{50}) at 0.77 mM and 0.26 mM, respectively, suggesting that zinc may be a more important contributor to low DNA-profiling success from brass objects.

However, their qPCR assay could not estimate DNA degradation, nor provide independent evidence for PCR inhibition (via an internal PCR control). Importantly, Kuffel et al. (2021) noted that directly adding metal ions to qPCR reactions is not analogous to normal casework

samples, because crime scene DNA is often present as encapsulated cellular material (i.e., DNA may be protected from metal ion effects), and collected samples are subjected to DNA extraction and purification that may remove metal ion inhibition prior to qPCR and STR genotyping. Any attempts to improve DNA recovery and profiling from brass substrates needs a better understanding of the relative roles of copper and zinc, as well as their combined effect, on DNA degradation and PCR inhibition.

In this study we aimed to assess the individual impact of Cu and Zn and their combined contribution (as brass) on DNA recovery. We attempted to disentangle the roles of copper, zinc and brass on PCR inhibition versus DNA degradation, by examining 1. direct inhibition of DNA quantitation in qPCR and 2. DNA degradation resulting from exposure of cellular DNA to metal ions, followed by DNA extraction and purification to reduce or eliminate PCR inhibitory effects.

7.2 Methods

7.2.1 Metal ions

Stock solutions (50 mM) of metal ions: copper (II) sulfate (puris p.a., anhydrous ≥ 99.0 % (RT)) and zinc chloride (reagent grade ≥98%), all purchased from Sigma-Aldrich, were prepared in DNA-free water and then diluted to working stocks of 10 mM using DNA-free water. Brass typically has a 1:2 Zn/Cu ratio, but to facilitate direct comparisons, the alloy was simulated by mixing equal volumes of Cu and Zn stock solutions of the same concentration.

7.2.2 Inhibitory effects of copper, zinc, and brass on DNA quantitation

We assessed the direct inhibitory effect of Cu, Zn, and brass on DNA quantitation 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 ng and 0.2 ng of Human Male Genomic DNA (Promega, cat#: G1471). To

minimise opportunities for DNA damage the metal ions were directly added to the PCR reaction mixture immediately prior to thermocycling using an automated Tecan™ Liquid Handling Platform (LHP) (Tecan Group Ltd., Männedorf, Switzerland). Quantification was done with the Quantifiler Trio™ DNA Quantification Kit (Thermo Fisher Scientific, Waltham, MA, USA) 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 metal ion (or water), and 1 µL of DNA template. No STR profiling was performed at this stage.

7.2.3 DNA damage and inhibitory effects of copper, zinc and brass on cellular DNA recovery, quantitation and STR profiling

We also assessed the impact of Cu, Zn, and brass on DNA degradation and inhibition under biologically more realistic conditions by adding 10 µL of 5 mM of each metal ion to a 10 µL aliquot of neat, 1:20 and 1:50 diluted saliva samples in triplicate. The saliva samples were then incubated on a lab bench for 2 h, after which extractions were completed using the DNA IQ™ System (Promega, Madison, WA, USA) on a Hamilton AutoLys LHP with a final elution volume of 60 µL. Each extraction batch included two reagent blanks and one positive (quality) control (2 µL of whole human blood spotted on a 5 mm x 5 mm square FTA card (Whatman, GE Healthcare) according to the standard operating procedure of Forensic Science South Australia (FSSA). Quantifiler™ Trio quantitation was performed as described above. STR profiling was performed for saliva extracts with the GlobalFiler™ PCR Amplification Kit (Thermo Fisher Scientific) using either 400 pg of DNA or 15 µL of DNA extract (if less than 400 pg DNA was available) on a ProFlex thermocycler (Thermo Fisher Scientific) with 29 cycles of PCR. 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).

7.2.4 Data analysis

DNA quantity was estimated using the small autosomal (SA) DNA yield. DNA quality was assessed using the degradation index (DI), calculated as the ratio of the small to the large autosomal target concentrations. A degradation value of < 1 indicates no degradation, 1–10 indicates slight to moderate degradation, and > 10 indicates severe degradation. Samples, where no large autosomal target was amplified, were considered severely degraded. PCR inhibition was assessed using the internal PCR control (IPC) cycle threshold (C_T) values, with values above 30 indicating inhibition. The small autosomal (SA) DNA yield, degradation index (DI) and internal PCR control (IPC C_T) data over the established concentration range (0 to 5 mM) were used to test PCR inhibition and DNA degradation across the three metals and DNA input amounts using the Mann-Whitney U test. The concentration of the metal that gives 50% inhibition of PCR (IC_{50}) values was determined for each metal ion using non-linear regression of '[inhibitor] vs. response -- Variable slope (four parameters)' with GraphPad Prism version 9.3.1 (350) (GraphPad Software, San Diego, California, USA). The response variable is the SA yield at the given metal ion concentration.

For data analysis purposes, samples reported by the HID Real-time PCR Analysis software showing undetectable amplification were assigned a SA target yield value of zero and an IPC C_T value of 40. STR profiles were assessed qualitatively and quantitatively for any indications of inhibition. 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. The Kruskal-Wallis test was used to assess any differences in mean RFUs of metal-

treated saliva samples compared to the non-treated control samples, followed by Dunn's multiple comparison test for $p < 0.05$ to identify where any significant differences occurred.

7.2.5 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 (NHMRC) National Statement of Ethical Conduct in Human Research [10]. In addition, written informed consent was obtained from the donor of the saliva samples.

7.3 Results

7.3.1 Inhibitory effects of copper, zinc, and brass on DNA quantitation

Copper, zinc, and brass added directly to qPCR reactions caused a substantial decrease in the estimated DNA concentration and showed strong PCR inhibition on the internal PCR control. For the higher DNA input (0.5 ng), brass showed the lowest IC_{50} (0.04 mM), followed by Zn (0.06 mM) and copper (1.81 mM) (Table 1). A similar trend was observed for the three metals when utilising 0.2 ng template (Table 1), but the IC_{50} for each metal was ~1.5-6 times lower. Across both DNA input amounts zinc and brass inhibited the qPCR at concentrations 10-40 times lower than copper (Table 1). IC_{50} values were influenced by DNA input amount (1.5-6 times lower for 0.2 ng input DNA) suggesting that inhibition involves some interaction between the metal ions and DNA.

