Genotoxicity and cytotoxicity of particulates - dependence on composition and source

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A549	Human adenocarcinomic human alveolar basal epithelial cells
AA	Ascorbic acid Assay
AAS	Atomic Absorption Spectrophotometry
Al_2O_3	Aluminium oxide
Ames test	*Salmonella typhimurium* reverse mutation assay
As	Arsenic
ATP	Adenosine Tri-Phosphate Assay
Ba	Barium
BC	Black Carbon
BCR723	Road dust
BEAS-2B	Human normal bronchial epithelium cells
Bi	Bismuth
CaO	Calcium oxide
Cd	Cadmium
Ce	Cerium
CMB	Chemical Mass Balance
Co	Cobalt
CPC	Condensation Particle Counters
Cr	Chromium
CRM	Certified Reference Material
CRPs	Crushed Rock Powders
Cu	Copper
D10	The 10^{th} percentile particle size
D50	The median particle size
D90	The 90^{th} percentile particle size
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	Dithiothreitol assay
ERMCZ100	Fine dust (PM10-like) (PAH's)
ERMCZ129	Fine dust (PM10-like) (trace elements)
Fe_2O_3	Iron(III) oxide
H2DCFDA	Reactive Oxygen Species Assay
H2O2	Hydrogen peroxide
HaCaT	Human aneuploid immortal keratinocyte cell
HepG2	Human liver cancer cels
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
INAA	Instrumental Neutron Activation Analysis
K_2O	Potassium oxide
La	Lanthanum
LGT	Low Gelling Temperature
LMP	Low Melting Point
MEM	Minimum Essential Medium
MgO	Magnesium oxide
MN	Micronucleus test

Mn	Manganese
MnO	Manganese oxide
Mo	Molybdenum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Na_2O	Sodium oxide
Nb	Niobium
NGT	Normal Gelling Temperature
Ni	Nickel
NIST1648A	Urban particulate matter
NIST1649B	Urban dust
NIST1650B	Diesel particulate matter
NIST2786	Fine particulate matter
NMP	Normal Melting point
NRF2	Nuclear factors erythroid 2-related factor 2
OPC	Optical Particle Counter
P_2O_5	Phosphorus pentoxide
PAHs	Polycyclic Aromatic Hydrocarbons
Pb	Lead
PCBs	Polychlorinated Biphenyls
PM	Particulate Matter
PMF	Positive Matrix Factorization
PSD	Particle Size Distribution
QCM	Quartz Crystal Microbalance
Rb	Rubidium
S	Sulphur
Sb	Antimony
SCGE	Single Cell Gel Electrophoresis
SEM-EDX	Scanning Electron Microscopy with Energy-Dispersive X-Ray Spectroscopy
SiO_2	Silicon dioxide
Sn	Tin
Sr	Strontium
T75	Corning 75 cm^2
TBE	Tris / Borate / EDTA - soultion
TEOM	Tapered Element Oscillation Microbalance
Th	Thorium
TiO_2	Titanium dioxide
U	Uranium
V	Vanadium
W	Tungsten
XRD	X-Ray Diffraction
XRF	X-Ray Flourecence
Y	Yttrium
Zn	Zinc
Zr	Zirconium

Abstract

It is well established that exposure to Particulate Matter (PM) has negative effects on human health. Even though the current body of evidence provides much needed information on PM toxicity, there still remain key unanswered questions regarding the role of variations in its structural and compositional properties in inducing toxic effects. This research project aimed to determine the association of induced toxic effects in cell-free and cell-based assays with PM's physicochemical and mineralogical characteristics. To do so, we focused on three different particle types with specific characteristics needed for the purpose of this research project and hence, we used; (i) Crushed Rock Powders (CRPs) from the Panasqueira mine area in Portugal; (ii) PM_{10} from Makkah, Saudi Arabia; and a (iii) Certified Reference Material (CRM-ERMZC120). The genotoxicity and cytotoxicity of the CRPs were found to be dependent on their structural and compositional properties, in particular and perhaps specific to these samples, MnO, Zn, S, Cu, and clinochlore IIb2. The genotoxic and cytotoxic effects of Makkah PM_{10} were found to be associated with meteorological conditions on the day of sampling and which are used here as a proxy for PM_{10} sources and composition. We found that fine particles of whatever origin and anthropogenically-sourced secondary particles were associated with genotoxic and cytotoxic effects. Based on observations in cell-free and cell-based assays, it can be concluded that toxicity is dependent on (i) particles' chemistry and surface reactivity in cell-free assays; (ii) particle size and surface area in cell-based assays; and (iii) sometimes both factors in (i) and (ii). This study has highlighted the role of particularly chemical composition, and/or mineralogy in controlling the extent and/or nature of genotoxic and cytotoxic effects caused by particulates. Lastly toxicity assay data are reported for a widely available CRM, ERMCZ120, providing a common reference that can be used for comparative purposes for other researchers investigating the toxic effects of PM.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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A special thanks to my family who have always been supportive and available and have always remembered me in their prayers. Thank you, Mum and Dad, as without

your continuous guidance, I wouldn't have become the person I am today. You two are the only people I look up to in my life and the ones I can go to no matter what. I truly, can't imagine my life without you. Thank you to all my sisters and brother; Dr Ghada, the older sister whom I've always felt sorry for since she followed Dad's footsteps and sold her soul to mathematics, Dr Ahmed, the older brother that always reminded me that he is way cooler than me, Dr Raghda, the older sister whom I can never take seriously due to her marshmallowy appearance, Dr Asmaa, the younger sister that always reminded me that she is a doctor, and Dr Sara, my youngest sister and the only person I know that lives in a perfect utopian pink bubble.

In the end, I am deeply grateful for my princesses, my wife Reem and daughter Sabah. Thank you, Reem, for giving me your unconditional support, encouragement and motivation throughout countless late nights and early mornings and keeping me sane over the whole period of this PhD project. I'm truly lucky to have you in my life. Thank you, Sabah, for always cheering me up with your gossip about other naughty children that always reminded me that I had the best child ever, your imaginary cooked meals that I can never get enough of, your lovely dressing up games that gave me the chance to be an astronaut, your perfect impersonations of famous characters from countless movies, and for sharing your pitch-perfect voice with nursery rhymes that I memorised by heart along the way.

Chapter 1: Introduction

1.1 Overview

Exposure to air pollution, especially particulate matter (PM), poses great risks to human health. Previous studies have found that exposure to PM is associated with premature mortality and exacerbating existing health conditions such as asthma, chronic obstructive pulmonary disease, cardiovascular diseases and cancer (Schlesinger et al. 2006; Riediker et al. 2019). The association between PM and these diseases indicates that PM causes alterations in cellular function possibly due to damage to proteins, lipids, and/or DNA (Dumax Vorzet 2010).

The term PM does not refer to a self-contained pollutant but to a complex mixture of pollutants that include organic chemicals, heavy metals, and dust amongst many others (U.S. EPA. 2018). PM's physical characteristics and chemical components vary significantly and both can affect PM toxicity (Riediker et al. 2019). PM toxicity has been researched heavily in the past. Thanks to the body of evidence available, awareness regarding the effects of asbestos, silica, and coal particles is widespread (Donaldson and Seaton 2012). Nevertheless, modelled health risks associated with these particle types are mainly from occupational exposure while scientific attention has now shifted to environmental exposure (Riediker et al. 2019). In an environmental setting, understanding which component or group of components of the PM mixture is causing adverse health effects in humans is extremely difficult. Unlike occupational settings where PM sources are generally well known and can be controlled to protect workers, environmental settings have a variety of PM sources that sometimes cannot be controlled to protect living populations. The variability in the structure and composition of particles originating from different sources and places results in a variety of cytotoxic and genotoxic effects (Jia et al. 2017; Hime et al. 2018).

In this perspective, a comprehensive physical, chemical, mineralogical, and toxicological analysis of PM may assist in linking a component or group of components from the particle mixture with a cytotoxic or genotoxic effect. Unfortunately, that is extremely difficult due to limitations stemming from the amount of material typically obtained via PM samplers (Vuong et al. 2017). The low amount of material obtained, usually in milligrams, is insufficient for many types of analyses. For example, an investigation into PM's physical properties through particle size distribution analysis and chemical composition through X-ray fluorescence requires at least ~ 10 grams of material (Rothwell and Rack 2006). Therefore, to overcome this issue, a comparative analysis of different types of particles including; (i) a set of fine PM-like samples with plenty of material sufficient for all types of analyses and with known physicochemical and mineralogical characteristics, (ii) PM collected from an area that represents a proper setting for such an investigation with several natural and anthropogenic PM sources, and (iii) a widely available and reasonably priced Certified Reference Material (CRM) may assist in reaching that goal.

The first particle type needed must be available in amounts sufficient for the several types of analyses required, appropriate in terms of its size, contain a wide range of chemical and mineralogical components, and have a known range of chemical compositions. Therefore, for the purpose of this study, a set of 24 samples of Crushed Rock Powders (CRPs) from the Panasqueira mine area in Portugal were chosen. The important characteristics of these CRPs in relation to the aims and objectives of this thesis is their availability (~ > 100 g per sample) and their physical, chemical, and mineralogical characteristics enabling an investigation of chemical compositional controls on the toxicity of particles of similar grain size. Although exposure to

particulates is an issue of concern, we note that exposure was not the purpose of the research with these samples and in any event the grain size distribution of the samples does not reflect that in occupational or environmental settings in and around the mine.

The second particle type needed must be representative of PM pollution in urban environments. The term PM pollution in urban environments refers to the introduction of harmful substances such as organic chemicals, heavy metals, and dust originating from natural and/or anthropogenic sources to the atmosphere of built up areas with high population density. Many cities around the world fit this description. However, for the purpose of this study, PM_{10} from Makkah, Saudi Arabia was chosen as being of particular interest for the author's home institution and also because there exist more extensive gaps in knowledge in PM research in Saudi Arabia compared to the UK and other developed countries. An extensive literature search found that so much has already been done in developed countries such as the UK that it was hard to identify a feasible study there within the current research scene. The use of particles from Makkah, in particular, was based on the city's importance to Saudi Arabia and the Muslim world as a whole, the lack of a detailed investigation of PM's toxic effects, albeit that most large Saudi cities lack such an investigation, as concluded from our review of the available literature (*Figure 1.1*), and on the availability of connections and resources required for the obtainment of a PM_{10} sampler and permissions associated with sampling. No toxicological studies were found for large Saudi cities such as Riyadh, Rabigh, and Taif. The only studies found were for Makkah (El-Assouli 2011) and Jeddah (El-Assouli et al. 2007; Sun et al. 2012; Brocato et al. 2014). ElAssouli (2011), reported that extracted organic matter from PM_{10} from Makkah was found to be mutagenic in the *salmonella* TA98 test as well as damaging human blood

cells' DNA in the Single Cell Gel Electrophoresis (SCGE) comet assay (El-Assouli 2011). ElAssouli et al. (2007), also used the same methods on extracted organic matter from PM_{10} from Jeddah. Similarly, significant DNA damage was reported in the SCGE comet assay and indirect mutagenic responses have also been reported in the *Salmonella mutagenicity* (Ames) test (El-Assouli et al. 2007). Sun et al. (2012) reported that the pathways involved in lipid and cholesterol metabolism of BEAS-2B (human bronchial epithelial cells) experienced irregularities when they were exposed to PM from Jeddah. Exposure to PM from Jeddah also induced genes involved in NRF2-mediated response to oxidative stress in the cells (Sun et al. 2012). Brocato et al. (2014) found that genes involved in metabolic syndrome and atherosclerosis are overly expressed in mice after exposure to PM_{10} from Jeddah (Brocato et al. 2014).

The third particle type needed must be widely available, reasonably priced, and resembling PM in terms of its size and composition. Therefore, for the purpose of this study a CRM, ERMCZ120 fine dust (PM_{10}-like) (trace elements), purchased from Sigma was chosen. This CRM enables the investigation of a homogenous and stable PM mixture created to replicate atmospheric PM. Its use in this project over other available CRMs was due to its low price and novel use in PM research. This will also serve as a common reference for other researchers investigating the toxic effects of PM.

1.2 Aims and objectives

The main objective of this thesis has been to examine the cytotoxic and genotoxic effects induced by different types of particles using several toxicological assays and

attempt to associate a component or group of components from the particle mixture with the endpoints under investigation.

To fulfil this purpose, the following specific objectives were pursued:

- Determine the association between toxic effects induced by PM, specifically crushed rock powders from the Panasqueira mine area, Portugal, and their chemical and physical properties.
- Determine toxic effects induced by PM_{10} from Makkah, Saudi Arabia and the association of those effects with source and composition as indicated by meteorological parameters used as proxies.
- Obtain quantitative data for the toxic effects induced by a widely available standard material, specifically ERMCZ120 fine dust (PM10-like) (trace elements), as a preliminary basis for its use as a validation reference material for toxicity testing.

A summary of the steps taken and the experimental methods applied to reach these goals is shown in *Figure 1.2*.

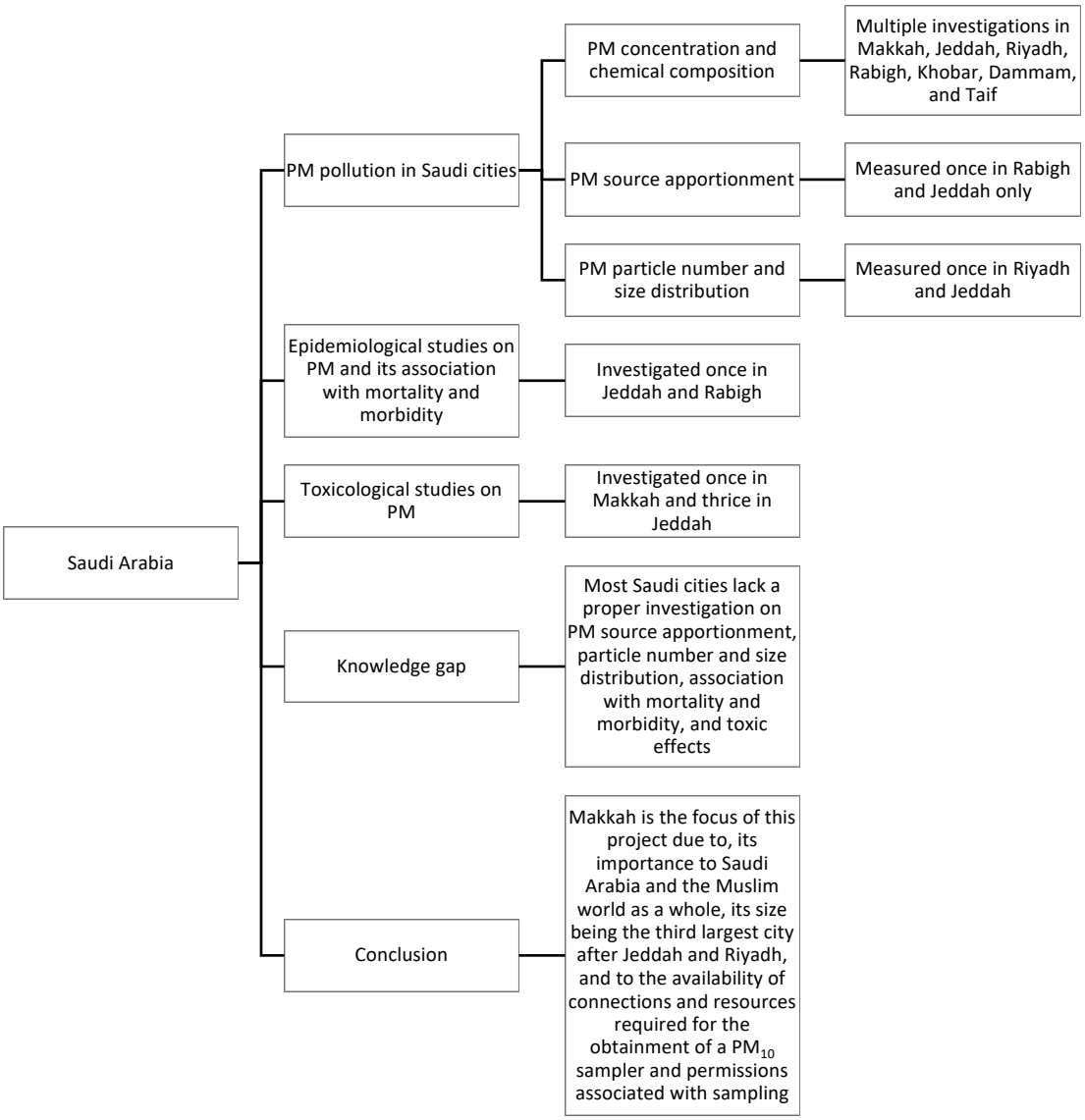

Figure 1.1, Methodological approach to finding the gap in knowledge in Saudi Arabia

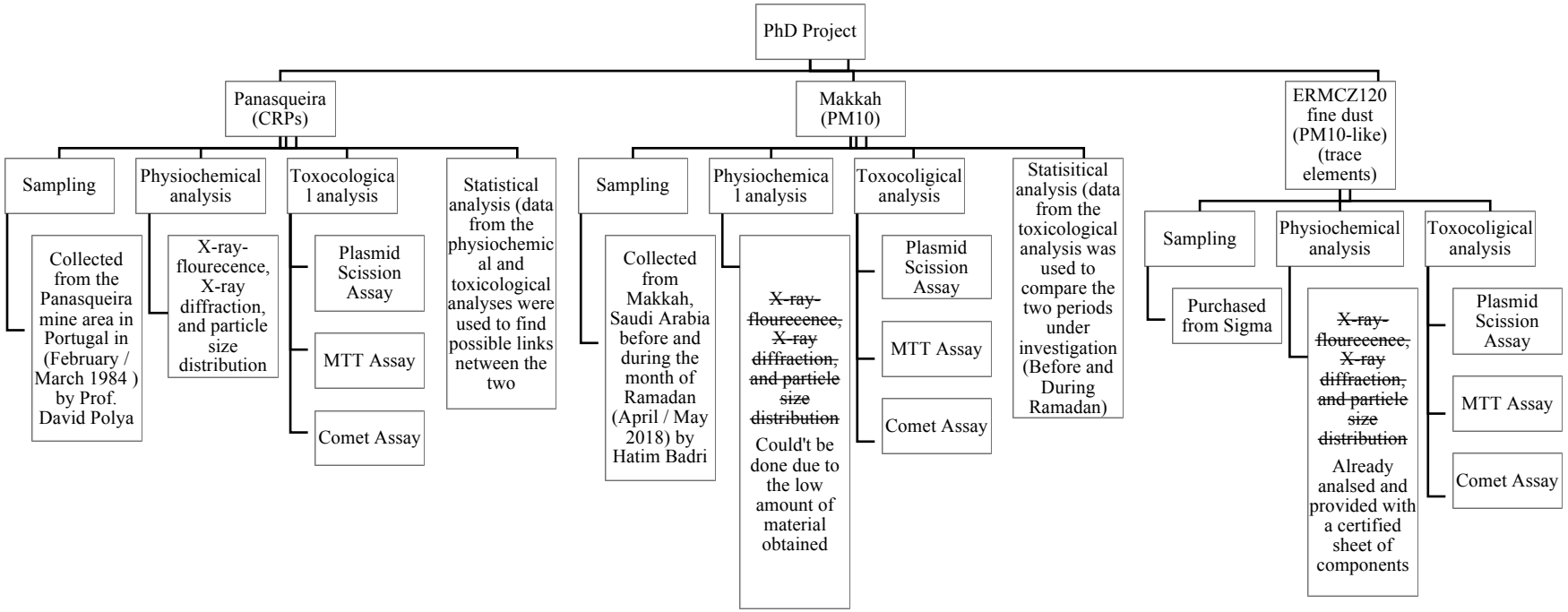

Figure 1.2, Summary of PhD Project Sub-Projects, Major Types of analysis undertaken and key analytical techniques used

1.3 Structure of the thesis

This thesis is presented in an "alternative format"; this means that results obtained from this project will be presented as series manuscripts in preparation, under review or published in peer-reviewed journals. Even though this may result in some repetition (particularly in relation to methodology) between chapters, this format will allow for an easier transfer of the thesis's chapters into manuscripts ready for submission to journals. The list of these manuscripts is given below. *Table 1.1* below provides a brief overview of each chapter of the thesis along with a summary of the contributions of co-authors other than this thesis' author, who was the lead (1st) author on each of the manuscripts.

- Badri, H., Polya, D.A., and Povey, A.C. Geochemical compositional controls on DNA strand breaks induced in in vitro cell-free assays by crushed rock powders from the Panasqueira mine area, Portugal. Manuscript, accepted (21st June 2020) for publication. Journal: Environmental Geochemistry and Health. (*Chapter 3*)
- Badri, H., Polya, D.A., and Povey, A.C. Geochemical compositional controls on DNA damage and cell viability induced in in vitro cell-based assays by crushed rock powders from the Panasqueira mine area, Portugal: A comparison with a cell-free assay. Manuscript, in preparation. Target journal: Toxicology letters. (*Chapter 4*)
- Badri, H., Polya, D.A., Povey, A.C. and Habeebullah, T.M. DNA strand breaks induced by PM_{10} from Makkah, Saudi Arabia: Association with meteorological proxies for source and composition. Manuscript, in

preparation. Target journal: International Journal of Environmental Research and Public Health. (*Chapter 5*)

- Badri, H., Polya, D.A., Povey, A.C. and Habeebullah, T.M. The cytotoxic and genotoxic effects of PM_{10} from Makkah, Saudi Arabia on A549 lung cells: A comparison with a cell-free assay. Manuscript, in preparation. Target journal: Mutagenesis. (*Chapter 6*)

Table 1.1, A summary of the thesis's structure and a brief overview of each chapter

Chapter	Title	Contents	Authors and their contributions
One	Introduction	An overview of the thesis, its perspective, and aims and objectives	• Hatim Badri
Two	Literature review	• An overview of air pollution in general and particulate matter in particular including the key processes affecting PM, PM size, PM chemical composition, and PM health effects. • An overview of PM measurement instruments and techniques and PM source apportionment. • An overview of techniques for investigating PM toxic effects. • A brief overview on the Panasequira mine area in Portugal is presented. A detailed overview is not provided because what's important about the CRPs in relation to the aims and objectives of	• Hatim Badri

		this thesis is their physical, chemical, and mineralogical characteristics. • An overview of PM pollution in Saudi Arabia and the toxicological and epidemiological studies conducted there. • A brief overview on CRMs, some of the types available and their respective prices.	
Three	Geochemical compositional controls on DNA strand breaks induced in an in-vitro cell-free assay by crushed rock powders from the Panasqueira mine area, Portugal (Badri et al. 2020a)	• Detailed information on the physical, chemical, and mineralogical composition of the CRPs. • Detailed information on the CRPs' effect on DNA damage using the cell-free plasmid scission assay. • A statistical analysis highlighting the most likely predictors of DNA damage.	• Hatim Badri: (Lead author – carried out the experimental work) • David Polya (collected and analysed the samples using XRF - overall supervision and helped with the chemical and particle number and size distribution analysis using XRD and particle sizers - supervised and helped in designing, planning, and writing) • Andy Povey (supervised and helped with the Plasmid Scission Assay)
Four	Geochemical compositional controls on DNA damage and cell viability induced in in vitro cell-based assays by crushed rock powders from the Panasqueira mine area, Portugal: A comparison with a	• Detailed information on the effect of CRPs on DNA damage and cell viability using the cell-based Neutral Comet and MTT assays. • A statistical analysis highlighting the most likely predictors of the endpoints under investigation.	• Hatim Badri: (Lead author – carried out the experimental work) • David Polya (collected and analysed the samples using XRF - overall supervision and helped with the chemical and

	cell-free assay (Badri et al. 2020b)	• A comparison of effects observed with those found a cell-free Plasmid Scission assay.	particle number and size distribution analysis using XRD particle sizers - supervised and helped in designing, planning, and writing) Andy Povey (supervised and helped with toxicological work using the Neutral Comet and MTT assays)
Five	DNA damage induced by PM_{10} from Makkah, Saudi Arabia: Association with meteorological proxies for source and composition (Badri et al. 2020c)	• An overview of the study area, sampling procedure, and PM_{10} extraction. • A statistical analysis into the relationship between the daily PM_{10} concentrations and individual meteorological parameters. • Detailed information on the effect of PM_{10} on DNA damage using the cell-free plasmid scission assay. • A statistical analysis into the relationship between the DNA damage and individual meteorological parameters.	• Hatim Badri: (Lead author – carried out the experimental work) • David Polya (overall supervision; helped in designing, planning, and writing) • Andy Povey (supervised and helped with the toxicological analysis using the Plasmid Scission Assay) • Turki Habeebullah (helped in reviewing and provided sampling equipment and assisted in acquiring permissions for sampling)
Six	The cytotoxic and genotoxic effects of PM_{10} from Makkah, Saudi Arabia on A549 lung cells: A comparison with a cell-free assay (Badri et al. 2020d)	• Detailed information on the methods and results for the effect of PM_{10} on DNA damage and cell viability using the cell-based comet and MTT assays. • A comparison of effects observed with	• Hatim Badri: (Lead author – carried out the experimental work) • David Polya (overall supervision and helped in designing, planning, and writing)

		those found a cell-free Plasmid Scission assay.	• Andy Povey (supervised and helped with the toxicological work using the Neutral Comet and MTT assays) Turki Habeebullah (provided sampling equipment and assisted in acquiring permissions for sampling)
Seven	Summary and findings	• A summary of the findings of the thesis including a general comparison between PM_{10} and CRPs, a general comparison between the assays used, and the difference between the cell-free and cell-based assays and how their results might be linked. • The limitations, potential impacts, and recommendations for future work.	• Hatim Badri

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Badri, H., Poyla, D., & Povey, A. (2020a). Geochemical compositional controls on DNA strand breaks induced in an in-vitro cell-free assay by crushed rock powders from the Panasqueira mine area, Portugal *Accepted (21st June 2020) for publication - Environmental Geochemistry and Health*

Badri, H., Poyla, D., & Povey, A. (2020b). Geochemical compositional controls on DNA damage and cell viability induced in in vitro cell-based assays by crushed rock powders from the Panasqueira mine area, Portugal: A comparison with a cell-free assay *In preparation, Toxicology letters*.