Table 1: IC_{50} values for qPCR inhibition of Quantifiler™ Trio relative to DNA quantity (0.5 ng and 0.2 ng).

Metal	IC_{50} (0.5 ng) ± SD (mM)	IC_{50} (0.2 ng) ± SD (mM)
Brass	0.04 ± 0.02	0.03 ± 0.02
Zn	0.06 ± 0.03	0.03 ± 0.01
Cu	1.81 ± 0.07	0.28 ± 0.01

SD = standard deviation.

Human DNA was completely undetectable at 1 mM for brass and 1.5 mM for Zn for both template DNA amounts, with the IPC C_T showing total inhibition at 1.5 mM (Fig 1B and 2B). In contrast, template DNA was still detectable at 3 mM (0.5 ng input DNA) and 1.5 mM (0.2 ng input DNA) with the IPC C_T not showing inhibition until 3 mM of Cu for both DNA input amounts (Table 1, Fig 1B). The apparent DNA concentration was always lowest and the IPC C_T always highest in the presence of brass suggesting additive effects of Cu and Zn.

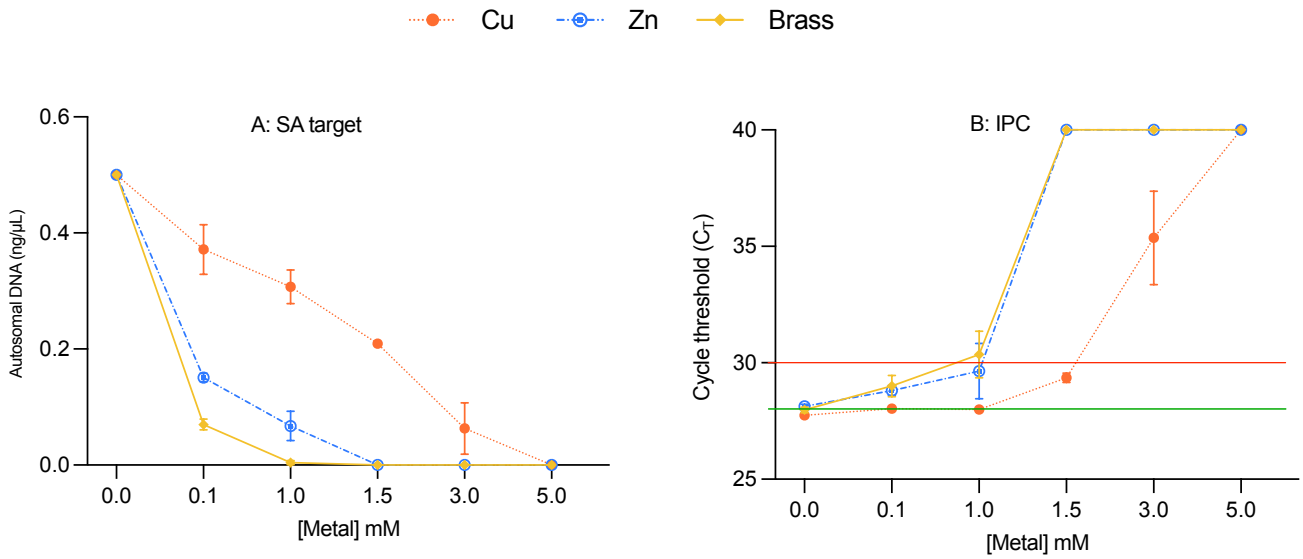

Fig 1. DNA recovery (A) and inhibition profile (B) for 0.5 ng template DNA samples treated with Cu, Zn, and brass and quantified using Quantifiler™ Trio. The green line represents the optimal IPC CT (~ 28). The red line is the upper limit of Quant Trio IPC C_T for no inhibition (range 20 – 30).

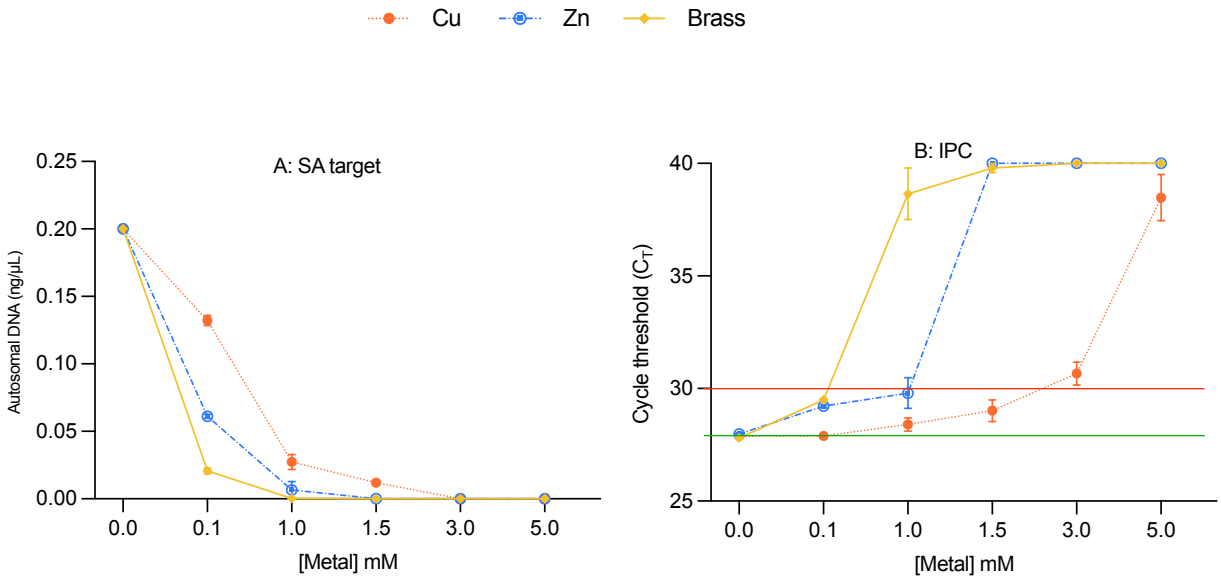

Fig 2. DNA recovery (A) and inhibition profile (B) for 0.2 ng template DNA samples treated with Cu, Zn, and brass and quantified using Quantifiler™ Trio. The green line represents the optimal IPC CT (~ 28). The red line is the upper limit of Quant Trio IPC C_T for no inhibition (range 20 – 30).