Badri, H., Poyla, D.,a Povey, A., & Habeebullah, T. (2020c). DNA strand breaks induced by PM10 from Makkah, Saudi Arabia: Association with meteorological proxies for source and composition *In preparation, International Journal of Environmental Research and Public Health*.

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Vuong, N. Q., Breznan, D., Goegan, P., O'Brien, J. S., Williams, A., Karthikeyan, S., et al. (2017). In vitro toxicoproteomic analysis of A549 human lung epithelial cells exposed to urban air particulate matter and its water-soluble and insoluble fractions. *Particle and fibre toxicology, 14*(1), 39, doi:10.1186/s12989-017-0220-6.

Chapter 2: Literature review

The nature of this project necessitates the need for a proper understanding of PM and the various issues associated with it. This chapter reviews literature on the following topics related to the research problem: (1) air pollution in general; (2) fine particles and particulate matter (PM) including information on the key processes affecting PM, physical and chemical characteristics of PM, PM and human health, common PM mass concentration and number and size distribution measurement methods, common PM source apportionment techniques, common methods for chemical and mineralogical analysis of PM, and common cell-free and cell based assays for PM toxicity testing (*Figure 2.1*); (3) a brief introduction on an area where samples of known chemical composition and consistent grain size distribution have been collected (Panasqueira mine area) including information on heavy metals and metalloids pollution in the Panasqueira mine area and the exposure studies conducted there (*Figure 2.2*); (4) PM pollution in an area representative of urban environments, Saudi cities including Makkah, including information on PM concentration, size distribution, chemical composition, and epidemiological and toxicological studies previously conducted there (*Figure 2.3*); (5) a brief introduction on Certified Reference Materials (CRMs) including information on the types available and their respective prices; (6) and a summary and conclusion.

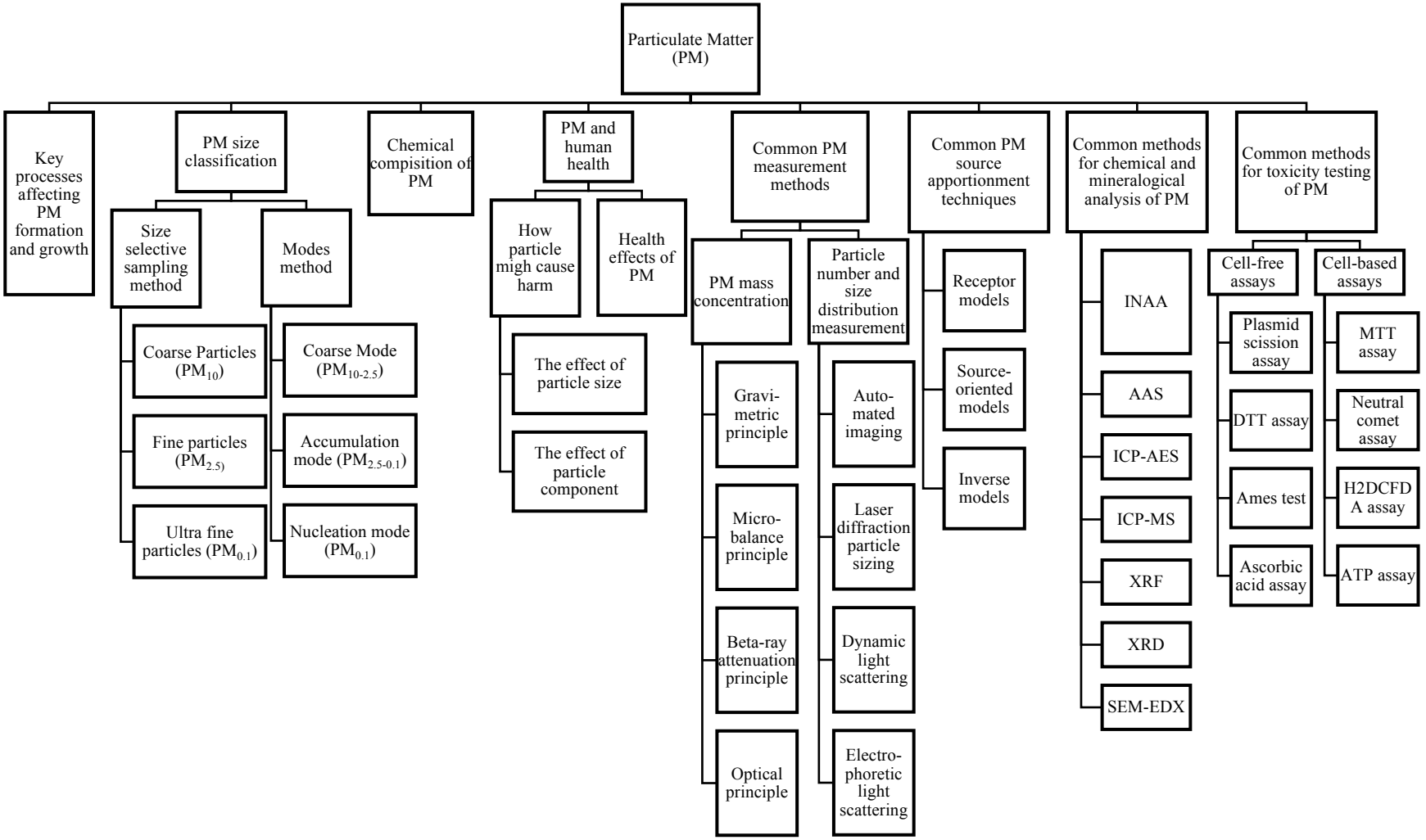


Figure 2.1, Structure of Literature Review of Relevant Issues Related to PM

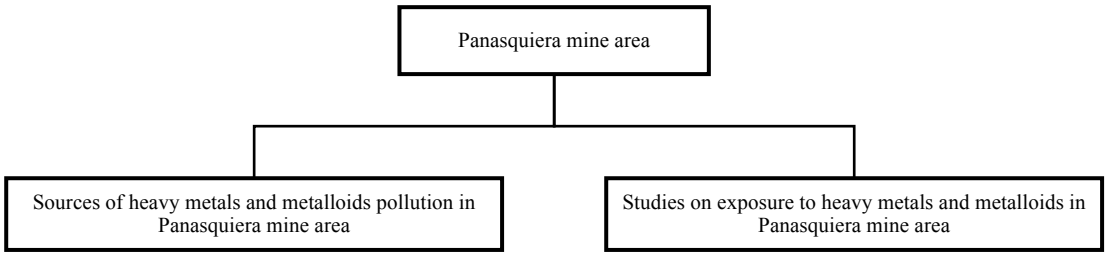


Figure 2.2, Methodological approach used for understanding heavy metal pollution in Panasquiera mine area

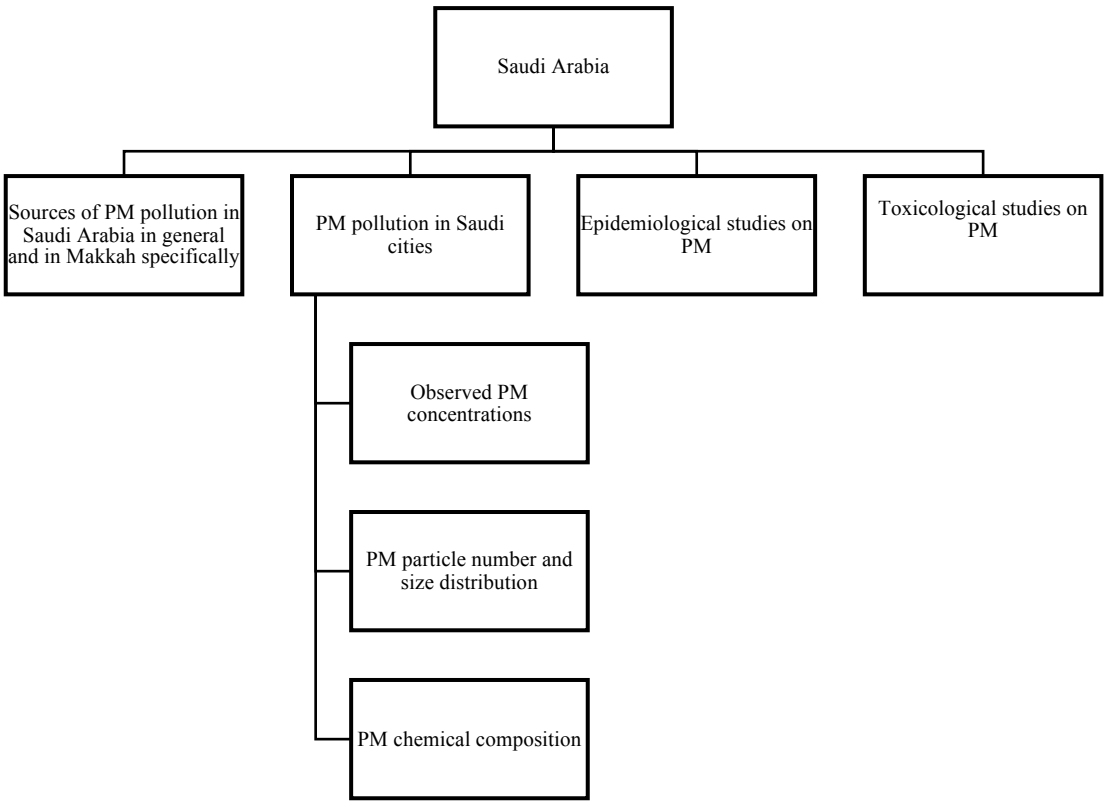


Figure 2.3, Methodological approach used for understanding PM pollution in Saudi Arabia

2.1 Air pollution

Clean air is a fundamental requirement for health and well-being (Lemes De Oliveira 2017). Unfortunately, air is continuously being polluted by anthropogenic and natural sources (Mabahwi et al. 2014). Air pollution is defined as the contamination of the outdoor or indoor environment by foreign substances that have harmful or poisonous effects (WHO 2017). It is an issue of serious concern to the international community due to its wide-ranging and deleterious effects on human health (Brunekreef and Holgate 2002; Du et al. 2016; Franchini and Mannucci 2015; Franchini et al. 2016; Chan-Yeung 2000). The main routes of exposure to air pollutants in humans are inhalation and ingestion, while exposure via dermal contact is possible, it is considered to be a minor route. (Thron 1996; Kampa and Castanas 2008; Salma et al. 2002).

Numerous studies have found that exposure to polluted air is associated with an increase in mortality and hospital admissions (Brunekreef and Holgate 2002; Achilleos et al. 2019; Zhang et al. 2020; Phosri et al. 2019). The impact polluted air has on human health varies depending on the dose, duration of exposure, composition of the air pollutants, and individual susceptibility with the severity of effects ranging from eye irritation to death (Kampa and Castanas 2008; Cohen et al. 2005). The World Health Organization describes air pollution as a public health emergency. It was classified as a *Group 1* carcinogen by the international agency for research on cancer (International Agency for Research on Cancer 2013). According to a report in 2006, there are more than 2 million premature deaths each year that can be attributed to air pollution (WHO 2006). In 2012, the death toll from exposure to polluted air reached around 7 million premature deaths, one in eight of total global deaths, per year worldwide which is a significant increase from previous findings (WHO 2014).

Lelieveld et al., (2015) independently estimated that 3.3 million premature deaths per year could be attributed to outdoor air pollution mostly from $PM_{2.5}$ (Lelieveld et al. 2015). In addition to that, it is estimated that Indoor air pollution causes around 3.5 million premature deaths per year with developing countries in Asia being mostly affected (Lim et al. 2013; Lelieveld et al. 2015).

Air pollutants can be emitted from mobile, stationary or indoor sources (Chan-Yeung 2000; WHO 2006). There are some naturally occurring processes, such as volcanos and forest fires which are considered stationary sources, that can release pollutants into the atmosphere (Misra et al. 2001; Kim et al. 2015; Atkinson et al. 2010; Hime et al. 2015). However, air pollution is also caused by anthropogenic activities (Srimuruganandam and Nagendra 2012). Some of the most common anthropogenic sources of air pollution are motor-vehicles, power stations, and household combustions devices which are considered mobile, stationary, and indoor sources respectively (Holman 1999; Kampa and Castanas 2008; WHO 2017; Chan-Yeung 2000; WHO 2006; Stern 2014). Pollutants emitted from these sources can either be particulate matter, gaseous pollutants, persistent organic pollutants, and/or heavy metals (Bernstein et al. 2004; Kampa and Castanas 2008; Franchini et al. 2016; He 2004).

Urban areas and areas with high population densities suffer from extremely high air pollution concentrations (Srimuruganandam and Nagendra 2012; Ozcan and Cubukcu 2018) with particulate matter (PM) specifically being one of the major air pollutants in such areas (Molina et al. 2017). The term PM refers to a complex mixture of solid particles, liquid droplets, and semi-volatile components with different sizes, chemical composition, and origin suspended in the air that can cause harm and suffering to

humans (Tsai et al. 2000; Colbeck and Lazaridis 2010; U.S. EPA. 2018b). Many epidemiological studies (Pope et al. 2008; Pope et al. 2014; Pope III and Dockery 2006; Riediker et al. 2019) and experimental studies (Kang et al. 2002; Wagner et al. 2012; Watkinson et al. 2003; Dumax-Vorzet et al. 2015) have shown that the risk for mortality and morbidity from pulmonary and cardiovascular disease significantly increases from exposure to PM. Moreover, PM contributes to the formation of smog, acid rain and other serious environmental problems that can affect human life (U.S. EPA. 2018a).

2.2 Fine particles and particulate matter (PM)

Particulate matter is formed by direct generation from natural and anthropogenic sources or by conversion of gaseous pollutants into particles (Zhang et al. 2015). Understanding the physical and chemical properties of PM and the processes affecting them is a crucial step in understanding their possible emission sources and their effects on human health (Davidson et al. 2005).

2.2.1 Key processes affecting PM formation and growth

Particles emitted directly from the source into the atmosphere are called primary PM while those that result from gas to particle conversion (condensation and nucleation of gaseous precursors) are called secondary – both types are subjected to chemical reactions, physical transformations, cloud processing, and transportation and removal from the atmosphere (Zhang et al. 2015; Pöschl 2005; Williams et al. 2002). Other than interacting with each other, particles react with gases, clouds, rain, and sunlight which results in changing their physical and chemical properties (Pöschl 2002). Particles would change in size, structure, and composition in a process referred to as

atmospheric aging (Rudich et al. 2007). This occurs through a number of ways including evaporation or condensation of vapour, coagulation, or by chemical reactions (Friebel et al. 2019; Kim and Park 2012; Friebel and Mensah 2019). The process of atmospheric aging occurs more effectively in clouds which are formed through condensation of water vapour on tiny particles floating in the air (Gunthe et al. 2011). Inside the clouds, aerosol particles are modified and released again into the atmosphere (Kim and Park 2012; Karydis et al. 2011). After their release into the atmosphere, particles are either scavenged by clouds and removed from the atmosphere in a process called wet deposition or fall from the atmosphere by gravity in a process called dry deposition (Williams et al. 2002; Pöschl 2005). *Figure 2.4* summarizes the key processes affecting PM formation and growth.

2.2.2 Size of PM

PM size is extremely important and is the one characteristic used for classification. Everything from atmospheric deposition rates and residence times to deposition patterns in the lungs are affected by PM size (Longhin 2012).

Since particles have irregular shapes, their size is described by the term Aerodynamic Equivalent Diameter (AED). This means that a particle's aerodynamic diameter is that of an idealised sphere whose density is 1 g/cm^{-3} and that would settle in still air the same way as the particle in question (Goodfellow 2001).

Classification of particles according to their size can be done using two methods; the size selective sampling method, which is based on the 50 % cut-off point of a sampling device and the modes method, which is based on the observed size distributions and

formation mechanisms (Longhin 2012). The size selective sampling method is the most commonly used in research due to its use in air quality standards and legislation.

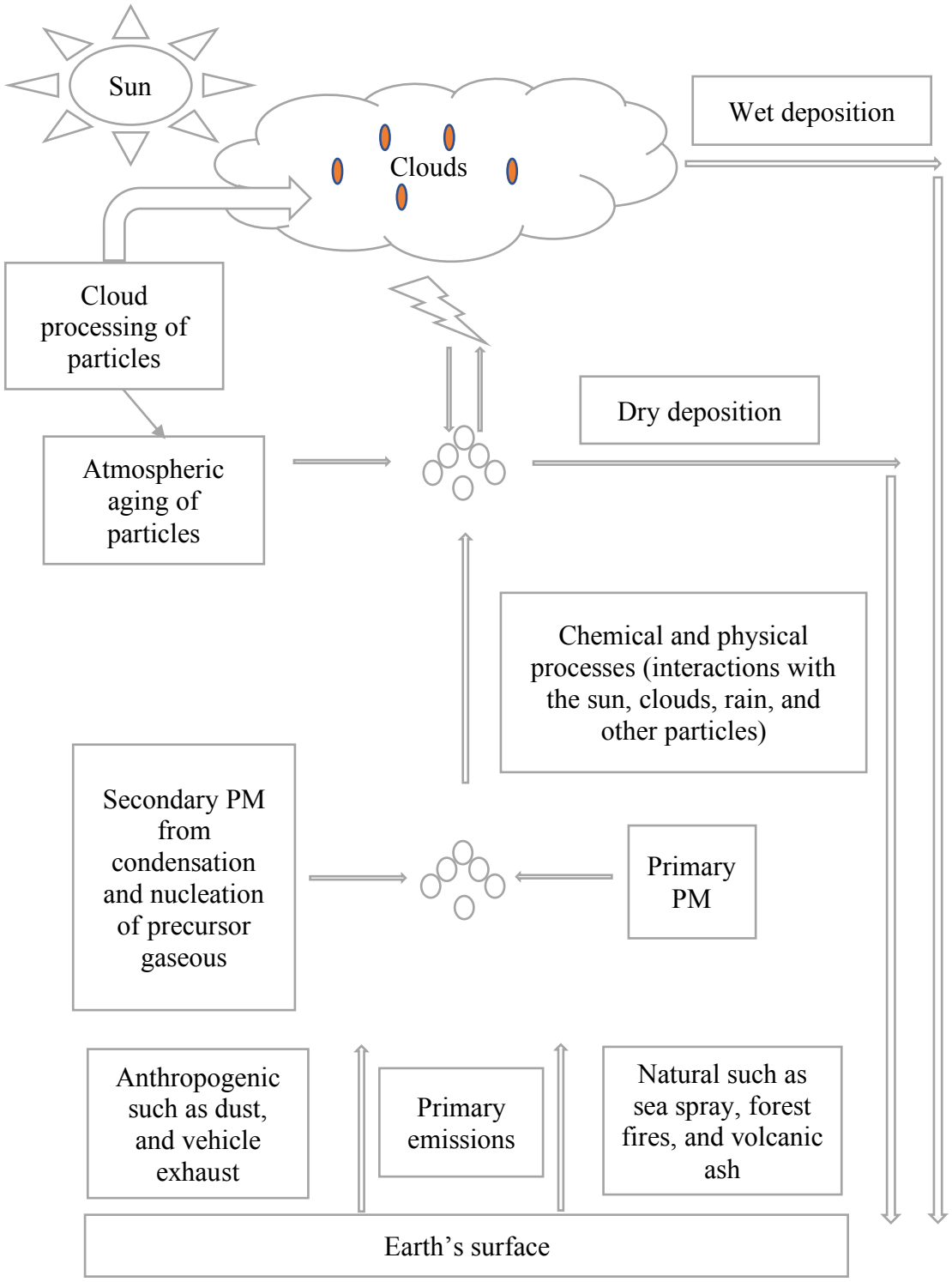


Figure 2.4 Summary of key processes affecting PM formation and growth

2.2.2.1 Classification of particles using the size selective sampling method

The term "total suspended particles" refers to particles of any size, suspended in the atmosphere (de Kok et al. 2006; Hime et al. 2015; Longhin 2012). Other than that, there are coarse, fine, and ultra-fine particles. The term PM_{10} is used to describe coarse particles or particles that are less than 10 to 2.5 µm in diameter. Fine particles are those that are 0.1 to 2.5 µm in diameter and are referred to as $PM_{2.5}$. Ultra-fine particles are those that are less than 0.1 µm in diameter and are known as $PM_{0.1}$ *(Figure 2.5)* (Valavanidis et al. 2008; Dockery and Pope 1994; Schwarze et al. 2006; Ibald-Mulli et al. 2002; Pöschl 2005)

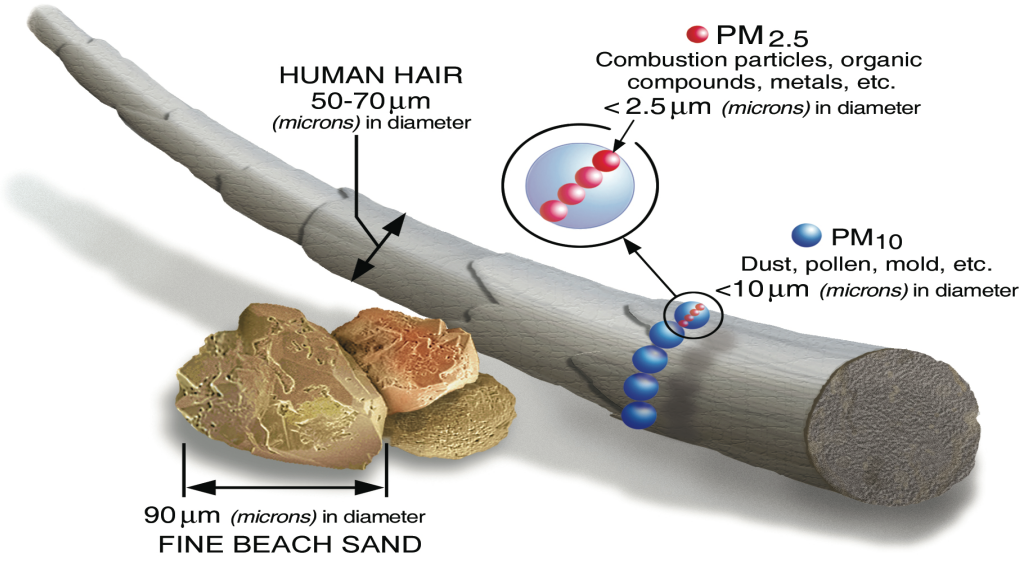


Figure 2.5 PM size fractions: showing the aerodynamic diameters in comparison to the diameter of a human hair, (From U.S. EPA. (2018b))

2.2.2.1.1 Coarse particles

Coarse airborne particles usually have a basic pH and are mostly formed from sea salt and soil (crustal material), pollens, fungal spores, and biological debris through mechanical processes such as wind erosion and re-suspension by traffic (Pye et al.

2019; Després et al. 2012). Due to their size, coarse particles tend to have a short life span (a few hours) in the atmosphere before being deposited on surfaces (Hinds 2012). Even though coarse particles can travel long distances and contribute to pollution on a regional and global level, their effects usually happen in proximity to their source (Salmond and McKendry 2009). Once inhaled, they are deposited in the upper respiratory tract (Dockery and Pope 1994; Schwarze et al. 2006; Hinds 2012).

2.2.2.1.2 Fine particles

Fine particles are usually acidic and result directly from several processes including emissions, condensation of sulphates, nitrates, and organic gaseous material, reaction in water droplets, and coagulation of smaller ultra-fine particles (Seinfeld et al. 1998; Hime et al. 2015; John et al. 1990; Pye et al. 2019). They include particles emitted from the combustion of fossil fuel and the photochemical reactions of volatile organic compounds and oxides in the presence of sunlight (Hinds 2012). Sulphate and nitrates usually make up the largest portion of small particles by mass (Dockery and Pope 1994). Unlike coarse particles, fine particles have a longer life span and can remain suspended in the atmosphere for weeks and can be transported for long distances (Brook et al. 1997; Yang et al. 2017). Therefore, fine particles are more consistently distributed on a regional scale compared to ultra-fine and coarse particles (Salmond and McKendry 2009). Fine particles have the ability to absorb trace elements and potentially toxic molecules due to their large mass and surface area (Hu et al. 2012; Bai et al. 2019). Once inhaled, fine particles are deposited in the lower respiratory tract (Hime et al. 2015).