7.3.2 DNA damage and inhibitory effects of copper, zinc and brass on cellular DNA

For untreated control saliva samples, detectable amounts of DNA were observed over the range of saliva dilutions consistent with the dilution factor (Fig. 4A). The mean ± SD of DNA yields were 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 dilution). The average DI was ≤ 1, with no significant differences between neat or diluted samples (Fig. 4B). For neat saliva samples treated with Cu, Zn, and brass, the DNA yield was lower than, but not significantly different to, the control sample ($p = 0.361$, Fig. 4A) while the degradation index was higher (mean of 1.3-1.7) than the control sample (0.9) for all three metals (Fig. 4B). In contrast, significantly lower DNA yields were recorded for 1:20 diluted saliva samples treated with Cu (0.0004 ± 0.0003 ng/µL), Zn (0.0005 ± 0.0002 ng/µL), and brass (0.0008 ± 0.0001 ng/µL) compared to the untreated control (Fig. 4A).

Degradation index for 1:20 diluted saliva treated with Cu (DI: 3.5) and Zn (DI: 6.8) were in the mildly degraded to degraded range, whilst for brass, the large autosomal (LA) target of two samples failed to amplify; hence degradation data (DI = 2.0) was available for only sample (see the asterisked bar for 1:20 in Fig. 4B) indicating severe DNA degradation [11]. For the 1:50 diluted saliva samples, only two (out of three) replicates of Cu-spiked samples gave a detectable, and very low, SA target quantification but failed to give a signal for the LA target indicating severe DNA degradation. No DNA was detectable for all Zn and brass treated samples (Fig 4A). The IPC C_T data for all metal-treated samples were not statistically different from the non-treated saliva sample ($p = 0.324$) and showed no evidence for inhibition of the internal PCR control.

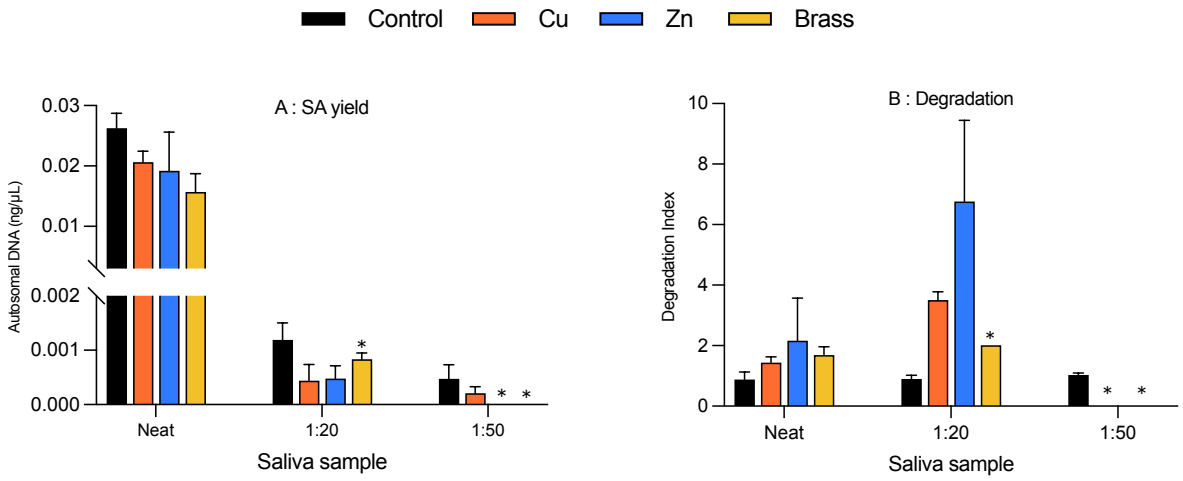

Fig 4. Effect of sample Cu, Zn, and brass on (A) autosomal DNA yield and (B) degradation of neat, 1:20 and 1:50 saliva samples. Asterisks indicate no SA target amplification or no DI data available for some or all replicates.

GlobalFiler™ STR profiling was not performed for 1:50 saliva extracts due to the extensive degradation seen from the quantification data. STR profiling was not successful for any of the 1:20 saliva dilutions treated with Cu, Zn, or brass, with no alleles detected at any loci. For neat saliva samples the average peak height was higher for the non-treated samples (5383 ±

3119) and significantly different from all but Cu-treated samples (4100 ± 2445, p = 0.3240). However, there was no statistically significant difference (p > 0.999) in the mean RFU data for neat saliva treated with Zn (3130 ± 2175) and brass (2938 ± 1679). All metal-ion treated samples yielded complete STR profiles that were 100% consistent with the untreated control sample, despite the reduced average peak height.

7.4 Discussion

Our results show that all three metals caused inhibition when added directly to quantitative PCR reactions. However, the extent of inhibition was influenced by the amount of template DNA in the reaction (Table 1) suggesting that higher amounts of input DNA can buffer the qPCR reaction against metal-ion inhibition. Copper showed the lowest inhibitory effect (highest IC_{50} and highest concentration to cause total inhibition of the qPCR reaction, Table1, Fig 1 and 2). Brass had greater inhibitory activity than each of the individual metals (Fig. 3). The inhibition curves (Fig. 1 and 2) and the IC_{50} data (Table 1) show that the PCR inhibition is predominantly driven by zinc, but with a synergistic or complementary impact of both metal constituents. This finding, therefore, constitutes the first evidence of brass-induced (instead of just copper) qPCR inhibition in forensic DNA analysis.

The impact of metal ions on the real-time quantitation of DNA has been assessed in only two previous studies. Kuffel et al. [2] and Combs et al [3]. These studies found an IC_{50} of 0.77 and 1.5 mM for Cu, respectively, compared to 1.8 mM for the Quantifiler Trio assay used in this study. The differences in IC_{50} values is likely due to differences in the qPCR assays used since Kuffel et al. observed variable inhibitory effects of metal ions on different *Taq* DNA polymerases. Combs et al. [3] did not assess Zn nor brass in their experiments, while Kuffel et al. [2] did not examine brass, but they reported a lower IC_{50} for Zn (IC_{50}: 0.26 mM)

compared to Cu (IC_{50}: 0.77 mM). Our results confirm this observation that Zn is a stronger PCR inhibitor than Cu, but we also demonstrate that PCR inhibition by brass is stronger than either metal in isolation.

DNA deposited on crime scene metal substrates is mostly present in nucleated cells [12] as opposed to being cell-free, so we tested the effect of the three metals in the presence of biological sample matrix to ascertain the extent of induced DNA degradation. After exposure to zinc, copper, and brass for two hours, followed by DNA extraction and purification we could not detect PCR inhibition using the internal PCR control. This indicates that the DNA extraction process efficiently removed metal ions since our first experiments showed that even low levels of brass and zinc in the qPCR resulted in elevated IPC C_T values. In contrast, extensive DNA degradation was indicated by reduced DNA yield and elevated degradation indexes for all three metals, possibly due to metal-DNA crosslinks impeding amplification or metal ions blocking the DNA polymerase. Generally, samples treated with brass showed the most extensive DNA degradation, with Cu showing the least impact on DNA yield (Fig 4A) and DI (Fig 4B). The foregoing outcome was reflective of the DNA profiles, where the profile intensity of Cu-treated saliva was comparable to the non-treated sample and significantly higher than Zn and brass. Prasad et al. [7]) also observed DNA degradation in saliva samples spiked onto brass cartridges, with no evidence of PCR inhibition following DNA extraction and purification. Interestingly they also observed differences in DNA degradation between sample collection methods - DNA collection from brass cartridges using "wet" methods (wet swabbing and vacuum filtration) yielded less DNA with higher degradation compared to "dry" methods (tape lifting).

Industrial brass alloys are only found in specific compositions, such as gilding metal (95Cu-5Zn), commercial bronze (90Cu-10Zn), red brass (85Cu-15Zn), low brass (80Cu-20Zn),

cartridge brass (70Cu-30Zn), and yellow brass (65Cu-35Zn) (the number before the elements denotes their weight percentage) [13,14]. Cu reportedly leaches at least five times higher than Zn [15] as a coating of a substrate, although the comparative rate of leaching of each metal, when combined as an alloy (brass) has not yet been studied. Consequently, if metal-ion co-purification occurs, samples recovered from such substrates may include a predominant amount of Cu, thereby validating Cu-mediated DNA degradation or inhibition. Patterson et al. [16] assessed the inhibitory and degradative impact of metal ions on DNA quantification and STR profiling by depositing blood on a range of Cu-containing substrates, including copper and brass sheets, cartridge casings, and domestic décor. They reported that direct PCR of swabbed blood stains yielded 88% more informative STR profiles than when samples were conventionally extracted before amplification (54%) from Cu substrates. After quantifying the % Cu composition of each substrate with scanning electron microscopy (SEM), the authors concluded that while Cu percentage in a substrate was an observable factor in allele dropout, it was not solely responsible for these events [16]. In the present study, Cu and Zn were employed in equal proportions to make the alloy and should be considered when interpreting the results.

7.5 Conclusions and further research consideration

A synergistic effect of brass alloy components, driven primarily by Zn, mediates PCR inhibition and DNA damage of samples recovered from brass substrates. In their investigation of Cu-Zn brass alloys, Hong et al. [17] found that many of the alloy's characteristics exhibited clear Zn-dependencies, supporting our results. DNA extraction appears to effectively remove inhibitory levels of copper and zinc, indicating that the low success rates for STR profiling of DNA recovered from brass are primarily caused by DNA damage prior to or during collection, sample storage or DNA extraction. Recent work

exploring strategies for improvement in trace DNA analysis from cartridges, bullets and casings has employed Cu-specific chelating agents as swabbing solutions [4] or extraction additives [18]. However, applying the same additives to samples recovered from Cu-containing cartridge cases did not enhance DNA yield or STR profiling success [19]. Future research should therefore consider chelating agents with higher affinity for both Cu and Zn to counteract the combined effects of both metals. In addition, it would be helpful to constitute brass solutions consistent with the Cu-Zn percentage compositions of industrial brass to further assess the effect on DNA in degradation and inhibition.

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Chapter 8

Titanium dioxide coatings on glass cause enhanced degradation of trace DNA when exposed to sunlight

Bonsu DOM, Higgins D, Austin JJ

Manuscript submitted to *Science & Justice*

Statement of Authorship

Title of Paper	Titanium dioxide coatings on glass causes enhanced degradation of trace DNA when exposed to sunlight
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Principal Author

Name of Principal Author (Candidate)	Dan Osei Mensah Bonsu		
Contribution to the Paper	Conceived the study, designed experimensts, collected, analysed and interpreted data, drafted manuscript and produced figures.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	1 November 2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Denice Higgins		
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Contribution to the Paper	Helped conceive and design experiments, supervised data collection and analysis and reviewed draft manuscript.		
Signature		Date	1 November 2022

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Chapter 9

Ethics reporting in forensic science research publications – A review

Manuscript Published in *Forensic Science International*

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Contribution to the Paper	Conceptualization, methodology, investigation, Data curation, formal analysis, visualisation, original draft preparation, reviewing and editing, project administration and correspondence with journal.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	1 November 2022

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iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	1 November 2022

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Chapter 10

General Discussion and Conclusion

10.1 Introduction

The desire to generate probative evidence from trace DNA deposited on metal substrates is of major operational interest. Law enforcement frequently requests DNA analysis from metal objects recovered from crime scenes. Interestingly, these metal objects, such as blades, jewellery, tools, etc., frequently produce no DNA likely due to the presence of metal ions. The low success rate of trace/touch DNA recovery and profiling from metal surfaces was recently re-confirmed using 5-years of firearm casework data relating to 731 criminal events (1,587 individual exhibits) from New South Wales, Australia [1]. The authors found that significantly less DNA was obtained from metallic cartridges, bullets and casings (CBCs); while 93% of STR profiles from CBCs samples were "unusable" [1]. As previously noted, the limited rates of trace DNA recovery from metal substrates make the cost of further downstream analysis prohibitively expensive [2]. As a result, published research in recent years has focused on improving sample collection techniques as extensively reviewed in Chapter 2, and the recently improved rinse-swab method utilising additives [3]. Only a few studies have attempted to explain the low success rate in trace DNA analysis of samples collected from metal substrates. However, these studies (e.g., [4,5]) have mainly focused on the quantitation aspect of the workflow, with often inconclusive or operationally non-adoptable/implementable results.