2.2.2.1.3 Ultra-fine particles

Ultra-fine particles are a result of combustion processes and the nucleation of atmospheric gases (Sioutas et al. 2005). An example of ultra-fine particles are diesel exhaust particles; these are largely insoluble due to their particulate carbon core and are present in the atmosphere as single particles or aggregates of particles (Kittelson et al. 2004). As a group, they tend to aggregate with sulphates, metals and hydrocarbons (Donaldson et al. 2001). Ultra-fine particles do not remain suspended in the atmosphere for a long time as they are removed through diffusion to surfaces, coagulation, and adsorption and condensation which is an indications of their inability to travel long distances and their great temporal and spatial variability (Salmond and McKendry 2009). Once inhaled, ultra-fine particles are deposited in tissues outside the lung due to their small size (Hime et al. 2015).

2.2.2.2 Classification of particles using the modes method

Particles in the atmosphere are classified into three populations or "modes" which are coarse mode ($PM_{10-2.5}$), accumulation mode ($PM_{2.5-0.1}$), and nucleation mode ($PM_{0.1}$) (Twigg and Phillips 2009). Each mode is unique as they all differ in total masses, volumes, surface areas and numbers of particles *(Figure 2.6)* (U.S. EPA. 2004). Coarse and accumulation modes represent the largest portion of total PM mass respectively. Coarse particles make up around 90 - 95 % of the total suspended particulate matter while smaller ones only make up around 1 - 8 % of the total mass (Brauer et al. 2001; Valavanidis et al. 2008). Even though the percentage of fine and ultra-fine particles is quite low, their presence in the environment is higher in number than coarse particles. Also, their total surface area is bigger (Valavanidis et al. 2008; Cao et al. 2013; Whitby et al. 1972; Heintzenberg 1989; Hime et al. 2015).

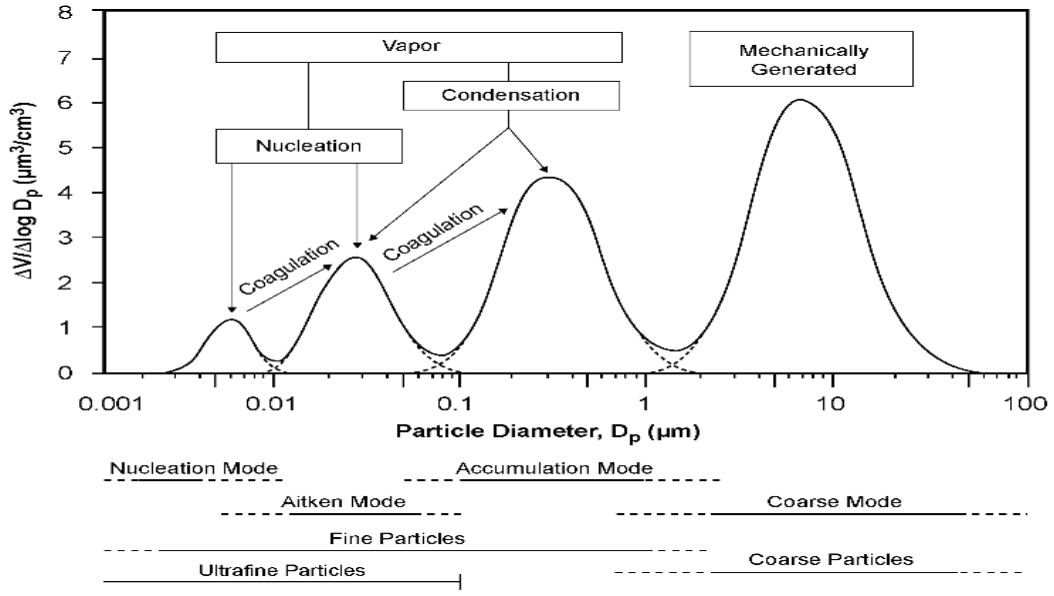


Figure 2.6 Idealized modal classification; coarse, accumulation, Aitken and nucleation, (From U.S. EPA. (2004))

2.2.3 Chemical composition of airborne particulate matter

The chemical components of PM are highly diverse. Several factors such as combustion sources, climate, season, and type of urban or industrial pollution influence that diversity (Valavanidis et al. 2008; Lighty et al. 2000; Hime et al. 2015; Pöschl 2005).

Harrison and Jones (1995), Harrison and Yin (2000) and (2004), and Harrison et al. (2004) reviewed the "bulk chemical composition" for urban areas in the UK and globally. The term refers to the abundance of the main chemical components in PM. These main chemical components are sulphate, nitrate, ammonium, chloride, elemental and organic carbon, and crustal and biological material. Nevertheless, there are other minor components such as trace metals and trace organic compounds (Harrison and Jones 1995; Harrison and Yin 2000; Harrison and Yin 2004; Harrison et al. 2004).

Sulphate and nitrate are formed through the oxidation of sulphur dioxide and nitrogen oxides in the atmosphere respectively (Kong et al. 2014). Sulphate would then be present as fine fractions of ammonium sulphate and sodium sulphate while nitrate would be present as ammonium nitrate and sodium nitrate (Petetin et al. 2016; Wang et al. 2015). The neutralisation of sulphuric acid and nitric acids by atmospheric ammonia results in the formation of ammonium (Wang et al. 2015). Chloride is present in the atmosphere from sea salt. Also, it can be present in the form of ammonium chloride (Wang et al. 2017). Carbon compounds mainly occur in two forms which are Organic Carbon and Elemental or Black Carbon. These two are present in the atmosphere because of incomplete combustion processes of which road traffic is its main source in urban areas (Kucbel et al. 2016). Crustal material, which is mainly present in coarse particles, is usually soil and wind-blown dust (Clements et al. 2014). Biological material is present in different sizes and shapes in the atmosphere of which pollens, spores, bacteria, and viruses are examples of such material (Després et al. 2012). Metals have anthropogenic and natural sources such as industrial emissions and sea spray (Popoola et al. 2018). Sodium, magnesium, potassium, and calcium mostly originate from sea spray and terrestrial dust and are mainly found in the coarse fraction while heavy metals such as Lead on the other hand mostly originate from industrial emissions and are mainly found in the fine fraction (Harrison and Yin 2004; Harrison et al. 2004; Harrison and Jones 1995; Harrison and Yin 2000).

2.2.4 Particulate matter and human health

Legislation and proper control on vehicle emissions and industries contributed to the reduction of PM pollution episodes in urban areas. Unfortunately, however, the issue of adverse health effects and increasing number of deaths yearly has not been resolved

(Holland 1979; Valavanidis et al. 2008; WHO 2006, 2014; Lelieveld et al. 2015). This is mainly due to the change in air pollution in current times. Better and cleaner technologies have been applied to the combustion of fossil fuels and their emission concentrations are much lower than they used to be (Lipman 2020). Nevertheless, the problem still exists due to the sharp increase in motor vehicle use in urban areas world-wide and the regulatory non-compliance or misleading information from car manufactures over emissions (Hooftman et al. 2018; Havaei-Ahary and Heyworth 2019). This led to fine and ultra-fine PM to gain prominence among other pollutants. Although PM is not the only air pollutant, it is considered as the most associated with adverse health effects (Valavanidis et al. 2008; Anderson et al. 2012; Rückerl et al. 2011; Lippmann and Chen 2009).

Numerous health effects have been associated with exposure to PM and they are well documented in the scientific literature (Dockery et al. 1993; Pope III et al. 1995; Abbey et al. 1999; Harrison and Yin 2004; Schwartz et al. 1999; Pope 3rd et al. 1999; Schwartz and Neas 2000; Castillejos et al. 2000; Choudhury et al. 1997; Ostro et al. 2000; Mar et al. 2000; Pope III et al. 2002; Schwarze et al. 2006; Laden et al. 2000; Pope 3rd 1989). In urban areas, research has indicated that both short term (hours to days) and long term (months to years) exposure has been associated with increased hospital admissions, respiratory symptoms, aggravation of already existing cardiovascular and respiratory diseases, and premature mortality from cardiovascular and respiratory diseases and lung cancer (WHO 2013; Dominici et al. 2006; Wellenius et al. 2005; Wellenius et al. 2006; Zanobetti and Schwartz 2005; Brook et al. 2010; Dockery et al. 1993; Laden et al. 2000; Pope 3rd 1989; Pope III et al. 1995). Also, symptoms such as low birth weight in infants, pre-term deliveries, and potentially

foetal and infant death have been associated with exposure to high levels of PM (Guaita et al. 2011; Halonen et al. 2009; Perez et al. 2012; Samoli et al. 2008; Dockery et al. 1993; Pope III et al. 1995).

Vulnerable groups such those with existing heart or lung disease, the elderly, and children are at a higher risk from exposure to PM than healthy people (U.S. EPA. 2018a). For example, lung development is affected in children exposed to PM, including reversible deficits in lung function, chronically reduced lung growth rate, and a deficit in long-term lung function (Brauer et al. 2012; WHO 2013; Kim et al. 2015).

2.2.4.1 How particles may cause harm

The pathogenicity of PM depends on their deposition in the respiratory tract, which depends on and the aerodynamic diameter of the particle, and on their transportation around the body and interaction with other tissues (WHO 2000). The main cause of PM related health effects has been linked to the size and components of particles (Akhtar et al. 2014).

2.2.4.1.1 The effect of particle size

The human respiratory tract is divided into two regions, the extrathoracic and intrathoracic region, based on size, structure, and function (Cheng and Swift 1995). The extrathoracic region acts as the first line of defence in protecting critical gas exchange processes occurring in the intrathoracic region (Sarangapani 2000). It consists of the nose, pharynx, mouth, and larynx. The deep part of the lung is protected by the intrathoracic region in which it acts as the conducting airways and comprised

of the tracheobronchial and alveolar regions (Brown et al. 2013; Dockery and Pope 1994; Plummer 2011).

Coarse particles with a diameter greater than 10 µm are easily filtered by the cilia and mucus and they usually end up either in the trachea or bronchi (Cadelis et al. 2014a). Small particles are known to have a greater effect on human health compared to coarse particles (CDC 2019). Particles with an aerodynamic diameter of 5 - 10 µm are usually deposited in the tracheobronchial region. On the other hand, those with an aerodynamic diameter between 1 - 5 µm are usually deposited in the alveolar region where the gas exchange occurs (Löndahl et al. 2006). Such particles can affect gas exchange, penetrate the lung, and eventually enter the circulatory system (Fu et al. 2011). Particles with an aerodynamic diameter below 1 µm have the ability to penetrate alveoli and then translocate into other cell tissues or circulation systems (Dockery and Pope 1994; Kim et al. 2015; Valavanidis et al. 2008). *Figure 2.7* shows the deposition potential for particles of different sizes.

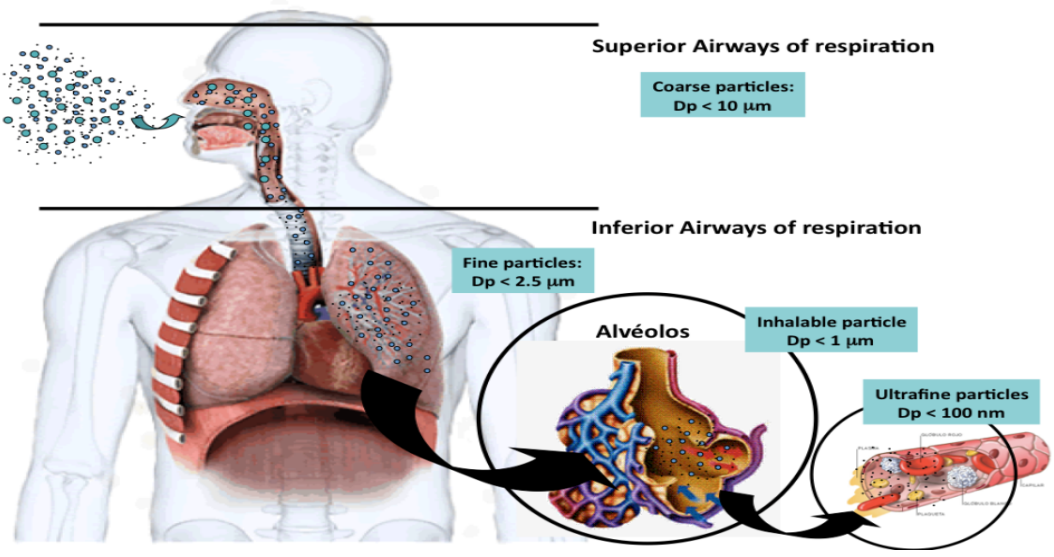


Figure 2.7 Deposition potential for particles of varying sizes, (From Guarieiro and Guarieiro (2013))

Particles can be eliminated from the human body through several mechanisms. Particles deposited in the upper respiratory tract (nasal and tracheobronchial regions) can be cleared by coughing, sneezing, or swallowing (Cadelis et al. 2014b). Lung macrophages also contribute to PM removal and transportation through phagocytosis (Kim et al. 2015; Dockery and Pope 1994; Plummer 2011).

Clearance pathways for coarse particles are known and documented. However, the same could not be said about ultra-fine particles. Many studies (Roth et al. 1997; Roth et al. 1994, 1993; Joshi et al. 2016; Terzano et al. 2010; Cassee et al. 2011) suggested that ultra-fine particle have the potential to evade phagocytosis. Therefore, they are usually retained in the lung. A series of pulmonary and systemic pro-inflammatory reactions are triggered by oxidative stress and pro-inflammatory influences which result from the prolonged interaction with epithelial cells that is caused by retention (Donaldson et al. 2001; Donaldson et al. 2004). Prolonged retention time might also lead to the translocation of ultra-fine particles throughout the internal environment (Semmler-Behnke et al. 2007; Ferin 1994; Ferin et al. 1992; Oberdörster et al. 2002). This leads to the direct interaction with interstitial and endothelial cells and in return triggers pro-inflammatory and pro-coagulant effects (Plummer 2011). Although there is some controversy regarding ultra-fine particles accessing the circulation and targeting organs, several animal model studies have proved it (Nemmar et al. 2002; Nemmar et al. 2001; Oberdörster et al. 2002; Kreyling et al. 2002; Kreyling et al. 2009; Saber et al. 2013). It hypothesized that developing systemic and cardiovascular responses after exposure to PM is a consequence of PM translocation from the lung into the systemic circulation and other target organs (Plummer 2011). *Figure 2.8* shows the pathways linking PM exposure and biological effects.

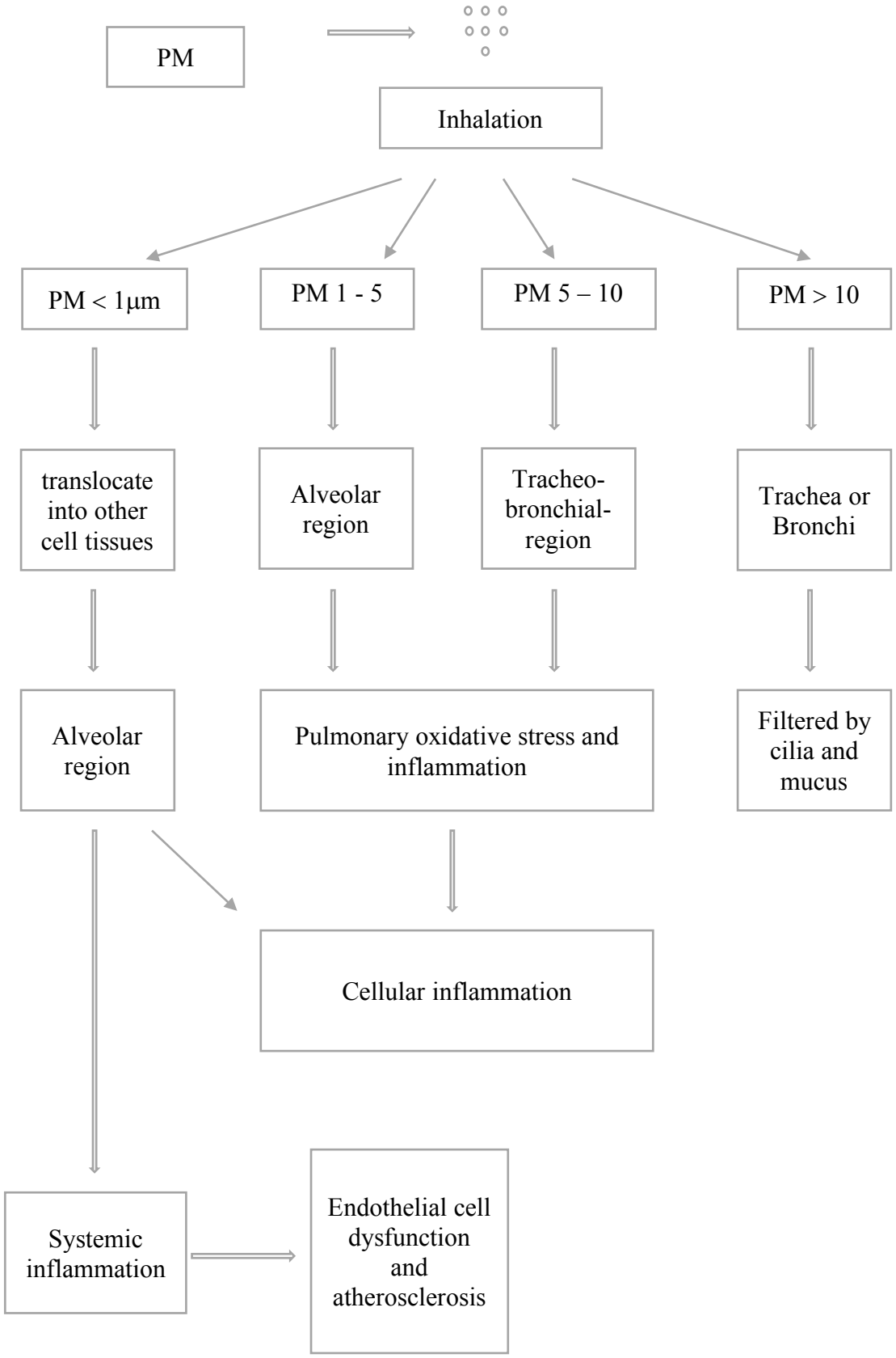


Figure 2.8 The pathways linking PM exposure and biological effects

2.2.4.1.2 The effect of particle components

Cellular and tissue damage within the pulmonary and systemic environments occur as a result of two mechanisms; oxidative stress and inflammation (Donaldson et al. 2005; Magnani et al. 2016). There is a biological link between these two mechanisms as inflammatory processes might cause oxidative stress and are driven by it (Biswas 2016). Acute inflammation is the primary response to any foreign agent introduced to the lung. The initiation of inflammation could potentially cause cellular injury and may induce vascular effects. It can also contribute to systemic inflammations through leukocyte production from cytokines and chemokines (Van Eeden et al. 2001; Eeden and Hogg 2002; Plummer 2011). Oxidative stress causes inflammation through reactive oxygen species. Cell and tissue injury can occur from reactive oxygen species that are produced by a variety of chemical and mineralogical components present in PM through the Fenton reaction. As the damage takes place, inflammation begins or worsens if already existed (Cho et al. 2018; Hong et al. 2016; Valavanidis et al. 2013).

Iron is a transition metal commonly present in PM. It is one of the transition metals that is abundant in the earth's crust. Previous studies have indicated that excessive exposure to iron could cause symptoms such as persistent airway inflammation and compromised lung function leading to cardiovascular and respiratory diseases (Cloonan et al. 2017). Also, excessive exposure could result in its accumulation in the brain which eventually might lead to the development of Parkinson's and Alzheimer diseases (Quintana et al. 2006; Chen et al. 1990; Patra et al. 2016). Copper is also a transition metal that can be released in the air from a number of industries. High levels of copper in the human body can cause coughs, chills and muscle aches and result in Wilson's disease (Romo-Kroger et al. 1989; Patra et al. 2016; U.S. AF 1990). Lead,

another type of transition metals, can accumulate in the environment from human activities such as fossil fuel burning and mining. Acute exposure to lead can lead to a number of health effects including loss of appetite and fatigue amongst others. Chronic exposure on the other hand causes more serious effects such as birth defects and a variety of cancers including lung, stomach, brain, kidney, and bladder amongst others (Martin and Griswold 2009; Silbergeld 2003). Just like iron, copper, and lead; mercury is also a transition metal. However, it is the most toxic in the environment. Many industries such as pharmaceuticals and the agricultural industry release mercury in the environment. Exposure to mercury in general can cause many health problems such as memory problems and changes in vision and hearing. Exposure to metallic mercury in particular can cause lung damage and increased heart rate and or blood pressure even if it was at low concentrations and for a short period of time. Excessive exposure can result in mercury poisoning and lead to a disease mostly referred to as pink disease or acrodynia (Morais et al. 2012; Jaishankar et al. 2014). Beryllium is a mineral found in nature and can also be emitted from the activities of certain industries. Exposure to beryllium has been associated with a variety of symptoms including inflammation of the respiratory tract tissues, hepatic necrosis, kidney stones, and weight loss (Smith et al. 2002). Also, it has been associated with a number of adverse health outcomes including Beryllium disease and cancer (Smith et al. 2002; Cooper and Harrison 2009; Middleton and Kowalski 2010; Kreiss et al. 2007; Patra et al. 2016). Mica is a complex silicate to which exposure to could cause varying degrees of lung damage. This could range from pulmonary overload, cough, and shortness of breath to sever cases of pneumoconiosis and fibrosis (Heimann et al. 1953; Falgayrac et al. 2011; Patra et al. 2016; Hulo et al. 2013). Crystalline silica is a basic component of soil, sand, and many other materials. Excessive exposure to crystalline silica can cause symptoms such as

shortness of breath, chest pain, or respiratory failure and can lead to silicosis, lung cancer, obstruction of airways, and lymph node fibrosis amongst others (Donoghue 2004; Hendryx and Ahern 2008; Patra et al. 2016; OSHA 2002). Asbestos is composed of a number of naturally occurring fibrous minerals that can easily become airborne and inhaled. It is used in a number of products and industries. Exposure to asbestos could cause symptoms such as shortness of breath, chest pain, and dry cough and might lead to lung cancer and mesothelioma (Ramanathan and Subramanian 2001; Donoghue 2004; Patra et al. 2016; ATSDR 2001). Arsenic occurs naturally in soil and minerals and can also enter the air and easily become inhaled. It is a well-known carcinogen to which excessive exposure to can cause irritations in the nose and respiratory airways and lead to cardiovascular and respiratory diseases and lung cancers (Hong et al. 2014; Martin et al. 2014). There are many other components of PM that could initiate adverse health effects or exacerbate existing ones. In this review however, the effects of some of the most important ones were highlighted above and summarised below in *Table 2.1*.

Table 2.1, A summary of common hazardous PM components and their effects on human health. Adopted from Cloonan et al. (2017), Quintana et al. (2006), Chen et al. (1990), Patra et al. (2016), Romo-Kroger et al. (1989), U.S. AF (1990), Martin and Griswold (2009), Silbergeld (2003), Morais et al. (2012), Jaishankar et al. (2014), Smith et al. (2002), Cooper and Harrison (2009), Middleton and Kowalski (2010), Kreiss et al. (2007), Heimann et al. (1953), Falgayrac et al. (2011), Hulo et al. (2013), Donoghue (2004), Hendryx and Ahern (2008), OSHA (2002), Ramanathan and Subramanian (2001), ATSDR (2001), Hong et al. (2014), and Martin et al. (2014).

PM component	Associated symptoms	Associated diseases
Iron	Persistent airway inflammation and compromised lung function	Cardiovascular and respiratory diseases, Parkinson's and Alzheimer diseases
Copper	Cough, chills, muscle ache	Wilson's disease
Lead	Loss of appetite, fatigue	A variety of cancers including lung, stomach, brain, kidney, and bladder
Mercury	Memory problems, changes in vision and hearing	Pink disease or acrodynia
Beryllium	Inflammation of the respiratory tract tissues, hepatic necrosis, kidney stones, weight loss	Beryllium disease, cancer
Mica	Pulmonary overload, cough and shortness of breath	Pneumoconiosis and fibrosis
Crystalline silica	Shortness of breath, chest pain, or respiratory failure	Silicosis, lung cancer, obstruction of the airways, lymph node fibrosis
Asbestos	Shortness of breath, chest pain, dry cough	Lung cancer, and mesothelioma
Arsenic	Irritations in the nose and respiratory airways,	Cardiovascular and respiratory diseases, lung cancers

2.2.5 Common particulate matter physical measurement methods

There are many options available on the market designed for measuring different physical aspects of PM. Most PM measuring equipment are designed for either (i) mass concentration measurement and/or (ii) measurement of particle numbers and sizes (Amaral et al. 2015).

2.2.5.1 PM mass concentration

In concentration methods, instruments depend on different principles, for example, gravimetric (filter-based samples), microbalance (tapered element oscillating microbalance or TEOM), optical (photometry), or Beta-ray attenuation (Winkel et al. 2014). These are outlined below and the advantages and disadvantages of each method compared in *Table 2.2.*

2.2.5.1.1 Gravimetric principle

This method depends on weighing the filter before and after sampling to determine the mass concentration of particles. All fractions of PM are collected on the filter unless a cyclone or impactor is used to eliminate unwanted size fractions. One of the advantages of this method is that it allows for the chemical analysis of particles collected on the filters (Giechaskiel et al. 2014; Winkel et al. 2014). It is important to note that the filters must be handled with care under controlled conditions of relative humidity and temperature. Otherwise, the final results could be altered from changes in the weight of filters due to the absorbance of water vapour (Su et al. 2008; Anderson and Albert 1998).