My research investigated trace DNA samples recovered from metal substrates in respect of the typical workflow from sample recovery through to profiling. In order to make the outcome more operationally useful, I explored and evaluated new techniques for improving sample collection and DNA profiling outcomes from various metal substrates. Overall, the thesis sought to gain insight into four fundamental issues:

1. To investigate the efficiency of swabs for trace DNA recovery from metal substrates that are relevant to forensic casework and to comprehend the influence of substrate type and sample collecting technique on such DNA recovery.
2. To investigate the impact of metal ions on sample purification, quantitative PCR (qPCR) amplification and DNA profiling.
3. To investigate the effect of metal-coated photocatalytic self-cleaning surfaces on trace DNA analysis and the implication for sample recovery at the scene of crime.
4. To examine the reporting rates of ethical compliance in published forensic science research utilising human and/or animal subjects.

The main objective of this thesis was to identify the issues that arise in the recovery and analysis of DNA from metal surfaces, regardless of whether they are due to a failure to collect and extract DNA from the surface, by the co-extraction of inhibitory factors with the DNA, or by metal ions interfering with quantitation which, ultimately adversely impact DNA profiling. Understanding the specific interactions of metals with each stage of the analytical workflow will assist forensic scientists in prioritising metal exhibits, employing optimal sampling, purification techniques and quantitation techniques to maximise the imperative requirement of obtaining DNA profiles that can be used for individual identification.

10.2 Empirical findings, implications, and recommendations for further research

The research contained in this thesis adds to existing knowledge by providing insights into how metal-DNA interactions occur at various stages of the trace DNA analytical process, with an industry focus and perspective that address gaps that have not been looked at previously.

To achieve this, it was essential to review the existing scientific literature, including from non-forensic science sources, on metal-DNA interactions, as presented in Chapter 2, in order

to identify the mechanisms underlying the difficulties in recovering and amplifying trace DNA from metal substrates as has been widely reported in forensic contexts (for example, [3,6–9]). I found that metals impact trace DNA recovery and amplification in one of seven ways: (i) damage to DNA integrity has been reported for samples left on brass cartridge casings simply by contact with the metal surface [9]; (ii) strong interactions between the metal surface ions and the DNA, which hinder the ability to dislodge and retrieve bonded DNA from the substrate (iii) enhanced trace biomaterial persistence on rough-textured metal surfaces, limiting sample collection efficiency, (iv) sampled metal contaminants can compromise trace DNA integrity during the extraction process, (v) co-extracted metal inhibitors may degrade DNA during storage or interfere with (vi) polymerase processivity and/or other (vi) qPCR assay components during amplification, which could lead to over or underestimation of DNA. This suggested that deciding on the optimal techniques to employ at each stage of processing samples from metal surfaces was vital, and it informed subsequent experiments, which began with exploring the sample recovery methods in Chapters 3 & 4.

A cursory assessment of the existing sample recovery techniques showed no consensus on the best system and approach for metal surfaces. While there has been a lot of research on DNA recovery, there are also many conflicting findings and emerging recovery strategies, with no specific methodology reported for metal surfaces. This lack of consensus was confirmed by information I obtained from two Australian forensic science laboratories, based on their knowledge of processes in other forensic laboratories (personal communication – Dr Julianne Henry, Forensic Science SA; Dr Jennifer Raymond, NSW Police Force). Swabbing was the preferred method due to its cost, convenience of use, and minimal training requirements. Hence, in Chapter 3, I tested two swabbing systems; Isohelix™ swab moistened with isopropanol and Rayon swab wetted with water for their sample collection and release

efficiencies for DNA recovered from metal substrates. Using the Isohelix™ swabbing system for trace DNA recovery in this study, which was not empirically tested in previous forensic literature, is innovative, as is its practical value for casework related to problematic metal surfaces. The Isohelix™ system was found to be a better sampling device than the Rayon system.

While an interesting 'proof-of-concept', the study in Chapter 3 had two limitations on its applicability to casework: (i) the experiments were performed in controlled conditions using non-trace quantities of acellular DNA on sterile metal substrates, unlike what prevails in a typical crime scene; (ii) the quantity of DNA used did not reflect that typically recovered from trace samples. Consequently, I designed the study presented in Chapter 4 to simulate a realistic casework scenario by testing the same two swabbing systems on commonly touched metal surfaces in a building. The result was the same as previously reported, with less DNA yielded from brass. However, the experiments were conducted at the onset of the COVID-19 pandemic, when enhanced cleaning had been implemented (before lockdowns). The study showed that apart from the physicochemical effects of metals, supplemental and touchpoint cleaning, as was widely used around the world during the pandemic, can impact touch DNA persistence and recovery.

Before publishing Chapter 2, one recent study [5] investigated the influence of metal on DNA quantitation. The authors used a commercial qPCR assay, the Quantifiler® Human DNA Quantification Kit (Thermofisher Scientific). However, the kit was being phased out by the manufacturer in favour of more robust assays with improved buffer systems and more tolerance to the presence of inhibitors (personal communication – Goodwin Corey, Field Applications Specialist – Genetic Sciences, Thermofisher Scientific). Furthermore, most

operational forensic laboratories had implemented the Quantifiler™ Trio DNA Quantification Kit for casework purposes. Later, Kuffel et al. [4] published their research assessing the impact of metal ions on qPCR efficiency. However, their work also had a few shortfalls: (i) operational forensic labs use commercial, well optimised, and extensively validated qPCR kits instead of the in-house assay used in their research; and the authors used (ii) template DNA at least 125-fold higher than the recommended limit for qPCR. Therefore, in Chapter 5, an 'inhibitory study' utilising different metal ions was performed for the first time for Quantifiler™ Trio DNA Quantification Kit and a custom assay for comparison. The results demonstrated that brass was the most potent inhibitor of the eleven metals tested and that there was no consistent inhibition pattern for different qPCR assays.