2.2.5.1.2 Microbalance principle

In this method, PM is determined through the use of changes in resonance frequency after the collection of a sample on the surface of an oscillatory microbalance element (Ward and Buttry 1990; Patashnick and Rupprecht 1991). The most common instruments based on the microbalance method are the Quartz Crystal Microbalance (QCM) and the Tapered Element Oscillation Microbalance (TEOM). In the QCM, the deposition of particles on a thin quartz crystal resonator occurs through electrostatic

precipitation. When mass is added to the quartz crystal resonator, its resonance frequency changes. This allows for the calculation of the accumulated mass. In the TEOM, the measurement of PM mass is dependent on changes in resonance frequency of a tapered quartz wand. The changes in resonance frequency occurs when particles are accumulated on the sampling filter that is connected to the wand (Amaral et al. 2015; Giechaskiel et al. 2014).

2.2.5.1.3 Beta-ray attenuation principle

In this method, PM is collected onto a filter. However, PM mass is determined via beta-rays emitted from a radioactive source directed towards the filter and the PM in it. The intensity of attenuation of beta-rays in time is indicative of PM mass (Winkel et al. 2014; Amaral et al. 2015).

2.2.5.1.4 Optical principle (photometry)

In this method, the interaction between PM and visible infrared or laser light is utilised to measure PM mass. Instruments based on light scattering either operate on single particles (Optical Particle Counter (OPC)) or an ensemble of particles (scattering photometer). The OPC has two major components, a light source and a photodetector. The light source illuminates the particle sample while the photodetector measures the light scattered from that sample as an electrical pulse. The measurement of particle size depends on the height of that electrical pulse (Giechaskiel et al. 2014). The OPC can only measure particles with a diameter > 100 nm. One of the advantages of the OPC is its ability to measure concentrations of up to 10^3 cm^{-3} for particles that are large enough to be detected (Gao et al. 2013). One of its disadvantages is the uncertainty in refractive index which could lead to significantly inconsistent size

distributions (Hering and McMurry 1991). The scattering photometer has the same major components of the OPC. The only difference between the two is that the OPC has a smaller optical detection volume. The higher optical detection volume enables the scattering photometer to measure the combined scattered light from all particles at different angles (Amaral et al. 2015). There is another special category that is also based on light scattering. In this category, particles are grown to micron sizes by condensation then counted by light scattering. An example of this category is the Condensation Particle Counters (CPC). The CPC is mainly used for measuring small particles (Giechaskiel et al. 2014).

Table 2.2, Advantages and disadvantages of PM mass concentration methods. Adapted from Amaral et al. (2015) and Keywood and Selleck (2016).

Technique	Advantages	Disadvantages	Estimated precision
Gravimetric	Moderately priced, simple and reliable, allows for the chemical analysis and source identification of collected samples	Poor time resolution, requires infrastructure and lots of work, relatively time intensive	± 2 μg m^{-3}
Microbalance	Allows for real time measurement, high time resolution, works well with filter samples	Loss of volatile mass from heating of sample	± 0.5 μg m^{-3}
Beta-ray attenuation	Allows for real time measurement with short time resolution	Loss of semi volatile compounds due to heating and interference due to the presence of water when heating is not used	±3 μg m^{-3}
Optical	Portable, measures several size fractions simultaneously	Depends on a number of factors such as particle characteristics which can vary depending on time and place	Depends on analyser type

2.2.5.2 PM particle number and size distribution measurement

The measurement of particle size occurs on the basis of some particle properties such as geometrical size and electrical mobility. The measurement of particle size distribution is achieved by combining charging, size classification, and detection from several instruments (Giechaskiel et al. 2014). Several techniques are available for this

type of analysis and each has its strengths and limitations (Malvern. Instruments 2015). *Table 2.3* below shows the advantages and disadvantages of each method.

2.2.5.2.1 Automated imaging

The use of automated imaging enables researchers to examine solid particle size, morphology, and other physical characteristics (Amaral et al. 2015; Malvern. Instruments 2015). Collecting a sample for imaging analysis basically involves collecting particles on a filter and prepare that filter for microscopic examination (Vincent 2007). Image analysis under the microscope provides useful information such as the size distribution of aggregates, the size distribution of primary particles, and the number of primary particles per aggregate (Wentzel et al. 2003). One of the disadvantages of this instrument is that its time consuming (Giechaskiel et al. 2014).

2.2.5.2.2 Laser diffraction particle sizing

The use of laser diffraction for particle sizing is common for PM ranging from hundreds of nanometres to several millimetres in size (Malvern. Instruments 2015). The measurement of particle size distribution in this method involves applying the Mie scattering theory for determining the angular distribution of light scattered by particles (Hinds 2012; Wang-Li et al. 2013).

2.2.5.2.3 Dynamic light scattering

Particle size distribution measurement using dynamic light scattering is non-invasive and common for particles ranging from micrometres to nanometres. Particles undergo Brownian motion as a result of temperature induced collisions between suspended particles and solvent molecules (Malvern. Instruments 2015). When the particles are

illuminated with laser, this causes light to scatter. The intensity of light scatter causes fluctuations which area measured to determine particle motion. Once the Brownian motion is determined, particle size is obtained using the Stokes-Einstein relationship (Berne and Pecora 2000; Malvern. Instruments 2015).

2.2.5.2.4 Electrophoretic light scattering

This method is based on two principles; electrophoresis and laser Doppler shift spectroscopy (Josefowicz 1979). It is used to measure the electrophoretic mobility of particles in a dispersion. Particles are introduced into a chamber containing two electrodes. When an electrical field is applied, charged particles move into the direction of the oppositely charged electrode. Their movement speed is known as the electrophoretic mobility and its related to their zeta potential. The laser Doppler technique is used to determine that movement speed (Varenne et al. 2015; Malvern. Instruments 2015).

Table 2.3, Advantages and disadvantages of PM particle number and size distribution methods. Adapted from Malvern Instruments (2015), Uskoković (2012), American laboratory (2005) and Azonano (2013).

Technique	Advantages	Disadvantages
Automated imaging	Allows for analysing particle shape and size, allows for the detection of agglomerates and oversized particles	Time consuming and exhausting, small number of particles analysed, requires sample preparation
Laser diffraction particle sizing	Allows for the analysis of different types of materials (aerosols, dry powders, liquid suspensions), easy to use, provides rapid, reproducible and precise measurements, non-destructive and non-invasive, calibration not required	incorrect refractive indices may lead to erroneous results, based on the assumption of optical homogeneity, resolution is limited by the number of available detectors
Dynamic light scattering	Suitable for nano and biomaterial, small amount of material needed, provides rapid measurements, non-invasive	Low resolution, accuracy is compromised in concentrated samples, large particle aggregates negatively affect measurements
Electrophoretic light scattering	Provides rapid measurements, high resolution, small amount of material needed	Inability to differentiate between particle surfaces with different charges because of rotational diffusion and particle orientation averaging

2.2.6 Common methods for chemical and mineralogical analysis of PM

A wide range of methods are available for the chemical and mineralogical characterisation of PM. Below is a brief description of some of the most common methods found. Also, the advantages and disadvantages of each method are shown in *Table 2.4* below.

2.2.6.1 Instrumental neutron activation analysis (INAA)

The INAA can be used for determining the concentration of trace and major elements in atmospheric aerosols (Maenhaut 1989; Sansoni 1987). A sample of PM would be

subjected to a neutron flux from a nuclear reactor which results in radioactive nuclides. After a suitable time period of cooling, gamma rays are emitted from the decaying radioactive nuclides which are then measured by a germanium (-lithium) detector. The frequency or energy of the gamma rays is used to detect certain elements (Malainey 2011; Salma and Zemplén-Papp 1999; Eby 2013).

2.2.6.2 Atomic absorption spectrophotometry (AAS)

The AAS is a very common technique for detecting metals and metalloids in PM samples (Araujo et al. 2011; Araujo et al. 2010). A sample of PM would undergo vaporization which results in its disassociation into its gaseous phase elements (Welz and Sperling 2008). Elements are then subjected to a flame or a graphite furnace and atomized. The atoms absorb energy at very specific wavelengths which enables the analysis of an elements concentrations (Mainey and William 1999).

2.2.6.3 Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

The ICP-AES is a widely used method for the analysis of trace elements in atmospheric PM samples (Boevski et al. 2000). The method is based on emission spectroscopy which uses inductively coupled plasma to excite atoms that emit electromagnetic radiations at specific wavelengths. The concentration of an element in a given PM samples is proportional to the intensity of the emission emitted (Pramanik and Das 2019; Smith and Nordberg 2015; Neikov et al. 2009).

2.2.6.4 Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

The ICP-MS is a commonly used method for the elemental and isotopic characterisation of PM samples (Gioda et al. 2011; Mateus et al. 2013). Samples of PM undergo digestion in acid and then introduced to radio-frequency-generated argon

plasma via nebulization. The occurring energy transfer processes result in desolvation, atomization, and ionization. Ions are then extracted from the plasma and separated based on their mass to charge ratio. Ions transmitted are registered and their information is then processed (Mainey and William 1999; Espinoa et al. 1998; Bazilio and Weinrich 2012).

2.2.6.5 X-Ray Fluorescence (XRF)

XRF is a powerful multi-elemental technique used for the analysis of PM samples (Calzolai et al. 2008). It is based on measuring X-rays produced by the ejection of inner shell electrons from the atoms of each element in a PM sample. As high energy electrons fill the vacant lower energy orbital, a fluorescent x-ray photon is released. Its energy is unique for each element and its numbers are proportional to the element's concentration (Potts 1987; Chow and Watson 1998).

2.2.6.6 X-Ray Diffraction (XRD)

XRD is used for the mineralogical characterisation and crystalline phase identification of PM (Satsangi and Yadav 2014; Bontempi et al. 2008). It is based on constructive interference of monochromatic X-rays and a crystalline sample. In an X-ray diffractometer, X-rays are produced by a cathode ray tube and undergo filtration to produce monochromatic radiation. The radiation is then concentrated and directed towards the sample. The interaction between the incident rays and the samples produces diffracted rays. These are then detected, processed, and counted. The conversion of diffraction peaks to d-spacings enable the identification of minerals (Ramachandran and Beaudoin 2000).

2.2.6.7 Scanning Electron Microscopy (SEM) with Energy-Dispersive X-Ray (EDX) Spectroscopy

The SEM can be used for the morphological characterisation of PM while the EDX system is used for the chemical identification of its components (Grassi et al. 2004). The SEM is basically a high magnification microscope that utilizes focused electron beams for the generation of single or multiple particle images. The interaction between the electron beams and the PM sample results in the production of secondary electrons, backscattered electrons, and characteristic X-ray. Since elements have different X-ray emission spectrums, these can be differentiated and the concentration of an element in a single and/or group of particles can be measured (Mutalib et al. 2017).

Table 2.4, Advantages and disadvantages of PM chemical and mineralogical analysis methods. Adopted from INAA Services at Washington University (2007), Harvey (2000), Skoog et al. (1998), Chemistry Net (2013), Jarvis and Jarvis (1992), Montaser (1998), Yang and Wei (2019), Taylor (2010), Oyedotun (2018), Nakai and Abe (2012), SERC (2020), and Choudhary and Choudhary (2017).

Method	Advantages	Disadvantages
INAA	Multi-elemental, high sensitivity, accuracy, and precision, allows for bulk sample analysis, sample preparation not necessary, low sample volume required, non-destructive	Inability to determine all elements, time consuming
AAS	Accurate, sensitive, relatively inexpensive	Only analyses solutions, large sample volumes required, lacks precision sometimes
ICP-AES	Allows for rapid and easy multi-elemental analysis, low chemical interference, low detection limit, good accuracy and precision	Destructive to the sample, poor sensitivity to certain elements, samples must be dissolved in a solution, sample preparation is time consuming, large sample volumes required
ICP-MS	Allows for rapid and easy multi-elemental analysis, high sensitivity, extremely low detection limits, allows for detection of isotope composition of elements, large linear range	Destructive to the sample, time consuming, high measurement cost, large sample volumes required
XRF	Allows for rapid and easy multi-elemental analysis, non-destructive, low cost of ownership	Strong matrix effects must be considered, inability to measure radiation for all elements, radiation exposure hazard, large sample volumes required
XRD	Rapid and powerful, minimal sample preparation, small sample volumes required	Homogenous and single-phase materials are best used, occurrence of peak overlay, not sensitive to trace elements
SEM-EDX	Allows for rapid and easy analysis of high-resolution 3D images of particles and their respective elemental components, minimal sample preparation required, small sample volumes required	Extremely expensive, requires high maintenance, sample preparation may result in artefacts, inability to provide an accurate estimation of a sample's composition

2.2.7 Common particulate matter source apportionment techniques

The aim of source apportionment methods is to provide information about the contribution of a source to the total concentration of a pollutant at a receptor (Viana et al. 2008; Belis et al. 2014b). There are three main groups of source apportionment methods, with *Table 2.5* below summarising the advantages and disadvantages of each method.:

2.2.7.1 Receptor models

Receptor models are based on analysing of PM chemical data at the point of impact (Viana et al. 2008). They relate the concentration of atmospheric pollutants measured at monitoring stations to emission sources (Hopke 2016). There are a number of statistical models and modelling approaches currently available. The most common methods however, are Positive Matrix Factorization (PMF) and Chemical Mass Balance (CMB). The main difference between the two lies in the required level of information on the nature of pollution sources. While PMF requires little knowledge about the sources, CMB requires detailed and complete knowledge about them (Olaguer 2016).

2.2.7.2 Source-oriented (forward) models

Source oriented models are based on emission inventories and/or dispersion models (Viana et al. 2008). These models take complex chemical atmospheric reactions into account. However, they require detailed knowledge on emission inventories. Unfortunately, such detailed information is not always available and even if it is available it might not be that accurate. A variety of models are available in the

literature for this type of source apportionment such as Gaussian reactive plume models, Lagrangian trajectory models, and Eulerian grid models (Olaguer 2016).

2.2.7.3 Inverse models

Inverse models are based on evaluating monitoring data (Viana et al. 2008). They start with the measurements obtained from the monitoring stations and compute backwards to identify the sources (Olaguer 2016). Such models are used to identify the source associations by examining the correlations of gaseous pollutants with PM components (Viana et al. 2008). The can also be used to examine the level of contribution to PM from natural sources by comparing and subtracting PM levels at background and urban locations and certain days (Escudero et al. 2007).

Table 2.5, Advantages and disadvantages of PM source apportionment methods. Adapted from Belis et al. (2014a), Mircea M. et al. (2020), Borrego and Norman (2006), Thunis et al. (2019), and Office of Community Air Protection (2018).

Method	Advantages	Disadvantages
Receptor models	Provides information derived from real-world measurements, allows for the estimation of the source contribution of most PM chemical components, extensive input data sets and computing resources are not required, allows for the estimation of output uncertainty	Limited applicability to very reactive species
Source oriented (forward) models	Allows for the estimation of; source contributions in the absence of measurements, contributions from different emission sectors to secondary pollutants, and contribution of transported pollutants, has the ability to predict air quality changes in relation to emission changes, has the ability to isolate individual effects of meteorology, has the ability to provide high temporal resolution output, allows for exploring the variability of source contributions in time and space	Significant computations required, lacks dynamicity, unsuitable for air quality planning
Inverse models	Enables the assessment of agreement between monitored data and emission inventories	Significant computations required

2.2.8 Common methods for toxicity testing of PM

Particulate matter toxicity can be evaluated *in vitro* and *in v*ivo (Mirowsky et al. 2015). *In vitro* studies of PM involve evaluating their effects on components (e.g. cells, microorganisms, and biological molecules) that have been isolated from their biological systems. *In vivo* studies of PM involve evaluating their effects on whole living systems (e.g. animals, humans, and plants). For the purpose of this project, focus

will be on *in vitro* toxicity testing as it is much more cost-effective, ethically favourable, and yet informative compared to *in vivo* toxicity testing (National Research Council Committee on Methods of Producing Monoclonal Antibodies 1999; Schmidt et al. 2019).

In vitro, PM can cause a variety of effects including cytotoxicity, genotoxicity/mutagenicity, and oxidative stress. These effects form the basis for categorising assays used for *in vitro* assessment of PM toxicity (Schmidt et al. 2019). Cytotoxicity is defined as the ability of PM to cause cell death. Genotoxicity/mutagenicity is defined as the ability of PM to cause mutation (Jain et al. 2018b). Oxidative stress is defined as the imbalance between the antioxidant system and the production of reactive oxygen species (Betteridge 2000). A variety of toxicological assays are available for investigating the cytotoxic and genotoxic effects of PM and their ability to cause oxidative stress. Assays are usually either cell-based or cell-free. Below is a brief description of some of the most common assays found.

2.2.8.1 Cell-free assays

A cell-free assay enables the study of reactions that might occur within cells however, without the full cell system. This leads to the reduction of complex interactions associated with working on whole cells (Swartz 2006). The use of cell-free assays in PM research is common (Stoeger et al. 2009; Øvrevik 2019; Dumax-Vorzet et al. 2015). Below are some of the most common cell-free assays found in the literature. Also, the advantages and disadvantages of each assay are shown *Table 2.6* below.

2.2.8.1.1 The Plasmid Scission Assay

The Plasmid Scission Assay is a sensitive and simple method used to assess only one form of DNA damage which is the Fenton reaction-mediated oxidative damage to supercoiled plasmid DNA induced by free radicals generated by PM (Lü et al. 2006; Dumax-Vorzet et al. 2015). Supercoiled plasmid DNA is the native form (covalently closed circular DNA) where there is no DNA damage (no strand breaks). The nicking of the undamaged supercoiled DNA form by free radical activity leads to the relaxation of the coil. Nicked or relaxed plasmid DNA is where there is a single strand break and the DNA will become a large floppy open circle. Further damage to plasmid DNA leads to its linearization. Linear plasmid DNA is where there are double-strand breaks. When plasmid DNA runs through an agarose gel, these three forms have different migration speeds where supercoiled plasmid DNA is the fastest as it doesn't have any strand breaks and its compactness sustains less friction against the gel. Linear plasmid DNA runs through the gel slower than supercoiled plasmid DNA but faster than Nicked or relaxed plasmid DNA. Nicked or relaxed plasmid DNA is the slowest form and it migrates through the gel as a large floppy circle (Moreno et al. 2004; Shao et al. 2013).

2.2.8.1.2 Dithiothreitol (DTT) assay

The DTT assay is a method commonly used for the measurement of oxidative potential of PM (Verma et al. 2011; McWhinney et al. 2011; Charrier and Anastasio 2012). It is based on the conversion of DTT to its disulphide form via oxidation by redox-active chemical constituents of PM (Charrier and Anastasio 2012). The rate of DTT loss is an indication of the concentration of redox-active species available in PM. The residual DTT can be measured using a spectrometer (Bates et al. 2019).

2.2.8.1.3 *Salmonella typhimurium* reverse mutation assay (Ames test)

The Ames test is used for the identification of carcinogenicity using mutagenicity in the bacterial strain *salmonella typhimurium* as an endpoint (Hengstler and Oesch 2001; Jain et al. 2018a). The test is widely used for assessing the toxic effects of PM (El-Assouli 2011; Bocchi et al. 2017). It is based on the principle of reverse mutation; a strain of *salmonella* that lacks the ability to produce histidine, a gene that enables their growth, is cultured with PM. This leads to mutations in the histidine encoding gene enabling its synthesis again. Thus, making this reverse mutation (Jain et al. 2018a).

2.2.8.1.4 Ascorbic acid (AA) assay

The AA assay is a cell-free method used commonly for the determination of PM's oxidative potential (Conte et al. 2017; Øvrevik 2019). Ascorbic acid is an antioxidant that occurs naturally in biological systems. However, it can become oxidised, in the presence of reactive oxygen species produced by different PM constituents, when it reacts with free radicals. Its reduced form is an indication of reactive oxygen species formation (Hellack et al. 2017).

Table 2.6, Advantages and disadvantages of cell-free assays used for investigating PM toxic effects. Adapted from Lü et al. (2006), Dumax-Vorzet et al. (2015), Xiong et al. (2017), Słoczyńska et al. (2014), Frezzini et al. (2019), and Hellack et al. (2017).

Assay	Advantages	Disadvantages
Plasmid Scission Assay	Sensitive, simple, and reliable, low sample volumes required	Only detects one form of DNA damage and does not account for background DNA damage caused through other processes other than oxidative damage
DTT Assay	Responsive with a large number of chemical components including aromatic hydrocarbons and certain transition metals	Does not capture hydroxyl radical's generation
Ames test	Easy to perform, no special equipment is required, inexpensive	Tester organism could be pathogenic, several tester strains must be used, analysis is time consuming, inability to detect mutagens that interact with eukaryote-specific targets
AA Assay	Easy to apply	Lacks specificity (cannot identify different ROS types)

2.2.8.2 Cell-based assays

A cell-based assay enables the study of reactions taking place between specific agents, such as PM and its constituents, and the various components of living cells. A variety of techniques for investigating the effects of PM on cells are available. Below are some of the most common assays found in the literature. Also, *Table 2.7* shows the advantages and disadvantages of each assay. However, before exploring some of those techniques, some of the most common cell lines, which are permanently established cell cultures that if maintained properly will proliferate indefinitely, used in PM

research are explored (*Table 2.8* shows the advantage and disadvantage of each cell line) (Peixoto et al. 2017; Ulrich and Pour 2001).

A variety of cell lines have been used in the assessment of PM toxic effects including A549 adenocarcinomic human alveolar basal epithelial cells (Sánchez-Pérez et al. 2009; Akhtar et al. 2014; Bełcik et al. 2017), BEAS-2B normal human bronchial epithelium cells (Oh et al. 2011; Dergham et al. 2015), HepG2 human liver cancer cells (Jiang et al. 2011; Hanzalova et al. 2010), and HaCaT human aneuploid immortal keratinocyte cells (Piao et al. 2018; Li et al. 2017). The selection of a cell line for PM toxicity testing depends on a number of factors including how representative the cell line is in terms of exposure to PM (Peixoto et al. 2017). The lungs are the first organ affected by PM and its where most PM is retained. That is why most PM studies use A549 and BEAS-2B cell lines for toxicity testing (Asgharian et al. 2014; Ling et al. 2011).

2.2.8.2.1 The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) Assay

The MTT assay was first described by (Mosmann 1983). It is a colorimetric assay used for measuring cytotoxicity of PM (loss of viable cells) (Hsiao et al. 2000). It is known to be sensitive and reliable and is preferred over other methods investigating this endpoint. The assay is based on converting the yellow water-soluble MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) to purple insoluble formazan. Viable cells with active metabolism become purple while dead cells do not change colour and show no signal. The difference in colour intensity can be measured using a plate reader (Riss et al. 2016).

2.2.8.2.2 The Neutral Comet Assay

The Neutral Comet Assay, also known as single-cell gel electrophoresis assay (SCGE), is a method used for detecting cellular DNA damage caused by PM (Dumax Vorzet 2010). The cells are embedded in agarose on a microscopic slide and lysed to remove their cell membranes, cytoplasm, and nucleoplasm and dissolve their nucleosomes (Nandhakumar et al. 2011). This exposes their nucleoids which are then electrophoresed making any breaks in DNA move towards the anode when exposed to a current during electrophoresis which results in the formation of comet-like structures (Lorenzo et al. 2013). These comets can be viewed using a fluorescence microscope with a suitable stain (Collins 2004).

2.2.8.2.3 H2DCFDA – Reactive Oxygen Species Assay

The H2DCFDA is a method used for detecting reactive oxygen species production due to cellular exposure to PM and its constituents (Crobeddu et al. 2017; Montiel-Dávalos et al. 2010). It is based on the deacetylation of 2',7' – dichlorofluorescin diacetate, which is a fluorogenic dye, by cellular esterase to a non-fluorescent compound. The resulting compound becomes oxidised by reactive oxygen species and turn into 2', 7' –dichlorofluorescein, which is highly fluorescent and can be measured via a fluorescence plate reader (Li et al. 2019).

2.2.8.2.4 The Adenosine Tri-Phosphate (ATP) Assay

The ATP (adenosine tri-phosphate) assay is a robust and simple method for assessing cell viability after PM exposure (Breznan et al. 2015; Thomson et al. 2015). It is based on the conversion of luciferin to oxyluciferin via catalyses by luciferase in the presence of Mg^{2+} ions and ATP. This results in a luminescent signal of which its

intensity is linearly correlated with ATP concentration (Mueller et al. 2004; Aslantürk 2018). The ATP serves as a cellular chemical energy reservoir. It is used for many purposes including biological synthesis, signalling and transport. That is why it's an important parameter for cell viability testing (Maehara et al. 1987; Aslantürk 2018). Whenever cellular damage occurs and membrane integrity is compromised, ATP synthesis is lost. This leads to a significant decrease in its cellular levels (Riss et al. 2016; García and Massieu 2003; Aslantürk 2018).

Table 2.7, Advantages and disadvantages of cell-based assays used for investigating PM toxic effects. Adopted from Aslantürk (2018), Singh et al. (1988), Hartwig et al. (1996), and Volk and Moreland (2014).

Assay	Advantages	Disadvantages
MTT Assay	Superior to dye exclusion methods, easy, safe, highly reproducible results	Occurrence of false negative or false positive due to background interference from PM inclusion
Neutral Comet Assay	Allows for DNA repair estimation via other methods, provides some indication of apoptosis, inexpensive	Optimisation of protocol and experimental procedure is extremely important
H2DCFDA - Reactive Oxygen Species Assay	Allows for general estimation of redox state	Inability to provide specific or quantitative analysis of endosomal superoxide or hydrogen peroxide generation
ATP Assay	Fast, easy, sensitive, less prone to error	Its sensitivity is limited to reproducibility of pipetting replicate samples

Table 2.8, Advantages and disadvantages of different cell lines used for the assessment of PM toxicity. Adopted from Schürer et al. (1993) and Peixoto et al. (2017).

Cell line	Advantages	Disadvantages
A549	Easily maintained in the lab	Already transformed
BEAS-2B	Not genetically altered	Difficult and expensive to maintain
HepG2	Has the ability to metabolize chemical agents	Insensitive to PM
HaCaT	High differentiation and proliferation rates *in vitro*	N/A

2.3 Panasqueira mine area, Portugal

The Panasqueira mine is located in central Portugal and it includes several mining operations. Local topography ranges in altitude from 350 to 1080 m with deep valleys (Reis 1971). The streams are generally dry in the summer and flooded in the winter (Grangeia et al. 2011).

Climate is extremely aggressive with hot dry summers and cold, rainy, and windy winters. Throughout the year, mean rain precipitation ranges from 1200 to 1400 mm. Snow occasionally falls especially in altitudes above 700 m. The temperature ranges from 0 ^{o}C in the winter to 30 ^{o}C in the summer with annual means of around 12 ^{o}C. Evapotranspiration in the area is around 1080 mm (Grangeia et al. 2011; Candeias et al. 2014c).

Several studies have described the geology of the Panasqueira mine area in detail (Polya 1987; Ávila et al. 2008; Orey 1967). In general, the area contains large amounts of wolframite, arsenopyrite, cassiterite and chalcopyrite. Also, it is known to be one of the biggest W-Sn deposits in Western Europe. The deposit is a classic example of post-magmatic ore classified as hydrothermal mineralization associated with the Hercynian plutonism (Candeias et al. 2014b).

2.3.1 Sources of heavy metals and metalloids pollution in Panasqueira mine area

Just like any other industry, mining activities can have serious negative effects on the surrounding environment. The extraction and production processes result in crushed, milled waste rock and tailings that have the potential to cause significant damage to the environment when exposed to weathering (Nordstrom and Alpers 1999; Dold and Fontboté 2001; Grangeia et al. 2011). They undergo oxidisation forming ferric hydroxides and sulfuric acid which leads to the formation of acid mine drainage with elevated levels of metals and sulphates (Candeias et al. 2014a).

Soil is the main receptor of tailings drainage and hence, it is usually affected over large areas. The nature of the soil's fine fraction, it has a large surface area that enables absorption and metal binding to iron, manganese oxide, and organic matter, allows it to become enriched with metals from the tailings' drainage. Theses metals are then dispersed in the atmosphere by wind. Human exposure to the metals can be through ingestion, whether it was direct or indirect, and inhalation (Rasmussen 1998; Yukselen and Alpaslan 2001; Candeias et al. 2014a).

Several studies conducted previously have illustrated the type of impact the mining activities in the Panasqueira area could have on surrounding environments. The studies reported elevated levels of toxic metals and metalloids in stream sediments, superficial and ground waters from local water courses, road dust, soils, and plants grown specifically for human consumption (Ávila et al. 2008; Da Silva et al. 2013; Grangeia et al. 2011; Coelho et al. 2014b).

2.3.2 Studies on exposure to heavy metals and metalloids in Panasqueira mine area

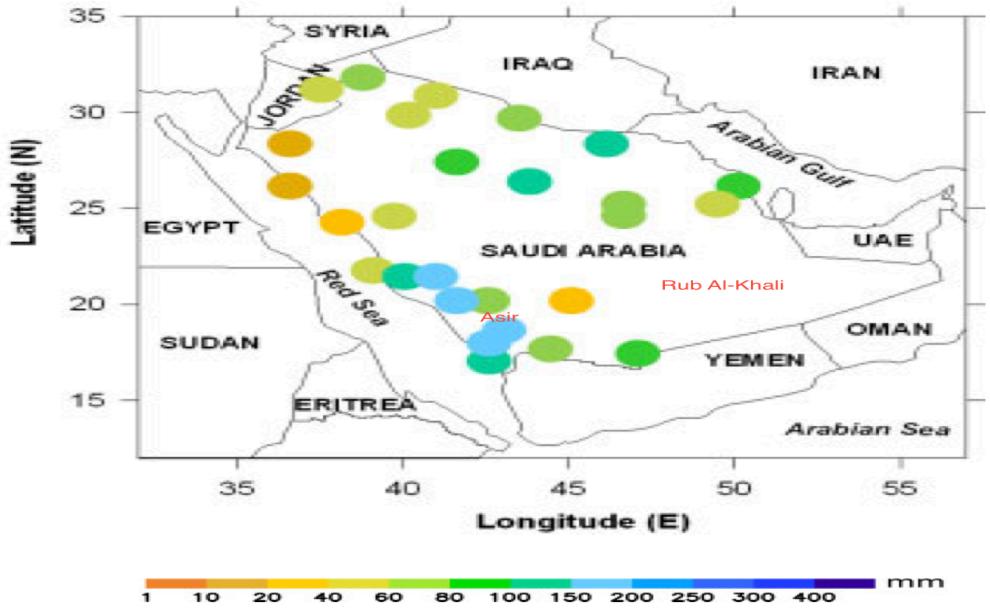
There are many studies in the literature that have examined the effect of exposure to heavy metals and metalloids on miners and surrounding populations in the Panasqueira mine area. Early studies performed on small groups of miners and people living nearby indicated that those exposed had increased levels of mutations in the micronucleus (MN) test and T-cell receptor mutation assay (Coelho et al. 2011; Coelho et al. 2012). Later studies such as the one by Coelho et al. (2013), tested miners and people living nearby for heavy metals and metalloids in a number of biological matrices (Coelho et al. 2014a). It was reported that populations involved in the study were exposed to significantly higher levels of As, Cr, Mn, Mo, Pb, and Zn when compared to their respective controls. Moreover, the results indicated that the environmentally exposed group showed significantly higher levels of heavy metals and metalloid than the one occupationally exposed. Avila et al. (2017) estimated the contents of As, Cd, Cr, Cu, Pb and Zn in rhizosphere soils, irrigation waters, road dusts and in potatoes, cabbages, lettuces and beans, collected from local gardens of villages close to the Panasqueira mine. The authors found that the local populations are exposed to are at risk due to the intake of heavy metals and metalloids through consuming their vegetables (Ávila et al. 2017).

2.4 Saudi Arabia

Saudi Arabia is located in Asia in the Middle East and is the largest country in the Arabian Peninsula. The country occupies around 80% of the peninsula and extends from the Red Sea in the west to the Arabian Gulf in the east (El-Nesr et al. 2010; Almazroui et al. 2013). It is the fifth largest state in Asia and the second largest in the Arab world with a land area of around 2,250,000 km^2 (Hasanean and Almazroui 2015).

The country's terrain is varied. However, it is mostly dominated by the Arabian Desert of which it takes around 647,500 km^2 of its total land area (Azorin-Molina et al. 2018). The Rub Al-Khali, which is the largest continuous sand desert in the world, is located in the south. Other than that, it has barren and harsh terrain with salt flats and gravel plains. There are a few man-made lakes but there are no rivers or other lakes in the country (U.S. Central Intelligence Agency 2017; Royal Embassy Of Saudi Arabia 2016).

The climate has a high spatial and temporal variability. However, it is generally harsh and dry with temperature extremes and frequent dust and sand storms (El-Nesr et al. 2010). Temperatures reach up to 52 °C during the day from June to August in the desert while in the winter, temperatures can drop to a freezing level (below 0 °C) in the north and central part of the country (Almazroui et al. 2014). Humidity is high in the coastal regions, and almost absent in other areas (MoE 2019). Strong sandstorms are frequent mostly in the summer due to the north wind (Shamal Wind) that blows over the country several times yearly (Yu et al. 2016). Mean annual rainfall in Saudi Arabia is typically < 100 mm/year (Azorin-Molina et al. 2018). There can be no rainfall in the Rub Al-Khali for long periods that sometimes last for 10 years. On the other hand, the region of Asir gets 300 mm (*Figure 2.9*) between October and March (Royal Embassy Of Saudi Arabia 2016; Azorin-Molina et al. 2018).



Figure, 2.9, The spatial distribution of the mean annual rainfall (mm) averaged over 1998–2009, (After Almazroui et al. (2012))

Since the discovery of oil, the Saudi population has become more settled. People became more concentrated around economic cities such as Dammam, Jeddah, Riyadh, and Makkah as shown in *Figure 2.10* Even though the country is trying to diversify its economy it still depends heavily on petroleum (Abbey et al. 1999; U.S. Central Intelligence Agency 2017; Farahat 2016).

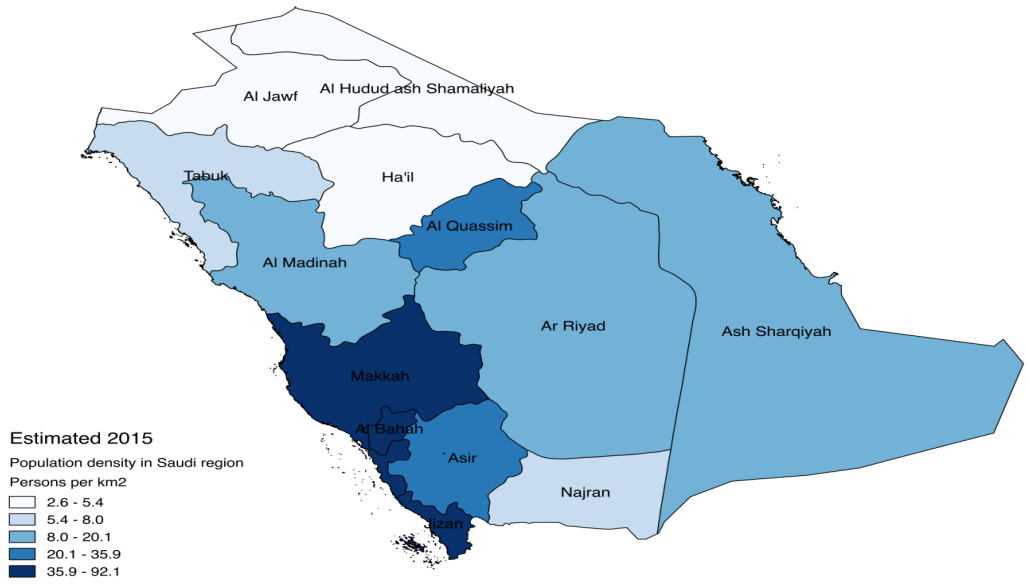


Figure 2.10 Population density in Saudi Arabia (map created by QGIS, the shapefile for Saudi Arabia was downloaded from http://www.gadm.org/country and population statistics from https://www.stats.gov.sa/ar/1070)

2.4.1 Makkah

The Holy City of Makkah is located south-western Saudi Arabia. At an elevation of 277 m above sea level and about 70 km inland towards the east from the coast, the city is located in a desert valley between mountains. Makkah is considered unique to all Muslims due to its Holy Mosque (Al-Haram). It attracts millions of visitors annually from around the world. As a result, the city becomes highly populated and very busy especially in terms of road traffic (Nayebare et al. 2018). This all leads to the exhaustion of the city's resources and air quality is expected to deteriorate under such conditions (Habeebullah 2016; Munir et al. 2017). Even though the local population in Makkah is about 1.6 million people (GASTAT 2015), that number rises significantly during Ramadan and Hajj (GASTAT 2018b, 2018a). Ramadan and Hajj are considered holy months to Muslims worldwide. In Ramadan, other than abstaining from food and drinks from dawn to dusk for the whole month, Muslims from across

the globe attempt to visit Makkah to perform the all year round allowed non-mandatory minor pilgrimage "Umrah" (Elmajnoun et al. 2020; Jafari and Scott 2014). The Umrah involves visiting Al-Haram and performing certain rituals that could take a few hours at any time of day (Alhogail et al. 2019). During the month of Ramadan in 2018, the total number of Umrah performers including Makkah's locals and outside visitors was about 7.8 million (GASTAT 2018b). As for Hajj, it is the major pilgrimage to Makkah and it is one of the five pillars of Islam. Unlike Umrah, it is a once in a life time mandatory religious duty for all physically and financially capable adult Muslims (Nafea et al. 2014). It involves performing certain acts of worship in specific holy places in Makkah including Al-Haram at specific times of day over five to six days (Alsafadi et al. 2011). During the month of Hajj in 2018, the number of Hajj performers including Makkah's locals and outside visitors was about 2.4 million (GASTAT 2018a).

2.4.2 Sources of PM air pollution in Saudi Arabia and in Makkah specifically

Saudi Arabia is considered to be a major anthropogenic and natural source of PM globally (Farahat et al. 2015; Rushdi et al. 2013; Rushdi et al. 2010). There has been a significant increase in the number and forms of air pollution sources since the discovery of oil in the Kingdom. There are several anthropogenic stationary and mobile sources of PM there (Khodeir et al. 2012). Some of the most important stationary sources of PM are oil refineries, cement and concrete factories, water desalination plants, and petrochemical industries (GAMEP 2017). Mobile sources of PM in the Kingdom include all forms of transportation such as motor vehicles and airplanes. Due to the country's arid climate, sand and dust storms, which are naturally occurring events that contain elevated levels of PM and usually affect arid and semi-

arid regions, are considered to be the most common natural source of PM there (Nayebare 2016; Khodeir et al. 2012; Alghamdi et al. 2015b).

In the case of Makkah, sources of PM pollution are mainly road traffic, construction work, re-suspended and windblown sand and dust particles (Habeebullah et al. 2010; Munir et al. 2013b). Nayebare et al. (2018), reported that anthropogenic sources including vehicular emissions, fossil-fuels/oil combustion, and industrial mixed dust accounted for 75 % of total $PM_{2.5}$ emissions using source apportionment with positive matrix factorisation (Nayebare et al. 2018). Habeebullah (2013), reported that PM_{10} concentration is mostly associated with re-suspended and windblown dust and sand particles which are more related to natural sources given the arid nature of the region rather than anthropogenic sources using polar plots, time variation plots and correlation analysis (Habeebullah 2013b). Munir et al. (2013), reported weak associations between traffic related air pollutants and PM_{10} concentration using statistical modelling. This indicates that anthropogenic sources are not the main contributor to PM_{10} in Makkah (Munir et al. 2013b). Findings from these studies (Habeebullah 2013b; Munir et al. 2013b; Nayebare et al. 2018) suggest that in Makkah, smaller sized particles originate from anthropogenic sources while larger sized particles originate from natural sources.

2.4.3 Particulate air pollution in Saudi cities

Studies investigating PM in Saudi Arabia were identified in Web of Science (http://www.webofknowledge.com) and Google Scholar (https://scholar.google.co.uk) by using the following search terms in different combinations; “Particulate matter”, “Air pollution”, “Sources”, “Causes”, “Concentration”, “Composition”, “Source apportionment”, “Health effects”,

"Cardiopulmonary health", "Respiratory diseases", "Cardiovascular diseases", "Saudi Arabia", "Riyadh", "Jeddah", "Makkah", "Rabigh", and "Taif". A total of 41 studies were found to be relevant for this literature review as they concentrated on PM concentration, composition, source apportionment, and cardiopulmonary health effects. The bibliographies of the studies found were also examined to see if there were any further relevant studies. *Table 2.9* below summarizes what has been conducted and in which part of the country was it conducted in.

Table 2.9, A summary of investigations conducted on PM in major Saudi cities

City	PM concentration	PM size and number distribution	PM composition	PM source apportionment	PM association with cardiopulmonary health effects
Jeddah	✓ (Nasralla 1983; El-Assouli et al. 2007; Abulfaraj et al. 1990; Nasralla 1984; Khodeir et al. 2012; Hussein et al. 2014; Alghamdi et al. 2015b; Lim et al. 2018)	✓ (Lihavainen et al. 2016; Hyvärinen et al. 2013)	✓ (Nasralla 1983; Alghamdi et al. 2015b; Khodeir et al. 2012) (El-Assouli et al. 2007; Hasnain et al. 1995)	✓ (Khodeir et al. 2012; Lim et al. 2018)	✓ (Nayebare et al. 2016)
Makkah	✓ (Nasralla and Seroji 2008; Othman et al. 2010; Munir et al. 2013a; Munir et al. 2013b; Habeebullah 2014; Habeebullah et al. 2015; Adly et al. 2019)		✓ (Habeebullah 2016; El-Assouli 2011; Habeebullah 2013c)	✓ (Nayebare et al. 2016; Nayebare et al. 2018)	**X**
Riyadh	✓ (El-Shobokshy 1984; Rushdi et al. 2013; Modaihsh and Mahjoub 2013; Alharbi et al. 2015)	✓ (Ahmed et al. 1987)	✓ (Al-Shayeb 2001; Alharbi et al. 2015; Rushdi et al. 2013; El-Mubarak et al. 2014; Bian et al.	**X**	**X**

			2016; Hasnain et al. 1995; Al-Suwaine et al. 1999)		
Taif	✓ (Shaltout et al. 2013; Shaltout et al. 2015)	**X**	✓ (Shaltout et al. 2013; Shaltout et al. 2015)	**X**	**X**
Rabigh	✓ (Nayebare et al. 2016)	**X**	✓ (Nayebare et al. 2016)	✓ (Nayebare et al. 2016)	**X**

In one of the early studies on PM in Saudi Arabia, Nasralla, (1989) investigated dust fall over the city of Jeddah, which is one of the major urban cities in Saudi Arabia. He found high dust deposition rates in three areas. The highest was close to a cement plant in which the measured rate (45 g/m^2/month) was much higher than that set-in air quality standards. High dust-fall rates (20 g/m^2/month) were also found in Jeddah's commercial centre and which is thought to be attributed to the high number of motor vehicles and commercial activities in that area. The industrial part of Jeddah situated downwind of the city and the oil refinery in that area, also received high rates of dust fall (18.3 g/m^2/month). It is important to note that this study was conducted in the 1980s. At that time, the author expressed his great concern regarding land use and selection of industrial and urban locations (Nasralla 1983).

In the past few decades, particulate air pollution in Saudi Arabia has gained much needed attention (Alghamdi et al. 2015b; Alharbi et al. 2015; El-Mubarak et al. 2014; El-Shobokshy et al. 1990; El-Assouli 2011; Habeebullah 2014, 2016; Habeebullah et al. 2015; Khodeir et al. 2012; Munir et al. 2013a; Munir et al. 2013b; Nayebare et al. 2016; Rushdi et al. 2013; Lim et al. 2018). The country experienced rapid development socially and economically from industrialization and urbanization that led to the expansion of cities. As a result, areas with high population densities are now adjacent or surrounded by industrial facilities such as oil refineries and cement and concrete factories that used to be outside the cities. Hence, people are now living in close proximity to PM emission sources (Khodeir et al. 2012). Moreover, due to the low means of rainfall, the atmosphere is not being regularly cleaned from PM. As a result, it remains suspended in the atmosphere for a long time. This would have serious

effects on a local and regional level and would increase the exposure to PM and its associated health risks (Nasralla 1986; Seroji 2011).

2.4.3.1 Particulate mass concentration measurement

A large air quality network has been recently developed for monitoring PM_{10} and $PM_{2.5}$ concentrations in major Saudi cities (*Figure 2.11*) (Munir et al. 2017; PME 2020). Unfortunately, however, even though there are studies that have used data from these stations, for this project, raw data from these stations couldn't be obtained directly from the Saudi Presidency of Meteorology and Environment (PME). The only data available was daily average air quality values (AQI) of PM_{10}, NO_2, SO_2, CO, and O_3 for different times of 2019 / 2020 which will be further explored in *Chapter 5* (Badri et al. 2020c). Therefore, the available data on PM concentrations in Saudi Arabia was mostly obtained from published studies. A number of published studies that examined PM in Saudi Arabia have been found in the scientific literature. All of those studies have been conducted in major urban cities, in particular those located in the western part of the Kingdom. *Table 2.10* below is a reference to PM standards in Saudi Arabia, the UK, EU, WHO and USA and is used to compare measurements mentioned below.

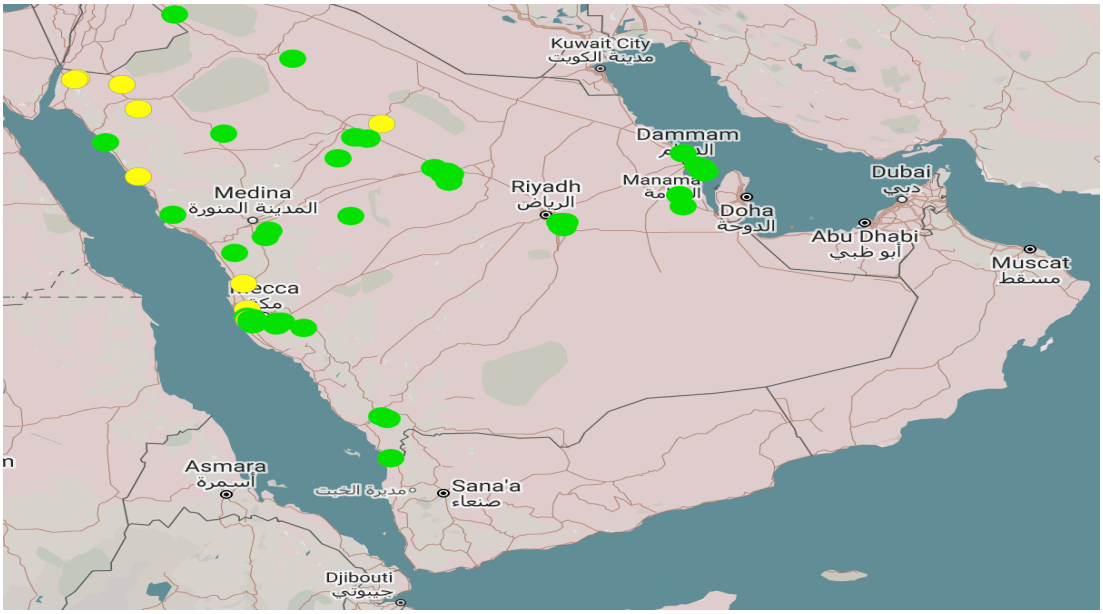


Figure 2.11, Location of PM_{10} and $PM_{2.5}$ monitoring stations in Saudi Arabia (From PME (2020))

Table 2.10, Air quality standards of different countries and organizations. Adapted from Habeebullah (2013a), DEFRA (2005), ECE (2019), and U.S. EPA (2019).

Country	PM_{10}	$PM_{2.5}$
UK	40 µg/m^3 (annual mean)	25 µg/m^3 (annual mean)
EU	40 µg/m^3 (annual mean)	25 µg/m^3 (annual mean)
USA	50 µg/m^3 (annual mean)	12 µg/m^3 (annual mean)
KSA	80 µg/m^3 (annual mean)	15 µg/m^3 (annual mean)
WHO	20 µg/m^3 (annual mean)	10 µg/m^3 (annual mean)

2.4.3.1.1 Jeddah

Khodeir et al. (2012) was the first to comprehensively investigate particulate matter concentration and composition in Jeddah (Khodeir et al. 2012). The authors conducted a multi-week sampling campaign at multiple sites in Jeddah between June and September, 2011 and reported that the overall daily mean concentration of $PM_{2.5}$ and PM_{10} at seven sampling sites was 28 ± 25 µg/m^3 and 87 ± 47 µg/m^3 respectively however, with a high level of spatial and temporal variability. Another study by

Hussein et al. (2014) found similar results for the overall daily mean concentration of $PM_{2.5}$ and PM_{10} being 34 ± 45 μg/m^3 and 104 ± 162 μg/m^3 respectively. A seasonal variation was also reported with higher $PM_{2.5}$ and PM_{10} concentrations during February-April which was expected since it's the dust-storms season (Hussein et al. 2014). It is important to consider dust-storms when measuring concentrations of PM especially in Jeddah since it is frequently exposed to such phenomena. Alghamdi et al. (2015) measured $PM_{1.0}$, $PM_{2.5}$, and PM_{10}, during dust-storms and non-dust-storms periods. Their results indicated that PM concentrations were higher in dust-storms environments compared to non-dust-storms environments. The daily mean concentration of $PM_{1.0}$, $PM_{2.5}$, $PM_{2.5-10}$ and PM_{10} was 16, 49, 116, and 165 μg/m^3 respectively in the non-dust-storms environment. In dust-storms environments, overall mean concentration for $PM_{1.0}$, $PM_{2.5}$, $PM_{2.5-10}$ and PM_{10} was 41, 247, 663, and 909 μg/m^3 respectively. As for the whole period of study the overall mean concentration for $PM_{1.0}$, $PM_{2.5}$, $PM_{2.5-10}$ and PM_{10} was 25, 126, 328, and 453 μg/m^3 respectively. It is obvious that coarse particles are higher in concentration than fine particles in Jeddah. The increase in coarse particle concentrations is attributed to dust-storms (Alghamdi et al. 2015b). Lim et al. (2018), measured $PM_{2.5}$ and PM_{10} for over a year, from June 2011 to May 2012 and reported annual mean concentrations of $PM_{2.5}$ (21 ± 11 μg/m^3) and PM_{10} (107 ± 72 μg/m^3). The authors also compared $PM_{2.5}$ and PM_{10} in weekdays and weekends and found the concentrations to be consistently higher during weekdays compared to weekends (Lim et al. 2018)

2.4.3.1.2 Makkah

The first study in Makkah conducted by Nasralla & Seroji (2008) reported that daily PM_{10} concentration in Mina valley during Hajj season ranged from 191-262 μg/m^3.