The extent of inhibition was found to be matrix-dependent (metals showed little to no inhibition in neat cellular DNA than diluted cellular samples and purified DNA). Inhibition was also influenced by the quantity of template in the qPCR reaction, suggesting that if additional clean-up for samples with potentially low amounts of DNA is not recommended (due to further loss of DNA), a larger sample volume may potentially offset co-extracted inhibitor action, especially when using the Quantifiler™ Trio kit, which is optimised to take up to 15 µL template, when DNA concentration is less than 400 pg [10]. Another important finding from this study that is critical for operational casework relates to the use of the IPC C_T values of the Quantifiler™ Trio as a metric for the presence of metal inhibitors. I have provided empirical data, using cellular and purified DNA, that cautions that while target autosomal DNA yield and cycle threshold (SA C_T) may reflect inhibition, the IPC C_T can remain normal unless triggered by relatively higher inhibitor concentration. Finally, the more popular affinity-based paramagnetic sample purification may not be ideal for samples potentially contaminated with ferrous or other magnetisable metals. Perhaps, in an operational sense, other extraction techniques may improve DNA yield from metal substrates.

A rare discovery of lowered IPC C_T values in tandem with an overestimation of DNA in samples spiked with tin (Sn) ions was examined in detail in Chapter 6. The overestimation of DNA was unique to Sn spiked samples and conspicuous only with the Quantifiler™ Trio kit. The excess and inaccurate estimation of DNA, several folds over the template quantity, was due to the reduced fluorescence of the Mustang Purple passive reference dye. Interestingly, Sn was found to exhibit two complex modes of interference in qPCR: (i) conventional inhibition, leading to limited DNA yield at very low concentrations of up to 0.1 mM and (ii) overestimation of DNA quantity through fluorescence quenching of the passive reference dye at Sn amounts greater than 0.1 mM. This is especially important given the recent surge in interest in direct lysis or direct amplification kits, which are being extensively researched to circumvent DNA loss during conventional DNA extraction of touch DNA samples [11–13]. Overestimation of a sample's DNA content results in insufficient input DNA used in the qPCR procedure. This can result in poor amplification and signal intensity. Sub-optimum DNA levels supplied to downstream STR amplification can cause casework outcomes to be negatively impacted and sample processing time and expenses to soar.

It is documented that low recovery and amplification success of touch DNA from CBCs were due to the deleterious effect of the copper (Cu) in the brass. This is supported by the historical data where the least DNA yield and profiles were obtained for brass CBCs [1]. About two-thirds of the ammunition casework samples in NSW are brass cartridges or cases, with the remainder being nickel or other materials [1]. Even though brass is a Cu-Zn alloy, the potential contribution of Zn to the overall negative effect on touch DNA samples retrieved from the alloy has never been examined [3,9]. As a result, in Chapter 7, I investigated the individual effects of Cu and Zn and their combined contribution as a brass alloy on DNA recovery. Interestingly, whereas Zn was found to be a significantly more potent inhibitor than

Cu, samples treated with brass showed the most extensive DNA degradation of the three metals. Consequently, in contrast to the long-held belief that Cu-induced damage causes PCR inhibition and DNA damage for samples recovered from brass substrates, I propose that a Zn-driven synergistic Cu-Zn interaction mediates these effects.

These findings have vital implications for the recently proposed techniques that use chelating agents either as wetting agents for sampling or additives in the extraction medium. For instance, the rinse-swab technique by Bille et al. [3] reportedly improves trace DNA yield from CBCs by at least threefold and a 67% STR profiling success rate compared to the traditional double swab [14] method. The method combines Bovine serum albumin (BSA) and the tripeptide, Gly-Gly-His (GGH), both of which have Cu binding specificity [20–22], into a solution called BTmix, an additive that supposedly reduces the degradative effects that copper (from brass) has on DNA [3]. Given the Zn directed inhibition/degradation model discovered in this study, the use of additives that chelate both elements may be the most ideal and might explain why other work [15] could not reproduce rinse-swab results.

Brass is industrially produced in precise elemental composition percentages, which instructively affect the element's physicochemical properties. As pure metals, Cu is known to have a higher leaching ability than Zn [16]. An adequate basis for choosing the most efficient additive to utilise during sample extraction would be provided by analysing the leaching propensity of Cu and Zn when used as an alloy (brass). Alternatively, the following agents like TPEN [N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine], DTPA (diethylenetriaminepentaacetic acid), BAPTA [1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetra acetic acid] and its derivatives, EDTA (Ethylenediaminetetraacetic acid) and EGTA (ethylene glycol tetraacetic acid) that are known to efficiently chelate both Cu and Zn [17], simultaneously, could be investigated for their utility as trace DNA sample purification additives.

Titania has been utilised for decades, but no scientific data exists on its effect on trace DNA analysis. In Chapter 8, I investigated the extent to which the capability to clear organic debris and microbes on photocatalytic self-cleaning surfaces could impact trace DNA on titania-coated surfaces. DNA from trace cellular samples was shown to degrade significantly faster on BioClean® and SaniTise™ self-cleaning glasses than on the control substrate after sunlight exposure. Notably, fluorescent light could also trigger biomaterial breakdown although at a slower rate. These findings, which have significant ramifications for trace DNA recovery and analysis, can be thought of as an "unintended side effect" of exploiting the photocatalytic characteristics of titania-coated substrates.