Also, it was reported that TSP concentrations reached 665 μg/m^3 (Nasralla and Seroji 2008). High PM_{10} concentrations during Hajj season were also reported by Othman et al. (2010) using satellite imagery as means of estimation (Othman et al. 2010). Munir et al. (2013), investigated PM_{10} from 1997 - 2012 and concluded that PM_{10} had a positive temporal trend in Makkah and a daily mean value of 113 μg/m^3 (Munir et al. 2013a). Munir et al. (2013), investigated PM_{10} concentrations from November 2011 to June 2012 and reported that it had a daily mean concentration of 174.6 μg/m^3 (Munir et al. 2013b). Habeebullah (2014), investigated PM_{10} and found that its annual concentration of 195 μg/m^3 (Habeebullah 2014). Habeebullah et al. (2015), investigated PM_{10} in 4 different sites and reported that the daily mean concentration for all 4 sites were 255, 185, 162, and 56 μg/m^3 (Habeebullah et al. 2015). Nayebare et al. (2018), investigated $PM_{2.5}$ concentrations from February 26, 2014 to January 27, 2015 and reported daily means of 113 ± 67, 88 ± 36, 67 ± 24, and 67 ± 36 during spring, summer, autumn, and winter respectively (Nayebare et al. 2018). Adly et al. (2019), investigated PM_{10} concentrations at six different locations and reported concentrations of 120 ± 52 μg/m^3, 223 ± 30 μg/m^3,77 ± 36 μg/m^3, and 89 ± 62 μg/m^3 during spring, summer, autumn, and winter respectively. Higher levels of PM_{10} during spring and summer are thought to be attributed to weak air dispersion during that period. Higher levels were also found during August and September , which coincides with Hajj, and May and June, which coincides with Ramadan and are thought to be caused by higher volumes of traffic during those months (Adly et al. 2019).

2.4.3.1.3 Taif

Only two studies (Shaltout et al. 2013; Shaltout et al. 2015) have been found to be conducted in Taif. Shaltout et al. (2013), investigated the concentration of $PM_{2.5}$ at a

residential and industrial site in the summer of 2011. The industrial site was located in proximity to the city's largest industrial area. The residential site was located in the heart of the city's most crowded area. The authors reported daily mean $PM_{2.5}$ concentrations to be 47 ± 15 μg/m^3 at the industrial site and 46 ± 31 μg/m^3 at the residential site. Shaltout et al. (2015), investigated the concentration of $PM_{2.5}$ at three different sites (industrial, residential, and traffic dominated) during 2011/2012. The authors reported daily mean $PM_{2.5}$ concentration to be 37 ± 22 μg/m^3, 57 ± 22 μg/m^3 and 50 ± 31 μg/m^3 at the residential, industrial and traffic sites, respectively (Shaltout et al. 2015).

2.4.3.1.4 Riyadh

Rushdi et al. (2013), investigated $PM_{2.5}$ and PM_{10} concentrations at 10 sites during June and November 2006 and February and May 2007. Daily mean concentration of $PM_{2.5}$ were 104 ± 61, 76 ± 66, 125 ± 70 and 190 ± 82 while PM_{10} concentrations were 180 ± 125, 146 ± 112, 268 ± 165, and 312 ± 147 for June, November, February, and May respectively. These findings show higher PM_{10} concentrations than $PM_{2.5}$ and indicate that the major PM source could have been local dust. Concentrations of PM were also found to be temporally and spatially variable (Rushdi et al. 2013). Modaihsh and Mahjoub (2013), measured mass concentrations of $PM_{2.5}$ and PM_{10} at Riyadh airport from January-April in 2012. The daily mean $PM_{2.5}$ and PM_{10} concentrations were reported to be 142 and 563 μg/m^3 respectively. Daily concentrations of $PM_{2.5}$ and PM_{10} were highly variable and the temporal variation was attributed to dust-storms events (Modaihsh and Mahjoub 2013). Alharbi et al. (2015), investigated PM_{10} concentrations in several locations from September 2011 to September 2012. The study reported annual PM_{10} concentrations ranging from 39.7 - 1803 μg/m^3 with an

overall mean of 289 ± 229 μg/m^3. A temporal and spatial variation was also reported. Concentrations were found to be 84 % higher in summer compared to winter which is expected since most dust-storms occur during the summer months. During the study period, a total of 15 dust-storms occurred and samples were collected during those events. A 200 % increase in concentrations on dust-storms days compared to non-storm days was reported. Also, concentrations were 17 % lower in weekends compared to weekdays. This is explained by the heavy traffic during weekdays. Moreover, concentrations in industrial areas were 60% higher than residential areas (Alharbi et al. 2015).

2.4.3.1.5 Rabigh

Rabigh is a heavily industrialised city that is located at the east coast of the Red Sea on the western part of Saudi Arabia. The study by Nayebare et al. (2016), measured $PM_{2.5}$ concentration at a fixed site during May - June 2013. It was reported that daily $PM_{2.5}$ concentrations ranged from 12 - 76 μg/m^3 and were spatially and temporally variable. The daily mean concentration of $PM_{2.5}$ (37 ± 16 μg/m^3) was found to be higher than the 24-h WHO limit (20 μg/m^3) of which 90 % of the collected samples were found to be exceeding that limit (Nayebare et al. 2016, 2017).

Table 2.11 below summarises the studies on PM mass concentration measurement in Saudi cities.

Table 2.11, A summary of studies on PM mass concentration in major Saudi cities

City	Parameter measured	Instrument	Time of measurement	Averaging period	Mean concentration ($\mu g/m^3$)	$PM_{2.5}/PM_{10}$ ($\mu g/m^3$)	Source
Jeddah	$PM_{2.5}$ PM_{10}	Automated Cartridge Collector Unit (ACCU)	June – September (2011)	24 hour	28 ± 25 87 ± 47	0.33 $\mu g/m^3$	(Khodeir et al. 2012)
	$PM_{2.5}$ PM_{1} PM_{10}	Optical scattering spectrometer (EDM-180D, Grimm Aerosol, Germany)	2011 – 2012	24 hour	34 ± 45 13 ± 11 104 ± 162	N/A	(Hussein et al. 2014)
	$PM_{1.0}$ $PM_{2.5}$ $PM_{2.5-10}$ PM_{10} (In non-dust-storms, Dust-storms, Whole study period)	Optical scattering spectrometer (Environmental Dust Monitor (EDM- 179), Grimm Aerosol, Germany)	March 2012	24 hour	16, 41, 25 49, 247, 126 116, 663, 328 165, 909, 453	N/A	(Alghamdi et al. 2015b)
	$PM_{2.5}$ PM_{10}	Harvard impactors connected to calibrated	June 2011 - May 2012	24 hour	21 107	0.20	(Lim et al. 2018)

		vacuum pump (Gast, USA)					
Makkah	PM_{10}	High volume samplers, PM_{10} sampler, and $PM_{2.5}$ sampler (Staplex Co.)	In Ramadan and hajj periods (2004 – 2005)	24 hour	N/A	N/A	(Nasralla and Seroji 2008)
	PM_{10}	Data obtained from PME	1997 – 2012	24 hour	113	N/A	(Munir et al. 2013a)
	PM_{10}	N/A	November 2011 – June 2012	N/A	175	N/A	(Munir et al. 2013b)
	PM_{10}	IP Beta Gage Monitor device	March 2012 – February 2013	Annual	196	N/A	(Habeebullah 2014)
	PM_{10} (In Shebikah, Masfalah, Aziziyah, Awali)	High Volume Sampler (HVS)	August 2012 – September 2013	24 hour	255, 185, 162, and 56	N/A	(Habeebullah 2016)
	$PM_{2.5}$ (spring, summer, autumn, winter)	pre-weighed, sequentially numbered polypropylene ring supported Whatman 2 μm pore-size PTFE 46.2 mm filters, using a low volume air sampling pump	February 2014 – January 2015	24 hour	113, 88, 67, and 67	N/A	(Nayebare et al. 2018)

	PM_{10} (spring, summer, autumn, winter)	mini volume sampler (Airmetrics, USA)	March 2016 - February 2017	24 hour	120, 223,77, and 89	N/A	(Adly et al. 2019)
Taif	$PM_{2.5}$ (In Industrial site, Residential site)	Polycarbonate filters loaded inside a collection cartridge in a cyclone (CASELLA Company, UK).	Summer of 2011	24 hour	47 ± 15, 46 ± 31	N/A	(Shaltout et al. 2013)
Riyadh	$PM_{2.5}$ PM_{10} (In June, November, February, May)	MiniVol portable air samplers (Airmetrics, 4 Eugene, OR, USA)	June 2006 – to may 2007	24 hour	(104 ± 61, 76 ± 66, 125 ± 70, 189 ± 82) (180 ± 125, 146 ± 112, 268 ± 165, 312 ± 145)	N/A	(Rushdi et al. 2013)
	$PM_{2.5}$ PM_{10}	Grimm model EDM 365 aerosol spectrometer	January – to April 2012	24 hour	142 563	0.25	(Modaihsh and Mahjoub 2013)
	PM_{10}	PQ-100 particulate	2011 - 2012	Annual	289 ± 229	N/A	(Alharbi et al. 2015)

		samplers (BGI incorp. USA)					
Rabigh	$PM_{2.5}$	pre-weighed, sequentially numbered polypropylene ring supported Whatman 2 μm pore-size PTFE 46.2 mm filters, using a low volume air sampling pump	May – June 2013	24 hour	37 ± 16.	N/A	(Nayebare et al. 2016)

2.4.3.2 Particle number and size distribution measurement

Data regarding particle number and size distribution measurements in Saudi Arabia are limited. Only three studies have been found in the scientific literature. The first study by Ackerman & Cox (1982), used a Forward Scattering Spectrometer Probe to measure particle number concentration from data collected during 5 flight missions in the month of May 1979. The study found that the maximum number concentration occurred at a particle radius of approximately 3 μm. Moreover, the study reported that there was a rapid decrease in number concentration associated with the increase in particle size and a smaller decrease with the decrease in particle size (Ackerman and Cox 1982).

The second study by Ahmed et al. (1987), attempted to evaluate the particle size distribution functions during five dust-storms in Riyadh at different heights (1 - 21 m). The study concluded that the distribution depends on factors such as height and storm conditions. Moreover, the mean diameter was reported to decrease with the increase in height. At the height of 21 m, the mean diameter ranged from 16 - 19 μm. On the other hand, at the height of 1m the mean diameter ranged from 21 - 43 μm (Ahmed et al. 1987).

The third study by Hyvärinen et al. (2013), was conducted in 2012 at The Hada Al Sham site which is located around 60 km east of Jeddah. A Differential Mobility Particle Sizer was used to measure aerosol size distribution. The mean particle number concentration in December 2012 was found to be 1640 #/cm^3. Newly formed particles were behind high particle concentrations with a 1h- mean maximum of 11400 #/cm^3 which could be attributed to sulphate emissions from heavy oil combustion such as oil

refineries. Particle size distribution was found to be highly variable. Midnight to early morning hours were dominated by the accumulation mode in which the mean particle diameter was reported to be 60 nm during that time. Then, Aitken mode appeared with a mean particle diameter of 40 nm. After that nucleation mode appears and particles grow to Aitken mode with time with a mean particle diameter of 33 nm during this period (Hyvärinen et al. 2013).

2.4.3.3 The chemical properties of PM and measurements within Saudi Arabia's atmosphere

There have been a number of studies that examined PM composition in Saudi Arabia. In this literature review, the author attempts to summarize the information in such studies to provide an estimate of typical PM composition in the Kingdom's atmosphere. The studies have been conducted in different locations around the country. A number of studies have focused on certain components such as heavy metals or organic compounds. Fortunately, however, there are studies that investigated PM composition and sources comprehensively. It is important to note that comparing and combining information from different studies is difficult since different sampling and analytical techniques were used.

2.4.3.3.1 Sulphates

In Makkah, mean sulphate concentrations were reported to be 74, 17, and 6 $\mu g/m^3$ while percent concentrations were reported to be 23, 17, and 21 % for TSP, $PM_{10,}$ and $PM_{2.5}$ respectively. It appeared that secondary sulphate was one of the dominant anions in TSP, $PM_{10,}$ and $PM_{2.5}$ (Habeebullah 2016). That was found to be the same in Rabigh, as sulphate was the most dominant anion in $PM_{2.5}$ with a daily mean concentration of

7 ± 5 μg/m^3. As for Jeddah, high sulphate concentration in dust fall samples were reported near the industrial zone there (Nasralla 1983). High sulphate concentration is thought to be attributed to industrial activities in Rabigh and Jeddah. However, the likely origin of sulphate is fine particles of ammonium sulphate that are formed as secondary aerosols in the atmosphere from the reactions between sulfuric acid, water, and ammonia (Chen et al. 2018). This is confirmed by the high positive correlation between ammonium and sulphate found in Rabigh (Nayebare et al. 2016). Other forms of sulphate would include sulfuric acid, which is formed by gas-phase oxidation of primary gaseous oxides of sulphur, and calcium sulphate, which could originate from industrial activities such as cement factories.

2.4.3.3.2 Ammonium compounds

In Makkah, mean ammonium concentrations were reported to be 16, 3, and 0.60 μg/m^3 while percent concentrations were reported to be 5, 3, and 2 % for TSP, $PM_{10,}$ and $PM_{2.5}$ respectively. It was reported that there was a high positive correlation between ammonium and nitrate in Makkah (Habeebullah 2016). Moreover, that correlation existed for $PM_{2.5}$ in Rabigh as well even though being moderate. The strongest correlation ammonium had was with sulphate in Rabigh. Ammonium mean daily concentrations in Rabigh were reported to be 2 ± 1.5 μg/m^3 (Nayebare et al. 2016). The likely origin of ammonium is fine particles of ammonium sulphate and ammonium nitrate formed by atmospheric reactions between ammonia and acid gases.

2.4.3.3.3 Chloride

In Makkah, mean chloride concentrations were reported to be 55, 17, and 5 μg/m^3 while percent concentrations were reported to be 17, 17, and 20 % for TSP, $PM_{10,}$ and

$PM_{2.5}$ respectively (Habeebullah 2016). Alharbi et al. (2015), also reported high concentration of chloride in Riyadh which just like Makkah is far from the sea. High chloride concentrations in those two cities can be from calcium chloride which is used in new construction activities and in maintenance of new unpaved roads (Alharbi et al. 2015). In Rabigh, daily mean concentrations of chloride in $PM_{2.5}$ were reported to be 0.64 ± 0.85 $\mu g/m^3$ (Nayebare et al. 2016). In Jeddah, the study by Nasralla (1983), reported high concentrations of chloride in samples of dust fall with an annual mean of 3.6%. Jeddah and Rabigh are coastal cities, thus being heavily influenced by sea sprays which explains the presence of chloride in the atmosphere. Chloride is mainly present in the atmosphere as sodium chloride but sometimes as ammonium chloride as well. It is expected that coastal areas would contain higher concentrations of chloride than inland areas as a result of sea spray (Nasralla 1983).

2.4.3.3.4 Nitrate

In Makkah, mean nitrate concentrations were reported to be 93, 40, and 8 $\mu g/m^3$ while percent concentrations were reported to be 29, 40 and 31 % for TSP, $PM10$, and $PM_{2.5}$ respectively. It appeared that secondary nitrate was one of the dominant anions in TSP, PM_{10}, and $PM_{2.5}$ (Habeebullah 2016). In Rabigh, daily mean concentrations of nitrate in $PM_{2.5}$ were reported to be 2 ± 1 $\mu g/m^3$ (Nayebare et al. 2016). Nitrate can be present in the atmosphere in the form of ammonium nitrate and sodium nitrate. Most nitrate in coastal areas such as Jeddah and Rabigh is present as sodium nitrate due to the influence of sodium from sea sprays.

2.4.3.3.5 Metals

In Makkah, the study by Habeebullah (2016), considered lead, nickel, cadmium, chromium, vanadium, arsenic, mercury, and aluminium in their analysis of TSP, $PM_{2.5}$, and PM_{10}. It was reported that the highest amounts of mercury, cadmium, and chromium were found in $PM_{2.5}$ while the highest amounts of lead and arsenic were found in TSP. Arsenic was the most abundant heavy metal in TSP (42.25 %) and $PM_{2.5}$ (43.42 %) and the second most abundant heavy metal in PM_{10} (23 %). Among all heavy metals analysed in this study, arsenic was found to be the most prominent. The presence of such toxic element in the atmosphere of Makkah is extremely concerning. Exposure to high levels of arsenic for long periods results in in acute toxic effects. On the other hand, exposure to low levels for long periods would not result in acute toxic effect but can however cause cancer. Currently, it is still unclear if arsenic poses a health risk since the data used here was for a short period of time only. Therefore, further monitoring for longer periods in Makkah is required (Habeebullah 2016). Adly et al. (2019), considered cadmium, chromium, arsenic, beryllium, and nickel in their analysis of PM_{10} and found that the concentrations of the aforementioned PM components were 0.098, 0.008, 0.26, 0.03, and 0.012 μm^{-3} respectively (Adly et al. 2019).

In Jeddah, Khodeir et al. (2012), found that sulphur and silicon were the largest contributors to $PM_{2.5}$ mass with a mean value of 3.4 and 2.1 $\mu g/m^3$ respectively. As for PM_{10}, the largest mass contributors were found to be silicon and calcium with a mean value of 11 and 4.4 $\mu g/m^3$ respectively. The study reported a significant correlation between $PM_{2.5}$ and PM_{10} mass concentrations which indicates that they originate from the same sources. Using factor analysis, the study identified a number

of sources, which were characterised by high concentrations of certain elements, for $PM_{2.5}$ and PM_{10} and they are re-suspended soil (calcium, aluminium, silicon, and iron), emissions of oil combustion (vanadium and nickel), traffic (lead, bromine, and selenium), and a source mixed by industrial activates and marine aerosol (copper, zinc, sodium, chlorine) (Khodeir et al. 2012). Alghamdi et al. (2015), considered 23 elements of $PM_{2.5}$ in dust-storms and non-dust-storms periods in their analysis. The authors found that 45 and 68 % of the total concentration consisted of crustal elements (silicon, calcium, sodium, aluminium, iron, potassium, and magnesium) in non-dust-storms and dust-storms periods respectively. This is an indication to the level of contribution soil sourced species make to airborne particles during dust-storms as the increase in percentage was significant. Factor analysis was also used in this study and identified soil and re-suspended dust as the main source of sodium, magnesium, silicon, potassium, calcium, titanium, chromium, manganese, iron, rubidium, and strontium in dust-storms and non-dust-storms periods which is consistent with the findings of the previous study. On the other hand, anthropogenic sources were identified to be the source of sulphur, chlorine, cobalt, copper, zinc, gallium, arsenic, lead, and cadmium in both dust-storms and non-dust-storms periods. It seems that Jeddah is heavily affected from the deserts surrounding it. Even though anthropogenic sources are contributing, it is evident that the source of those mineral crust elements was the deserts (Alghamdi et al. 2015b).

In Riyadh, Al-Shayeb et al. (2001), examined metal content of roadside surface and sub-surface soils along Riyadh's ring road. The elements considered in the study were lead, zinc, copper, chromium, and nickel. It reported a positive correlation between traffic density and lead, zinc, and copper. Their concentration decreased as the

distance from the road increased. The study found no evidence of nickel contamination. The main source of lead pollution is considered auto mobile traffic. However, a substantial difference between levels in surface and sub-surface soils was reported which indicates that atmospheric deposition was the main source. Nevertheless, it is important to note that Saudi Arabia introduced the use of un-leaded fuel after 2001 (Al-Shayeb 2001). Rushdi et al. (2013), found high concentrations of nickel and sulphur which is contradictory to Al-shayeb et al. (2001) reporting's of no nickel contamination. Using factor analysis, only sulphur and nickel were found to be of anthropogenic origin (Rushdi et al. 2013). High sulphur concentration might have resulted from heavy construction activities in the city. The concentration of nickel could be attributed to automobile pollution. Other elements found in the study (sodium, magnesium, aluminium, potassium, calcium, and silicon) were attributed to soil and re-suspended dust (Rushdi et al. 2013). The influence of dust from the surrounding deserts is also emphasised in the study by Alharbi et al. (2015). Crustal matter components such as iron, magnesium, titanium, calcium, and manganese increased several folds in summer when dust-storms occur more frequently. Also, a strong correlation was reported between aluminium, iron, magnesium, and manganese which suggests their common source from soil and re-suspended dust. What is surprising however is that dust-storms might also be involved in decreasing the concentration of elements such as vanadium, chromium, copper, lithium, and lead in 2012 since the increase in crustal elements from dust-storms dilutes other elements. Nevertheless, anthropogenic source also contribute to the metal content of PM in the city as there was a significant increase in concentration of elements such as zinc, iron, and boron at industrial locations compared to residential ones. The contribution of anthropogenic sources is also confirmed by the reported correlation between arsenic,

cobalt, cadmium, and lithium and between arsenic, vanadium, and nickel that indicates their common industrial source (Alharbi et al. 2015).

In Taif, Shaltout et al. (2013), reported high concentrations of silicon, sulphur, potassium, iron, and calcium in both the industrial and residential area. Just like most cities in the country, these elements are thought to originate from the deserts surrounding Taif. Higher concentrations of zinc and copper were found in the industrial area compared to the residential area. This indicates that they probably originated from local anthropogenic sources in the industrial area. Interestingly, titanium concentration was found to be higher in the residential area. Fortunately, however, the study found that the measured concentrations of hazardous trace elements did not exceed the limit of national and international guidelines. The city seems to be not suffering from severe pollution compared to other urban areas even though it is located in a desert area. Nevertheless, more research still needs to be conducted in the city to confirm these findings (Shaltout et al. 2013).

In Rabigh, a study reported that the major contributor to a large proportion of measured trace elements was soil (Nayebare et al. 2016). With the exception of sulphur, the overall contribution of anthropogenic sources was relatively small. Using factor analysis, silicon, aluminium, magnesium, titanium, potassium, calcium and iron were found to be originating from a common natural source which is soil. Calcium, iron, silicon, and aluminium can also originate from industrial dust. Moreover, nickel, vanadium, zinc, lead, chlorine, sulphur, lutetium, and bromine were found to be originating from anthropogenic sources. Fossil fuel/oil combustion are thought to be the source of vanadium, nickel, lead, lutetium, copper and zinc. The previous findings show that the city is affected by several sources. Being the first study to evaluate fine

particulate air pollution in Rabigh, more comprehensive research needs to be conducted in order to better understand the sources of metals in the city and their impact on human health (Nayebare et al. 2016).

2.4.3.3.6 Carbon compounds

In Taif, a black smoke reflectometer was used to examine BC levels in the city. Mean concentrations of BC were found to be 860 ± 440 and 32 ± 240 ng/m^3 at the industrial and residential site respectively. Those results show that the concentration of BC at the industrial site was about three time higher than the residential one. However, this is not surprising as that significant increase would be attributed to a number of industrial activities such as blacksmithing and construction. Also, heavier traffic was reported at the industrial site compared to the residential one (Shaltout et al. 2013). In Rabigh, a non-destructive Dual-wavelength Optical Transmissometer Data Acquisition System was used to determine BC levels in the city. Mean daily BC concentrations were reported to be 1 ± 0.4 μg/m^3 for BC_{IR} (measurement at infrared wavelength) and 1 ± 0.3 μg/m^3 for BC_U (measurement at ultraviolet wavelength). The actual BC is represented by BC_{IR} while the presence of non-black organic compounds is represented by BC_U. Black Carbon represented only 3.4 % of total $PM_{2.5}$ in the city. Heavy oil combustion and vehicular emissions are mostly the sources of BC in Rabigh (Nayebare et al. 2016).

Combustion processes can result in BC being coated by organic matter such as Polycyclic Aromatic Hydrocarbons (PAHs) and Polychlorinated Biphenyls (PCBs). A number of studies investigated PAHs since they are known to be carcinogenic.

In Makkah, El-Assouli (2011), found that during the 2004 and 2006 Hajj season total amounts of PAHs extracted from PM_{10} in Arafat were 3 and 2 ng/m^3 respectively. On the other hand, concentrations in Muzdalifa were higher as total PAHs in 2004 and 2006 were 5 and 4.7 ng/m^3 respectively. The Major PAHs component in both sites was reported to be benzo(ghi)perylene with its concentration ranging from 13 - 63 % of the total PAHs in both sites during the study period (El-Assouli 2011). Habeebullah (2013), found that total PAHs concentrations ranged from 104 - 195 ng/m^3 with mean values of 165, 138 and 132 for TSP, PM_{10}, $PM_{2.5}$, respectively at three different sites. The high concentrations found in here could be attributed to the massive construction work being conducted there during the period of the study. It is also important to note that two of the sampling sites in this study were in close proximity to the construction sites (Habeebullah 2013c).

In Jeddah, El-Assouli et al. (2007), examined 16 PAHs compounds in eleven 24-h PM_{10} samples from different urban sites (El-Assouli et al. 2007). The study reported concentrations of PAHs ranging between 0.8 ng/m^3 at industrial and heavy traffic sites to 0.2 ng/m^3 at a residential area. The most abundant PAHs was reported to be benzo(ghi)perylene. Its concentration reached 65 % of total PAHs in one of the sites. Another study by Alghamdi et al. (2015), investigated vapour and particulate phase PAHs and reported that the sources of PAHs in the city might mainly be gasoline vehicles, industrial sources, and fuel/oil combustion (Alghamdi et al. 2015a).

In Riyadh, PM_{10} PAHs concentrations were investigated in 2010 by El-Mubarak et al. (2014) and it was reported that levels of individual PAHs congeners were extremely high. Total PAHs concentrations varied from 1,383 to 13,470 ng/m^3 with a mean of

5,871 ± 2,830 ng/m^3. Benzo[a]pyrene)/m^3 was detected and had a mean concentration of 451 ± 259 ng/m^3 and ranged from 30 - 1,224 ng/m^3 which is extremely high. The study concluded that traffic emissions were the main source of PAHs in the city (El-Mubarak et al. 2014). In another study, Bian et al. (2016), quantified 16 PAHs in 167 sample collected from 2011-2012. It was found that total particle-phase concentrations ranged between to 516 ng/m^3 and had a mean concentration of 18 ± 61 ng/m^3. The two most abundant PAHs were reported to be Pyrene and Flouranthene; their mean concentration was 3 ± 14 ng/m^3 and 8 ± 44 ng/m^3 respectively. Oil combustion emissions accounted for 96% of the total PAHs concentration which is expected in a city like Riyadh as oil fuels are heavily used there (Bian et al. 2016).

2.4.3.3.7 Biological particles

Hasnain et al. (1995), investigated outdoor allergens at different sites of Jeddah and Riyadh. The authors reported that the genus Ulocladium was found to be one of the major spore categories in the outdoor environment of Saudi Arabia. During the period of study higher concentration were reported in winter and spring. The maximum concentration was reported at the coastal city of Jeddah (1200 spore/m^3) (Hasnain et al. 1995). Another study in Riyadh (Al-Suwaine et al. 1999) attempted to identify allergic fungi at two different sites. Again, Ulocladium was reported to be one of the major airborne fungi in the city. Other major components found in the study included Cladosporium, Penicillium, Aspergillus, and Alternaria. The most abundant at both sites was Cladosporium. The same seasonal variation was also reported here as concentrations in winter were higher. Hasnain et al. (2005), investigated airborne pollen grains and Spores at three Saudi cities, Abha, Al-Khobar and Hofuf. Just like

the previous studies, Cladosporium and Ulocladium were among the major types found (Hasnain et al. 2005).

2.4.4 Epidemiological studies on PM and its health effects in Saudi Arabia

Not much data regarding the effect of PM on mortality and morbidity due to cardiovascular and respiratory diseases are available to the public in Saudi Arabia. Even though most studies have reported elevated levels of PM in several major cities of the country, their association with cardiopulmonary health outcomes was unclear until recently. The first known study to investigate the association between daily exposure to $PM_{2.5}$ and its chemical constituents and cardiopulmonary morbidity in Saudi Arabia has been recently published (Nayebare 2016; Nayebare et al. 2019).

The association was investigated using a generalized linear time-series model (GLM) with negative binominal distribution. In order to avoid errors associated with collinearity, the health effects associated with exposure to individual air pollutants were modelled separately in single pollutant and single lag models. Confounders such as temperature, humidity, and wind speed were adjusted for in the models as extreme weather conditions have been associated with increased mortality and it's important to differentiate between the effects of PM and extreme weather conditions (Fang et al. 2017). The daily and seasonal variations were also adjusted for in the models by including the day of the week and month. Personal information on life style and habits such as socio-economic status and smoking were not adjusted for as such information was not available. Time lags of up to six days (lag 0 – lag 6) were used to evaluate the time period between the exposure and health effects (Nayebare 2016; Nayebare et al. 2019).

Due to the lack of data as previously mentioned, the author of the study collected his data from physicians working in two hospitals in Jeddah and Rabigh. The physicians provided data for emergency room visits and outpatient and hospital admissions from cardiovascular and respiratory diseases (Nayebare 2016).

In Rabigh, the physicians recorded 2513 Emergency room visits, 268 Outpatients and 155 Inpatient from the 6th of May to the 17th of June 2013. Due to sample size limitations, relative risk analysis at age group level and for out-patients and in-patients was not conducted (Nayebare 2016).

In Jeddah, the physicians recorded 2,274 and 18,401 emergency room visits from cardiovascular and respiratory diseases respectively, 4,520 and 10,327 outpatients from cardiovascular and respiratory diseases respectively, and 1,993 and 1,022 inpatients from cardiovascular and respiratory diseases respectively (Nayebare 2016; Nayebare et al. 2019).

The study found that the morbidity risk was immediate most of the time for respiratory diseases at lags 0 – 1 (lag 0 effects means the same day effects while lag 1 means the effects after one day). For cardiovascular disease however, they were delayed at lags 3 – 6 (lag 3 effects mean the effects after three days while lag 6 means the effects after six days). It was reported that the prevalence of cardiovascular diseases increased significantly with age while the prevalence of respiratory diseases decreased significantly with age (Nayebare 2016; Nayebare et al. 2019).

The study concluded that in general, respiratory disease morbidity risk ranged from 1.081 (CI: 1.005 – 1.162) to 1.096 (CI: 1.023 – 1.173) at moving averages (MAs) 2 – 4 (moving average 2 means the effects on averaging lags 0 and 1 while moving averages 4 means the effects on averaging lags 0, 1, 2, and 3) male, 1.081 (CI: 1.019 –1.146) to 1.087 (CI: 1.020 – 1.159) at MAs_2 – 3, and female, 1.086 (CI: 1.007 – 1.172) to 1.093 (CI: 1.017 – 1.175) at MAs_2 – 4. In general, young females (0 – 14 years) were the most at risk for respiratory diseases with relative risk = 1.097 (CI: 1.025 – 1.174) to 1.148 (CI: 1.049 – 1.257). cardiovascular disease morbidity risk was highest in emergency room visits with overall relative risk = 1.057 (CI: 1.005 – 1.111) to 1.137 (CI: 1.065 – 1.213) across all MAs; male, 1.060 (CI: 1.007 – 1.204) to 1.131 (CI: 1.060 – 1.208); female, 1.065 (CI: 1.008 – 1.125) to 1.116 (CI: 1.045 – 1.192) (Nayebare 2016; Nayebare et al. 2019).

2.4.5 Toxicological studies on PM in Saudi Arabia

A number of toxicological studies on PM have been conducted in Saudi Arabia of which only one of them in Makkah (El-Assouli 2011). In this study, the author used organic matter extracted from PM_{10} to investigate PM genotoxicity. It was reported that the extracted organic matter was found to be mutagenic in the *salmonella* TA98 test as well as damaging human blood cells DNA in the comet assay. In Jeddah, El-Assouli et al. (2007), used organic matter extracted from PM_{10} to investigate PM genotoxicity. The authors of the study found significant DNA damage in the single cell gel electrophoresis (SCGE) comet assay. Indirect mutagenic responses have also been reported in the *Salmonella* mutagenicity (Ames) test (El-Assouli et al. 2007). Sun et al. (2012), conducted an *in vitro* exposure study (Sun, et al., 2012) using PM from Jeddah and reported that the pathways involved in lipid and

cholesterol metabolism of BEAS-2B (human bronchial epithelial cells) experienced irregularities when they were exposed to PM. Exposure to PM also induced genes involved in NRF2-mediated response to oxidative stress in the cells (Sun et al. 2012). Another *in vitro* exposure study in Jeddah by Brocato et al. (2014) found that PM_{10} induced genes involved in inflammation, cholesterol and lipid metabolism, as well as atherosclerosis (Brocato et al. 2014).

2.5 Certified Reference Materials (CRMs)

Certified Reference Materials (CRMs) are defined as a "reference material characterized by a metrologically valid procedure for one or more specified properties, accompanied by a reference material certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability" (Trapmann et al. 2017). They can be used for a variety of purposes including method validations, quality control, training, estimation of measurement uncertainty, and calibration (ILAC 2005). They can also be used as a common reference for other researchers investigating the toxic effects of PM. A variety of commercial CRMs are available; *Table 2.12* below includes some of the CRMs available and their respective prices.

Table 2.12, Commercial CRMs available and their respective prices. Adapted from Sigma Aldrich (2020).

CRM	Price (GBP)
Fine dust (PM10-like) (trace elements) - ERMCZ129	78.70
Fine dust (PM10-like) (PAH's) - ERMCZ100	78.70
Road dust – BCR723	267
Urban dust - NIST1649B	997
Fine particulate matter - NIST2786	1190
Diesel particulate matter – NIST1650B	1190
Urban particulate matter - NIST1648A	1260

2.6 Summary and conclusion

The sections in this chapter have shown the complexity of PM and the key processes affecting its physical and compositional properties of which negative effects in humans are thought to be dependent on. Even though it still is an issue of major concern to developed and developing countries, there still remains key un-answered questions regarding the role of differences in its structural and compositional properties in inducing toxic effects. Unfortunately, investigating such a relationship can be extremely difficult due to limitations arising from the amount of material obtained via PM samplers. That is why, as we previously mentioned in *Chapter 1*, different types of particles will be used in this research project to overcome this issue. Hence, in this chapter, we provided a; (i) brief introduction on an area where samples of known chemical composition and consistent grain size have been collected; (ii) a detailed review on PM in Saudi Arabia in general, and on a city, Makkah, that still lack a proper investigation of PM components and their relationship with toxic effects; and (iii) and a brief review on CRMs that can be obtained at reasonable prices.

The use of different particle types might assist in reaching an understanding of the most likely attribute or group of attributes responsible for causing toxic effects. We chose Crushed Rock Powders (CRPs) from Panasequeira due to their availability in large amounts and suitability in terms of its size and composition. In the case of PM from Makkah, even though $PM_{2.5}$ seemed to be much important, our choice of PM_{10} was only based on sampler availability as a $PM_{2.5}$ sampler was not available and purchasing such equipment and shipping it to Saudi Arabia would have been expensive and time consuming. As for the CRM, it was chosen due to its size, which was similar to particles collected from Makkah (PM_{10}), and it was the least expensive amongst a variety of CRMs available.

Future chapters will show that we analysed the CRPs by means of XRF, XRD, and particle number and size distributions. These techniques were chosen for their analytical capabilities in investigating all aspects that might affect the CRPs toxic effects. They provided detailed information on the following: (i) elements and major oxides present in the samples (XRF); (ii) minerology and crystalline phases (XRD); and (iii) the size and range of particles in the CRPs. For Makkah PM_{10} a complete physical, chemical, and mineralogical analysis was not feasible due to the low amount of material obtained. Nevertheless, since PM_{10} concentrations and compositions are mainly affected by weather conditions, the relationship between toxic effects of PM_{10} and meteorology was investigated in order to identify the most likely particle type and source associated with the effects found. As for the CRM, it was already analysed and provided with a certificate of analysis.

Future chapters will also show that all particle types were investigated for their toxic effects using three *in vitro* techniques; a cell-free plasmid scission assay and cell-based

Neutral Comet and MTT assays. The plasmid scission assay was chosen for its ability to investigate DNA damage in a cell-free environment that does not involve complex interactions associated with working on whole cells. The Neutral Comet assay was also chosen for its ability to investigate DNA damage however in a cell-based environment that involves interactions between particles and various components of living cells. The MTT assay was chosen for its ability to investigate loss of viable cells caused by particles.

In terms of achieving the aim of this project, results obtained from the toxicological assays in conjunction with physical, chemical and mineralogical characteristics for CRPs and individual meteorological parameters for PM_{10} from Makkah will be compared and their relationship investigated to identify the most likely cause of toxic effects of particles.

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Chapter 3: Geochemical compositional controls on DNA strand breaks induced in in vitro cell-free assays by crushed rock powders from the Panasqueira mine area, Portugal

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Abstract

DNA strand breaks are a common form of DNA damage that can contribute to chromosomal instability or gene mutations. Such strand breaks may be caused by exposure to heavy metals. The aim of this study was to assess the level of DNA strand breaks caused by µm-scale solid particles of known chemical composition with elevated heavy metals/metalloids, notably arsenic, using an *in-vitro* cell-free DNA Plasmid Scission Assay. These samples were incubated with and without H_2O_2 to see if damage occurs directly or indirectly through the Fenton reaction. Levels of DNA damage in the absence of H_2O_2 were < 10 % but in the presence of H_2O_2 all samples showed higher levels of damage ranging from 10 – 100 % suggesting that damage was being incurred through the Fenton reaction. Using bivariate correlation analysis and Multiple Linear Regression, Manganese Oxide (MnO), Sulphur (S), Copper (Cu), and Zinc (Zn) concentrations in the particulates were found to be the most significant predictors of DNA damage. The mechanism of this DNA damage formation has yet to be thoroughly investigated but is hypothesised to be due to reactive oxygen species formation. Further work is required to assess the extent of contribution of reactive oxygen species to this DNA damage but this study highlights the potential role of chemistry and/or mineralogy to the extent and/or nature of DNA damage caused by particulates.

3.1 Introduction

Heavy metals and metalloids are natural elements characterized by their high densities, atomic weights, or atomic numbers (Koller and Saleh 2018). Our natural environment contains a large number of heavy metals and metalloids, such as arsenic, cadmium, chromium, and nickel amongst others, that become sources of exposure to humans as a result of natural or anthropogenic processes (Alloway 2013; Tchounwou et al. 2012; Bhavani and Sujatha 2014).

It is well established that exposure to many heavy metals and metalloids causes adverse health effects in humans. Many heavy metals and metalloids are classified as human carcinogens by the International Agency for Research on Cancer (IARC 2018). A variety of signalling and cellular regulatory proteins that are involved in important processes such apoptosis, cell cycle regulation, DNA repair, DNA methylation, cell growth and differentiation are affected by exposure to heavy metals and metalloids (Kim et al. 2015; Engwa et al. 2019). Any disruptions to these processes can lead to cancer (Engwa et al. 2019). The main mechanism of inducing these disruptions is oxidative stress. Certain heavy metals and metalloids, such as arsenic, iron, copper, chromium, cobalt, and vanadium amongst others, are known for their ability to produce Reactive Oxygen Species (ROS) such as superoxide ion, hydrogen peroxide, and hydroxyl radical by utilizing the Fenton chemistry/Haber-Weiss reaction (Jaishankar et al. 2014; Manoj and Padhy 2013; Szivák et al. 2009). Their production results in oxidative stress, a state where cells have elevated levels of ROS, which causes damage to proteins (e.g. protein fragmentation), lipids (e.g. lipid peroxidation), and DNA (e.g. DNA strand breaks) (Schieber and Chandel 2014; Barrera 2012; Rehman et al. 2018; Engwa et al. 2019).

Given the known effects of exposure to heavy metals and metalloids, this study will focus on the effect of samples with known mineralogical, chemical, and physical characteristics on DNA strand breaks. Samples previously collected from inside and around the Panasqueira mine area in Portugal were selected for this study because they exhibited a wide range of heavy metals/metalloids and major oxide composition and also for being available in large amounts (250 g). The overall objectives of this study were to (i) determine the level of DNA damage induced in the presence and absence of H_2O_2 using the Plasmid Scission Assay and (ii) identify the main determinants of DNA damage formation using bivariate correlation analysis and multiple linear regression.

3.2 Materials and methods

Rock samples were collected from in and around the Panasqueira mine, Portugal (Polya 1987a; Ávila et al. 2008; Orey 1967; Kelly and Rye 1979; Bussink 1984; Foxford et al. 2000; Polya et al. 2000; Polya 1987b, 1989, 1988). It's a tin-tungsten mine located in central Portugal (latitude, 40° 10' 10" North / longitude, 7° 45' 33" West). Extensive studies including those by (Polya 1987b, 1989, 1988) show that the deposit is surrounded by an extensive hydrothermal alteration halo. The 200 + rock samples collected exhibit a wide range of chemical compositions, including with respect to a number of elements (e.g. arsenic, uranium, and silicon) suspected to be associated with increased toxicity. Accordingly, these samples represent an ideal set of samples to explore the associations between particulate chemical composition and toxic effects. After crushing, the resultant crushed rock powders (CRP) were analysed by means of X-ray diffraction (XRD), X-ray fluorescence (XRF), and particle size analyser to investigate their mineralogical, chemical, and particle size characteristics

respectively. The ability of CRPs to cause DNA damage was investigated using an *in-vitro* cell-free Plasmid Scission Assay. A more detailed description of each method is provided below.

3.2.1 Sample collection

Whole rock samples ranging from 0.5 – 1 kg in weight were collected from the Panasqueira mine area in Portugal in February / March 1984. Every sample was broken up with a carbide splitter and reduced to millimetre sized particles in a jaw crusher. A portion of the crushed material was then placed in a Cr-V stainless steel Tema Mill (TEMA Machinery Ltd, Woodford Halse, Northants, UK) and further crushed to < 50 μm powders. Pressed powder pellets were then prepared by standard techniques as outlined in (Polya 1987, 1988). Throughout the whole process every piece of equipment was thoroughly cleaned after each sample treatment. Around 250 Crushed Rock Powder (CRP) samples were eventually obtained and subsequently stored in sealed individual zip-bags at room temperature. For the purpose of this study, a sub-set of 24 samples, each weighing approximately 250 g, were selected on the basis of their chemical compositional variability, particular with respect to arsenic, to test their association with toxic effects.

3.2.2 XRD analysis

Sample preparation involved grinding ~ 0.1 g of crushed rock powder, mixing with ~ 1 ml of amyl acetate, using an agate pestle and mortar, transferring the resultant slurries to a glass microscope slide and air drying. Measurements were carried out on a Bruker D8 Advance diffractometer, equipped with a Göbel Mirror and a Lynxeye detector. The X-ray tube had a copper source, providing CuKα1 X-rays with a

wavelength of 1.5406 Å. Samples were scanned from 5 - 70^{o} -2θ, with a step size of 0.02^{o} - 2θ and a count time of 0.2 s per step. The resultant XRD patterns were evaluated using EVA version 4, which compares experimental data to standards from the ICDD (International Centre for Diffraction Data) Database.

3.2.3 XRF analysis

XRF analysis was conducted on the 24 samples at the University of Nottingham using a fully automated Phillips PW1400 XRF wavelength dispersive spectrometer. Summaries of the analytical method have been reported elsewhere (Polya 1987, 1988, 1989).

3.2.4 Particle size distribution

Particle size analysis was conducted at the British Geological Survey (BGS) in Keyworth by Thomas Walker (Walker, unpublished work). Each sample was weighed out into 2 vials of 0.25 g and suspended into 10 ml solution of Calgon (25 % sodium hexametaphosphate). The samples were then shaken and mixed for 30 seconds using a vortex mixer at 2500 rpm before being analysed using a Beckman Coulter LS 13 320 Particle Sizing Analyser. Each sample was analysed twice. Each resultant particle size distribution was characterised by three parameters, D90 (the 90th percentile particle size), D50 (the median particle size), and D10 (the 10th percentile particle size). Values were calculated from the distribution and statistical analysis was conducted using Gradistat© software on Microsoft Excel (Walker, unpublished).

3.2.5 Plasmid Scission Assay

The ability of CRPs to cause DNA strand breaks was investigated using the plasmid scission assay as described previously (Dumax-Vorzet et al. 2015; Dumax Vorzet 2010) with minor modifications. When plasmid DNA runs through an agarose gel, three bands are observed. Supercoiled plasmid DNA is the native form (covalently closed circular DNA) where there are no strand breaks. When one DNA strand is cut, the resulting nicked or relaxed plasmid DNA will have a floppy open circle structure. When both strands of the plasmid are cut, the result is linear plasmid DNA. These three forms have different migration speeds where supercoiled plasmid DNA is the fastest as it doesn't have any strand breaks and its compactness sustains less friction against the agarose gel. Linear plasmid DNA runs through the gel slower than supercoiled plasmid DNA but faster than nicked or relaxed plasmid DNA. Nicked or relaxed plasmid DNA is the slowest due to its large floppy circular nature. pchAT plasmid DNA purified from *E.coli* in lab using Miniprep (Qiagen, Netherlands) was kindly provided by Professor Geoff Margison. In brief, pchAT Plasmid DNA (5 ng) was diluted to 20 μl in an Elution Buffer (10 mM Tris-HCl pH 8.5) with different levels of CRPs and H_2O_2. Samples were incubated for 1 - 5 hours at 37 ^oC. The reaction was stopped by adding loading buffer (Promega blue/orange 6X loading dye) and the whole reaction mixture loaded onto 0.6 % TBE-agarose gel. Electrophoresis was conducted at 90 - 100 V for 45 min – 2 hours in 1x TBE buffer. The different forms of plasmid were visualised on a Typhoon 9200 variable mode imager. The intensity of the different forms of plasmid in each lane was analysed using ImageQuantTM (GE Healthcare Life Sciences) and the level of damaged plasmid in each sample was calculated as shown in Equation (1).

$$DP\ (\%) = \frac{R+L}{R+L+S}\ x\ 100 \quad (1)$$

Where;

DP is the percentage of DNA damage

R is the relaxed form of plasmid DNA

L is the linear form of plasmid DNA

S is the supercoiled form of plasmid DNA

In each experiment, positive and negative controls were added. The positive control was H_2O_2 (3.5 mM), pchAT plasmid DNA (5 ng), and $FeSO_4$ (25 μM) in elution buffer. The negative control was; H_2O_2 (3.5 mM) and pchAT plasmid DNA (5 ng) diluted in elution buffer.

3.2.6 Statistical analysis

Data obtained from each Plasmid Scission Assay were described using the mean, standard deviation, minimum, and maximum values. A one-way analysis of variance was used to compare each group individually to determine if the levels of DNA strand breaks varied significantly between samples. A bivariate correlation analysis was then conducted to examine possible associations between DNA strand breaks and the physiochemical composition of the samples. All the significant variables from this analysis were then plotted against the percentage of DNA strand breaks to examine the correlations and entered into a backward stepwise multiple linear regression model. The least significant variable was eliminated step by step and all the models were compared using Bayesian information criterion (BIC) to choose the best

explanatory model. Statistical analyses were performed using SPSS Statistics version 22. Graphs and scatterplots were created using Microsoft Excel 2010.

3.3 Results

3.3.1 XRD analysis

The crystalline minerals identified by XRD in the CRPs were mostly silicates with minor sulphides. These included quartz, muscovite 2M1, dravite tourmaline, and albite amongst others *(Table 3.1)*. The most abundant crystalline minerals found were quartz (Modal abundance = 40 %, *SD* = 17 %), muscovite 2M1 (*M* = 32 %, *SD* = 19 %) and dravite tourmaline (*M* = 11 %, *SD* = 16 %) with lesser amounts of albite (*M* = 9 %, *SD* = 12 %), phlogopite 1M mica (*M* = 4 %, *SD* = 8 %), clinochlore II2b (*M* = 3 % , *SD* = 5 %), and traces of microcline intermediate 1, magnetite , and pyrite. *(Table 3.2)*.

Table 3.1, Mineralogical composition of crushed rock powders from Panasqueira; as determined by XRD.