For example, several studies have suggested messenger RNA (mRNA) and micro RNA (miRNA) profiling as promising substitutes for routinely determining the source of forensically significant body fluids left at crime scenes [18–24]. One potential drawback of using a molecular-based approach for routine tissue source identification is that the RNA molecules in trace dried blood spatter stains on titania-coated glass walls, saliva or sperm stains on titania-coated floor tiles, and vaginal secretions or seminal fluid stains on titania-treated fabric can deteriorate via photocatalysis if exposed to enough light.

Conventional calorimetric and fluorometric presumptive testing for trace body fluids discovered at crime scenes can be complicated by the impact of photocatalytic degradation on organic molecules because these tests rely on chemical reactions with cellular or autofluorescent components that may already be degraded. It would be interesting to find out if the UV light in alternative light sources (ALS) for crime scene biofluids search may initiate photocatalysis, given that TiO_2-coated glasses are effectively activated by UV light sources [25]. Additionally, recent research efforts to use microbiome analysis as trace evidence [26–28] may encounter difficulties due to the capacity to eradicate microorganisms on self-cleaning substrates. It has also been shown that TiO_2 coating on substrates renders the

surfaces oleophobic [25,29], increasing the surface's resistance to oil. Because most enhancement approaches target organic components, including lipids, this could be a significant limitation for latent fingerprint analysis [30]. Given that touch DNA samples on self-cleaning substrates degrade quickly, as was found in this study, forensic scientists and crime scene investigation experts would be highly interested in a study of how photocatalysis and oleophobicity affect the performance of conventional powder-based procedures for latent fingerprint enhancement on these substrates.

The study presented in Chapter 9 was undertaken as a stopgap measure during the COVID-19 pandemic lockdowns when I could not continue with the practical aspects of my research and highlights the trends in ethics compliance reporting in forensic science research. Seeking ethical approval before research is a requirement in scientific research that is often seen as bureaucratic and onerous [31], especially in academia. Ethics reporting for research involving humans or animals has become mandatory for manuscript submission to journals. As I reviewed the literature for the experiments in this thesis, I noticed inconsistencies in ethical approval and informed consent statements in manuscripts published in highly regarded forensic science journals. As ethical research conduct is at the heart credibility of scientific credibility I saw an opportunity, albeit a daunting one, to delve into how prevalent adverse findings were. The result was that at least 63% of research published in six top forensic science journals examined did not report informed consent or ethical approval despite using human and/or animal samples. The results were even more surprising when compared to the over 90% reporting rate in published research in biomedical journals.

The lack of standardised nomenclature on ethics/informed consent requirements across forensic science journals and the disparities in ethics requirements in industry versus academia were notable lapses. Relevantly, the lack of ethical approval or an explicit

declaration of the non-requirement of ethics, for instance, in the case of scavenged parts, in research utilising animals, was brought to the notice of the forensic science community. The importance of this research lies in the personal feedback received from renowned scientists for their "unintentional omission" and the recognition offered by the receipt of the 'Best Poster' award in the 'Science, Justice and Legal Issues' at the 25th ANZFSS International Symposium held in Brisbane, Queensland from 11th to 15th September 2022.

10.3 Limitations

One question I initially sought to explore was how metals' surface and solid-state structure impact trace DNA analysis. I was keen on employing solid state characterisation techniques such as scanning transmission electron microscopy (STEM) and Energy Dispersive X-Ray Analysis (EDX) to examine how the metal type, gross and microscopic topography (e.g., roughness, smoothness, corrosion), elemental composition, structural defects, or impurities of the surface impact trace DNA persistence, recovery, and downstream processes. Due to normal wear and tear or mechanical force from contact with other objects, the surfaces of metal substrates frequently discovered at crime scenes might alter over time. Additionally, the salts and acids found on bare human hands from daily activities and the active compounds in cleaning products can cause metal substrates to corrode or weather (as noted in Chapter 4). These changes to the macro and microstructure of the metal substrate are vital to its affinity, which either promotes or inhibits biomaterial transfer, adhesion and persistence. For example, extensive casework data from NSW showed better trace DNA yields, and STR profiling success from unfired versus fired ammunition [1]. Unfired ammunition has a smoother texture than fired cartridges. The riflings created during the shooting process create crevices that encourage biomaterial persistence limiting DNA recovery, as explained in Chapter 2. Such changes, however minimal, can potentially affect the deposition and spread of

biomaterial on the surface, including the persistence of biomaterial even after sample collection. Xu et al. [2] indirectly illustrated this concept when they showed a high success rate of touch DNA yield and profiling from fired cartridges by initially modifying the smooth surfaces of standard unfired cartridges into microtextured surfaces to trap shed cells following gun loading.

Understanding how specific substrate composition and variables affect the transfer and persistence of trace biological material is vital to evidence search and discovery and, more importantly, developing the most appropriate methodology for optimal sample collection from such substrates. My inability to conduct this research, which would have enhanced the findings in Chapters 3 and 4 as part of the thesis, occasioned by time constraints and force majeure (pandemic), is a limitation of this thesis but a basis for future research.

Throughout this thesis it has been argued that metals exhibit non-patterned, complex interactions with DNA resulting in often unpredictable quantitation and DNA profiling results. As shown in Chapter 5, normal IPC C_T values do not necessarily indicate a total absence of inhibition. A study that found undetectable qPCR results combined with normal IPC C_T data from fired cartridge casings yielded an almost complete profile [32]. The complexity was further highlighted in recently published casework data from New South Wales, where some touch DNA samples collected from CBCs with DNA concentrations below the laboratory threshold yielded profiles that were uploadable to the database for identification or intelligence purposes. In contrast, some other samples with DNA concentrations above the established threshold did not produce informative DNA profiles [1]. The use of the Quantifiler™ Trio DNA Quantification Kit, which was used in this research, is one obvious similarity in the examples above. Together with the results of Chapter 6, metal inhibitors appear to have an unexplored impact on the target assays, passive reference, IPC,

and possibly other constituents of the Quantifiler™ Trio. To fully understand this, it would have been necessary to explore the effects of potential metal interferents on each constituent of the Quantifiler™ Trio kit compared with other commercial qPCR assays. This would have revealed whether the observations made in this thesis were unique to the Quantifiler™ Trio kit, a limitation that also suggests further investigation to inform the decision-making in the operational use of qPCR kits for samples recovered from metal substrates. It also suggests that STR profiling of all extracts from metal substrates may be warranted and brings into question the use of analytical thresholds and quality control metrics to exclude samples for downstream amplification.

Following the discovery of Sn ion quenching of the Mustang Purple (MP) passive reference dye of the Quantifiler™ Trio TaqMan™ assay, it would have been more appropriate to test another TaqMan assay that uses ROX dye as the passive reference, preferably, from the same manufacturer, rather than an in-house assay based on SYBR green chemistry that employed ROX. This would have provided insight into the specific mechanism underpinning the effects observed. However, I was unable to source a test sample of an alternative kit. Testing samples of MP dye would have been an alternative, but since Mustang Purple® is a patented dye, I was not able to further investigate a connection between Sn and its weaker signal.

In operational laboratories, affinity-binding sample purification techniques, such as the DNA IQ™ System [33], are the preferred methods because they have been demonstrated to outperform other DNA extraction methods including organic [34,35], Chelex® 100 [36,37], and silica spin columns [37] and the process is easily automated. In addition, contamination and PCR inhibitors are eliminated through several washing stages [13,23]. Nonetheless, I have demonstrated in this work that metal contaminants can interfere with the extraction efficiency of these extraction methods leading to enhanced co-purification and qPCR failure.

Alternative extraction techniques would have been just as crucial to understanding the metal's effects and the limitations of DNA extraction. This would have influenced any additional cleaning steps and helped to determine whether particular purification and cleanup procedures are more effective than others at removing the metal, as well as the concentration at which purification and cleanup become ineffective resulting in carryover in qPCR and STR profiling. The amount of co-extracted inhibitors and the amount of metal concentration eliminated by purification could have been estimated from an analysis of sample waste after extraction using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

I have presented for the first time evidence of the detrimental influence of self-cleaning surfaces on the persistence, recovery, and amplification of trace DNA. For a comprehensive evaluation of the photocatalytic effect on trace DNA analysis, it would be necessary to evaluate the same in a typical outdoor situation, for which Bioclean® and SaniTise™ glasses are optimised, under varying climatic conditions or during prolonged exposure to fluorescent light.

10.4 Concluding remarks

The primary motivation for this study was to determine how metals affect trace DNA analyses. This has been accomplished with new insights about the impacts of metals on the specific phases of the analytical workflow - sample recovery, extraction, quantitation, and DNA profiling - and mitigation actions proposed. This information can be used to triage metal exhibits, select appropriate sample collection and extraction systems, and interpret qPCR target and quality control data with caution. This research has demonstrated for the first time that, an Isohelix swab moistened with isopropanol maximises trace DNA sample collection, Zn rather than Cu, mediates the deleterious effects of brass on trace DNA, affinity extraction methods may not be optimal for metal-contaminated samples, metal interferents in

qPCR can induce target amplicon inhibition or overestimation of DNA concentration, co-purified metal inhibitors can create STR profile imbalance, and titania-coated glasses degrade biomaterial on their surfaces via photocatalysis. The findings have inspired casework techniques for analysing samples taken from metal substrates and have influenced future research paths, as seen by multiple citations of the published chapters. Overall, the evidence offered in this research highlights the need for further study into more sensitive and robust amplification methods and novel approaches to sample collection and quantitation when working with metal substrates.

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Appendix: Achievements

Grants applied for to support my research:

- The Chartered Society of Forensic Science Research Grant 2020 – this grant is announced annually and offered to only one most competitive applicant. I was awarded the maximum amount for my application.

- Bill Retalic Prize for Science and Justice – this is a competitive grant offered annually and administered on behalf of the family and friends of the late William "Bill" Retalic by the Faculty of Science, University of Adelaide. The grant support research projects which seek improved justice outcomes using science or technology. I was awarded the maximum amount for my application.

Collaborations established

- Forensic Science South Australia (FSSA). FSSA supported my research by providing reagents, STR profiling and technical assistance during my PhD. In return, I undertook a mutually beneficial internship where I worked on two internal projects, which have since had immense operational benefits to FSSA.
- NSW Police Force – My collaborator, Dr Jennifer Raymond, is the Research Coordinator, Science & Research Unit, Forensic Evidence and Technical Command at NSW Police Force. Her insights on their difficulties with profiling trace DNA recovered from metal substrates helped shape my PhD research.

Key Presentations

- 12 Sept 2022 “Investigation of Casework GO! For improved DNA recovery from touch DNA exhibits. ANZFSS 25th International Symposium, Brisbane. Oral Presentation
- 13 Sept 2022 “Investigation of Casework GO! For rapid detection of male DNA in sexual assault cases”. ANZFSS 25th International Symposium, Brisbane. Poster Presentation.
- 14 Sept 2022 “Ethics reporting in Forensic Science research publications”. ANZFSS 25th International Symposium, Brisbane. Poster Presentation.
- 10 Mar 2021 “Touch DNA recovery from metal surfaces”. Advances in Forensic Science Research. ANZFSS SA Branch Meeting. Oral Presentation.

Awards

- 15 Sept 2022 - Best Poster Display in Science, Justice and Legal Issues at the ANZFSS 25th International Symposium on the Forensic Sciences, Hilton, Brisbane
- 30 May 2022 - Royal Australian Navy Commendation in recognition of work identifying the HMAS Sydney unknown sailor using mitochondrial DNA at the Australian Centre for Ancient DNA (ACAD), presented by Chief of Navy, Vice Admiral Michael Noonan AO, Royal Australian Navy,
- 30 May 2022 - The Ross Vining Award. ANZFSS SA Branch award for the best Symposium award application to attend and present my research at the 25th International Symposium on the Forensic Sciences held in Brisbane in September 2022.
- 1 Apr 2022 - ANZFSS Symposium Award. An NZFSS Executive award to attend the 25th International Symposium on the Forensic Sciences held in Brisbane in September 2022.
- Industry Engaged PhD (IEP) Placement Scholarship. The University of Adelaide award to undertake a five-month industry placement at Forensic Science SA.