Sample	Mineral Phase																	
	Albite		Clinochlore IIb-2		Dravite Tourmaline		Magnetite		Microcline intermediate1		Muscovite 2M1		Phlogopite 1M Mica		Pyrite		Quartz	
	Weight %	Error %	Weight %	Error %	Weight %	Error %	Weight %	Error %	Weight %	Error %	Weight %	Error %	Weight %	Error %	Weight %	Error %	Weight %	Error %
A	1.0	0.2	0.8	0.3	37.7	0.8	ND	ND	2.4	0.5	19.4	0.7	ND	ND	ND	ND	38.9	0.8
B	16.4	0.8	ND	ND	11.8	0.7	ND	ND	ND	ND	17.3	1.0	ND	ND	ND	ND	54.5	1.3
C	15.8	0.7	2.6	0.6	ND	ND	ND	ND	3.4	0.8	18.9	0.9	ND	ND	ND	ND	59.3	1.2
D	0.6	0.2	1.5	0.4	49.8	0.9	ND	ND	2.4	0.6	10.1	0.6	ND	ND	ND	ND	35.6	0.8
E	ND	ND	ND	ND	47.0	0.9	ND	ND	ND	ND	20.4	0.7	ND	ND	ND	ND	ND	ND
F	26.3	1.6	17.6	1.2	1.4	0.8	ND	ND	ND	ND	54.8	1.9	ND	ND	ND	ND	32.7	0.8
G	ND	ND	3.1	1.0	ND	ND	ND	ND	ND	ND	85.1	1.2	ND	ND	ND	ND	11.8	0.8
H	8.0	0.5	5.9	0.4	ND	ND	ND	ND	2.6	0.6	17.7	0.8	ND	ND	ND	ND	65.7	1.0
I	11.4	0.4	3.3	0.3	ND	ND	ND	ND	2.6	0.4	12.5	0.5	ND	ND	ND	ND	70.1	0.7
J	24.6	0.9	8.7	0.5	ND	ND	ND	ND	ND	ND	34.9	1.1	ND	ND	ND	ND	31.8	1.0
K	ND	ND	ND	ND	23.4	0.7	ND	ND	2.6	0.5	22.2	0.8	ND	ND	ND	ND	51.7	0.9
L	ND	ND	1.1	0.9	7.3	1.0	ND	ND	ND	ND	48.0	1.6	ND	ND	ND	ND	43.6	1.6
M	ND	ND	2.2	0.6	ND	ND	ND	ND	ND	ND	53.5	1.5	ND	ND	ND	ND	44.3	1.5
N	0.4	0.9	0.6	0.8	7.0	1.0	ND	ND	ND	ND	37.4	1.6	12.2	0.8	ND	ND	42.5	1.6
O	15.9	0.9	7.8	0.7	ND	ND	ND	ND	ND	ND	41.7	1.3	ND	ND	ND	ND	34.7	1.2
P	8.9	1.0	ND	ND	5.4	0.9	ND	ND	ND	ND	32.1	1.5	20.6	1.1	ND	ND	33.1	1.4
Q	44.8	2.2	1.6	0.6	ND	ND	0.9	0.3	ND	ND	6.0	1.0	26.4	1.7	ND	ND	20.4	1.4
R	ND	ND	ND	ND	16.0	0.8	ND	ND	ND	ND	46.8	1.2	5.1	0.5	ND	ND	32.2	1.1
S	2.4	0.5	6.3	0.6	ND	ND	ND	ND	ND	ND	29.7	1.1	ND	ND	ND	ND	61.6	1.2
T	10.7	0.5	ND	ND	6.9	0.5	ND	ND	ND	ND	16.5	0.7	5.5	0.3	ND	ND	60.4	0.9
U	ND	ND	ND	ND	9.5	0.7	ND	ND	ND	ND	39.2	1.6	16.6	1.0	ND	ND	34.7	1.5
V	26.2	0.8	ND	ND	2.5	0.5	ND	ND	ND	ND	17.6	0.7	16.5	0.7	ND	ND	37.2	1.0
W	ND	ND	ND	ND	34.1	0.9	ND	ND	1.9	0.6	30.7	0.9	ND	ND	0.7	0.1	32.5	0.8
X	ND	ND	0.15	0.54	4.12	0.6	ND	ND	ND	ND	65.99	1.34	1.02	0.38	ND	ND	28.72	1.17

*ND; not detected

Table 3.2, Descriptive statistics for abundance (wt. %) of crystalline minerals identified by XRD in crushed rock powders from Panasqueira.

Crystalline mineral	Minimum	Maximum	Mean	Std. Deviation
Albite	ND	44.8	8.9	11.9
Clinochlore IIb2	ND	17.6	2.6	4.1
Dravite Tourmaline	ND	49.8	11.0	15.6
Magnetite	ND	0.9	0.0	0.2
Microcline intermediate1	ND	3.4	0.8	1.2
Muscovite 2M1	6.0	85.1	32.4	19.3
Phlogopite 1M Mica	ND	26.4	4.3	7.9
Pyrite	ND	0.7	0.0	0.2
Quartz	ND	70.1	39.9	16.8

*ND; not detected

3.3.2 XRF analysis

The chemical compositions of the CRPs are summarised in *Tables 3.3 to 3.6.* The compositions of these largely lower Greenschist facies meta-silstones and meta-sandstones are dominated by SiO_2 (M = 63 % , SD = 8 %), Al_2O_3 (M = 19 % , SD = 5 %) and Fe_2O_3 (M = 7 %, SD = 2 %). Notable traces included S (M = 1600 µg/g, SD = 3800 µg/g) Ba (M = 560 µg/g, SD = 250 µg/g) and As (M = 380 µg/g, SD = 650 µg/g).

Table 3.3, Chemical composition (major oxides) of crushed rock powders from Panasqueira; determined by XRF (Polya, 1987, 1988).

Sample	Oxide (Wt%)									
	Al_2O_3	**CaO**	**Fe_2O_3**	**K_2O**	**MgO**	**MnO**	**Na_2O**	**P_2O_5**	**SiO_2**	**TiO_2**
A	17.78	0.18	7.66	2.58	1.51	0.07	0.61	0.14	65.53	0.95
B	16.78	0.24	5.22	3.58	1.70	0.05	1.69	0.18	66.93	0.87
C	14.50	0.14	5.56	2.09	2.05	0.04	1.58	0.12	72.14	0.67
D	16.54	0.23	9.15	1.35	1.79	0.06	0.89	0.28	65.31	1.14
E	18.31	0.69	9.07	2.57	2.01	0.11	0.74	0.55	61.78	1.08
F	25.74	0.12	9.51	5.22	3.20	0.05	1.94	0.19	48.37	1.31
G	33.35	ND	5.38	7.39	1.34	0.02	0.81	0.06	46.13	1.89
H	13.88	ND	5.95	1.86	1.89	0.04	0.81	0.07	74.78	0.63
I	11.32	ND	3.19	1.26	1.29	ND	1.41	0.10	80.72	0.54
J	20.32	0.32	7.52	3.40	2.05	0.06	2.62	0.20	60.69	0.97
K	14.95	0.37	5.57	3.09	1.50	0.09	0.42	0.36	71.27	0.77
L	20.28	0.01	8.17	4.06	1.71	0.02	0.53	0.16	58.67	1.06
M	24.38	0.02	5.91	4.33	0.89	0.01	0.81	0.06	60.01	1.02
N	20.01	0.21	5.59	5.03	2.12	0.03	1.20	0.14	63.74	1.02
O	21.14	0.21	7.49	4.14	2.01	0.05	1.59	0.15	60.89	0.99
P	18.72	0.29	7.82	5.47	2.47	0.07	1.53	0.27	59.68	0.95
Q	17.25	3.73	7.62	3.19	4.37	0.11	3.16	0.59	55.25	1.18
R	20.41	0.22	8.27	5.96	2.52	0.17	0.42	0.17	57.36	1.08
S	19.26	ND	5.08	2.92	2.06	0.02	0.20	0.03	65.85	0.83
T	14.44	0.25	4.19	3.01	1.61	0.04	1.54	0.20	73.19	0.64
U	20.48	0.18	7.04	5.67	2.17	0.06	0.98	0.15	62.27	0.94
V	16.04	0.26	6.22	3.47	2.21	0.04	3.01	0.14	66.64	0.90
W	19.20	1.14	7.05	3.35	2.06	0.05	0.48	0.93	63.28	0.95
X	21.36	0.55	6.49	7.00	2.20	0.18	0.14	0.44	57.35	0.86

*ND; not detected

Table 3.4, Chemical composition (trace elements) of crushed rock powders from Panasqueira; as determined by XRF (Polya, 1987, 1988),

Sample	Element (μg/g)																							
	As	**Ba**	**Bi**	**Ce**	**Co**	**Cr**	**Cu**	**La**	**Mo**	**Nb**	**Ni**	**Pb**	**Rb**	**S**	**Sb**	**Sn**	**Sr**	**Th**	**U**	**V**	**W**	**Y**	**Zn**	**Zr**
A	3164	274	5	150	13	120	94	32	1	13	32	10	308	3733	3	122	58	8	3	116	64	31	338	190
B	113	564	2	48	11	99	8	30	ND	12	31	4	242	35	ND	35	51	10	2	96	14	33	78	258
C	10	402	ND	45	14	83	9	20	ND	11	23	9	62	49	ND	ND	44	7	3	76	4	23	63	177
D	730	419	12	99	7	130	17	42	ND	9	29	6	162	9	ND	73	80	12	3	134	16	31	360	193
E	883	365	14	100	15	135	197	35	1	14	44	7	237	4570	ND	66	90	13	4	141	17	40	368	189
F	99	928	ND	92	10	151	27	58	1	18	35	12	179	64	ND	ND	74	13	3	171	4	41	144	264
G	235	1330	3	125	10	218	40	62	2	26	21	130	236	40	1	38	64	20	5	222	19	64	140	496
H	94	354	ND	32	8	73	23	24	ND	8	18	37	56	61	ND	ND	36	7	ND	67	9	25	55	175
I	3	214	ND	37	10	66	4	17	ND	8	26	ND	43	25	ND	ND	33	3	3	59	5	30	33	178
J	330	633	ND	85	18	129	25	34	ND	14	45	3	217	1140	ND	14	72	11	2	141	7	37	158	193
K	550	257	1	63	11	89	104	25	1	10	32	55	426	2732	ND	87	57	7	2	78	16	27	460	221
L	417	608	1	69	13	127	51	38	1	14	29	23	250	28	ND	37	61	11	4	129	24	28	147	199
M	10	848	ND	68	7	136	44	44	1	16	8	33	175	201	ND	4	51	11	3	156	7	40	35	228
N	60	646	ND	68	16	116	46	43	2	14	42	18	546	956	4	46	54	5	3	122	45	42	209	248
O	44	688	4	71	12	130	29	38	1	15	52	4	273	487	ND	21	53	11	3	141	38	37	126	189
P	206	701	ND	61	8	121	6	37	1	13	39	5	589	32	ND	47	50	4	3	135	20	46	125	200
Q	17	596	ND	68	17	37	20	39	1	9	8	13	236	1362	ND	51	391	6	2	109	3	28	182	227
R	553	561	3	85	8	130	8	35	2	15	38	ND	763	249	ND	69	40	8	4	141	40	45	288	201
S	14	606	ND	64	10	88	18	37	ND	12	11	13	82	67	ND	0	31	9	2	92	5	27	164	196
T	137	442	ND	42	6	84	8	21	ND	9	19	4	214	83	ND	28	40	5	3	70	15	23	50	228
U	199	689	ND	65	15	124	26	36	1	13	53	3	785	515	ND	60	51	9	4	134	24	43	157	195
V	159	362	3	69	13	109	9	34	ND	13	42	35	396	459	ND	25	47	7	3	102	43	36	414	187
W	656	470	11	110	19	137	362	34	3	13	46	98	370	17822	5	257	135	9	4	145	82	52	248	184
X	462	550	3	64	10	116	86	43	2	22	46	16	1241	3764	6	162	333	9	4	130	66	46	166	174

*ND; not detected

Table 3.5, Descriptive statistics for chemical composition (major oxides) of Panasqueira crushed rock powders (n=24) determined by XRF.

Oxide	Minimum / Maximum (Wt%)	Mean / Std. Deviation (Wt%)
Al_2O_3	11.3 / 33.4	19.0 / 4.5
CaO	0.0 / 3.7	0.4 / 0.8
Fe_2O_3	3.2 / 9.5	6.7 / 1.6
K_2O	1.3 / 7.4	3.8 / 1.7
MgO	0.9 / 4.4	2.0 / 0.7
MnO	0.0 / 0.2	0.1 / 0.0
Na_2O	0.1 / 3.2	1.2 / 0.8
P_2O_5	0.0 / 0.9	0.2 / 0.2
SiO_2	46.1 / 80.7	63.2 / 7.9
TiO_2	0.5 / 1.9	1.0 / 0.3

Table 3.6, Descriptive statistics for chemical composition (trace elements) of Panasqueira crushed rock powders (n=24) determined by XRF. Zero used as default to indicate below detection limit.

Element	Minimum / Maximum (µg/g)	Mean / Std. Deviation (µg/g)
As	3 / 3164	381 / 646
Ba	214 / 1330	563 / 244
Bi	0 / 14	3 / 5
Ce	32 / 150	74 / 28
Co	6 / 19	12 / 4
Cr	37 / 218	115 / 36
Cu	4 / 362	53 / 80
La	17 / 62	36 / 11
Mo	0 / 3	1 / 1
Nb	8 / 26	13 / 5
Ni	8 / 53	32 / 14
Pb	0 / 130	22 / 32
Rb	43 / 1241	337 / 279
S	9 / 17822	1604 / 3714
Sb	0 / 6	1 / 2
Sn	0 / 257	52 / 60
Sr	31 / 391	83 / 89
Th	3 / 20	9 / 4
U	0 / 5	3 / 1
V	59 / 222	121 / 38
W	3 / 82	25 / 22
Y	23 / 64	37 / 10
Zn	33 / 460	188 / 124
Zr	174 / 496	216 / 65

3.3.3 Particle size distribution

The particles size distribution of CRPs is summarised in *Table 3.7.* Fine size fractions that compromised 10 % of the samples' mass ranged from 1.97 - 4.48 µm (D10 - *M* = 2.93 µm, *SD = 0.58*). Median size fractions that compromised 50 % of the samples' mass ranged from 7.59 - 27.66 µm (D50 - *M* = 13.42 µm, *SD = 5.15*). Coarse size fractions that compromised 90 % of the samples' mass ranged from 43.75 – 259.10 (D90 - *M* = 113.46 µm, *SD = 48.55*).

Table 3.7, Particle size distribution of crushed rock powders from Panasqueira; as determined by Beckman Coulter LS 13 320 Particle Sizing Analyser - values represent the mean of two experiments, data kindly supplied by Thomas Walker.

Sample	Particle size parameters		
	D10 (µm)	D50 (µm)	D90 (µm)
A	3.5	27.7	259.1
B	3.6	15.0	98.61
C	3.4	13.2	136.6
D	2.8	17.5	119
E	2.9	14.1	104.1
F	3.3	12.4	115.9
G	3.0	10.2	101.9
H	2.7	11.9	109.4
I	2.2	9.3	91.7
J	2.2	7.6	43.75
K	2.9	15.3	67.6
L	3.1	9.9	51.3
M	2.7	8.6	84.93
N	2.8	11.1	120.7
O	2.6	9.4	103.9
P	4.5	20.6	218.4
Q	2.2	21.9	171.4
R	3.5	15.7	86.55
S	2.6	9.6	121
T	3.8	21.5	134.2
U	2.7	9.7	104.5
V	2.0	8.5	50.69
W	2.4	9.6	106.6
X	3.0	12.0	121.1

3.3.4 Plasmid Scission Assay

Hydrogen peroxide (H_2O_2) alone did not induce substantial strand breaks but in combination with increasing $FeSO_4$ concentration, there was a dose-dependent increase in the proportion of damaged plasmid, (*Figure 3.1*). Negative controls (i.e. without particulates or $FeSO_4$) gave rise to DNA damage of less than 10 %, with this damage being independent of H_2O_2 concentration over the range 0 to 200 µM H_2O_2. These small levels of DNA damage are similar to those observed in earlier studies

using this technique (Dumaz-Vorzat et al, 2015) and we speculate reflect small amounts of damage caused by the DNA extraction procedure or during the incubation period.

The addition of increasing amounts of CRPs to a reaction mix containing, pchAT plasmid DNA, H_2O_2 and Elution Buffer resulted in a dose-dependent increase in the proportion of damaged plasmid (*Figure 3.2*).

For samples incubated at the highest concentration of CRP (1250 μg/ml), the percentage of DNA strand breaks found in the absence of H_2O_2 was between 0 - 10 %. However, when the samples were incubated with H_2O_2, the percentage of DNA strand breaks increased to between 10 – 100 depending upon the sample (*Figure 3.3*). When comparing the percentage of DNA damage in the two groups (with and without H_2O_2), a significant difference was found for all samples using a paired samples t-test (*Table 3.8*). Also, when comparing the percentage of DNA damage within each group individually, a significant difference was found using a one-way analysis of variance (with H_2O_2 $p < 0.001$ / without H_2O_2 $p < 0.001$).

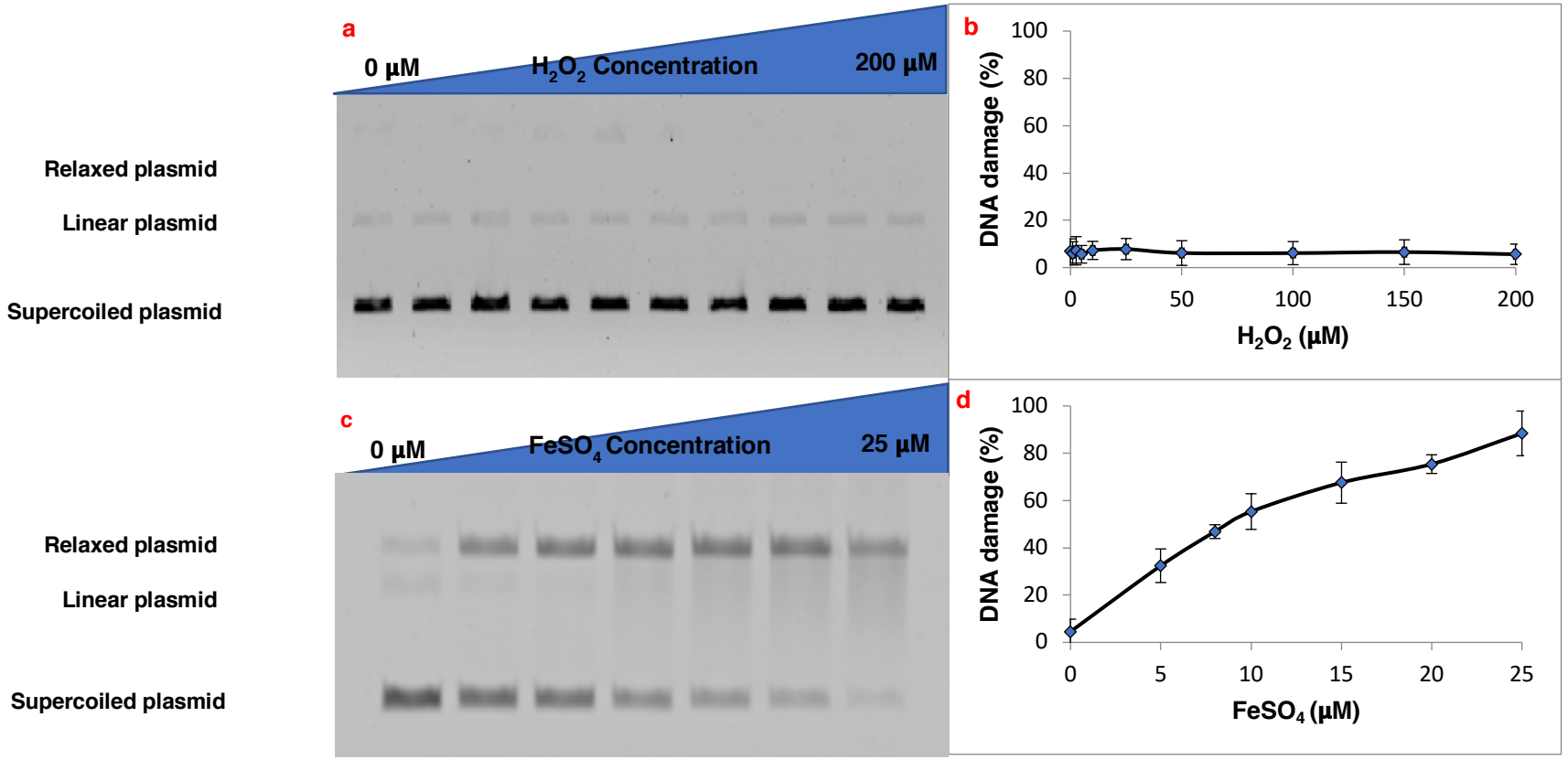

Figure 3.1, Plasmid Scission assay, Dose dependent DNA strand breaks arising from (a, b) H_2O_2 and (c, d) $FeSO_4$

(a, electrophoresis results) / (b, % DNA damage induced by increasing amounts of H_2O_2) – pchAT plasmid DNA was incubated at 37 °C for 1 hour with 0 - 200 µM H_2O_2 in Elution Buffer (10 mM Tris-HCl pH 8.5) (20 µl). (c, electrophoresis results) / (d, % DNA damage induced by increasing amounts of $FeSO_4$ in the presence of H_2O_2) – pchAT plasmid DNA was incubated at 37 °C for 1 hour with 3.5mM H_2O_2 and increasing concentration of $FeSO_4$ (0 - 25 µM). Both reactions were stopped by the addition of loading buffer (Promega blue/orange 6X loading dye). The samples were separated on 0.6% TBE-agarose gel at 90 V for 2 h. The different forms of plasmid were visualised on a Typhoon 9200 variable mode imager. The intensity of the different forms of plasmid (relaxed, linear, and supercoiled) in each lane was analysed using ImageQuantTM and the level of damaged plasmid in each sample was calculated. Error bars represent the standard deviation (SD) of three independent experiments.

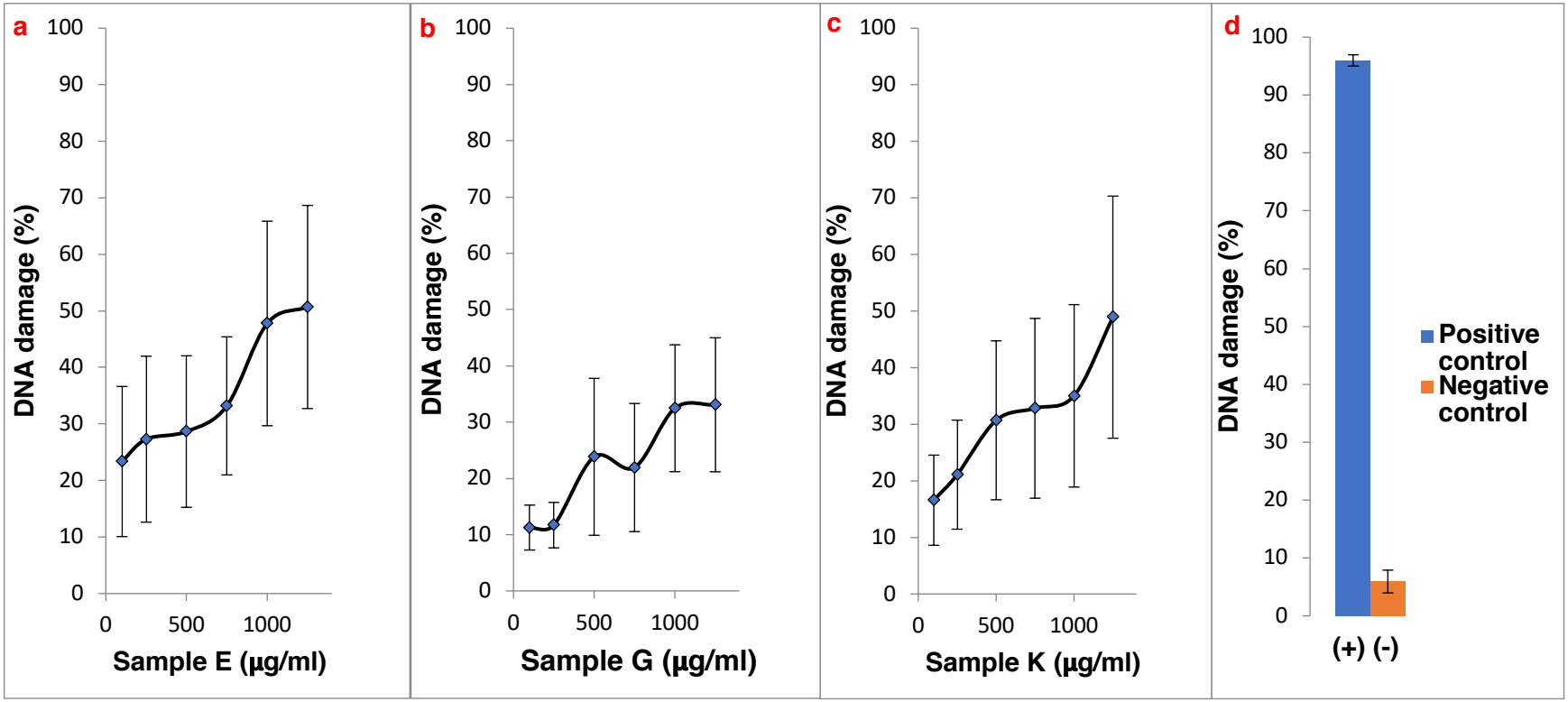

Figure 3.2, Plasmid Scission assay of Panasequeira CRPs, Dose dependent DNA strand breaks arising from CRPs (100 – 1250 µg/ml)

(a, b, c - % DNA damage induced by increasing amounts of CRPs in the presence of H_2O_2 for three representative samples E, G & K) Samples were suspended in distilled water (5 mg/ml) by sonication for a total of 3 mins. Sonication was performed at 80% amplitude. The samples were used directly after being suspended without centrifugation. Plasmid DNA (5 ng) was incubated at 37 °C for 5 hours with 3.5 mM H_2O_2 and increasing concentrations (100 µg/ml - 1250 µg/ml) of the CRPs. (d - % DNA damage induced by negative and positive controls) positive ($FeSO_4$ 25 µM, Plasmid DNA (5 ng), H_2O_2 3.5 mM, and Elution Buffer (10 mM Tris-HCl pH 8.5)) and negative (Plasmid DNA (5 ng), H_2O_2 3.5 mM, and Elution Buffer (10 mM Tris-HCl pH 8.5)) controls were also added. The reaction was stopped by the addition of loading buffer (Promega blue/orange 6X loading dye). The samples were separated on 0.6% TBE-agarose gel at 100 V for 45 mins. Three independents were carried out and the mean for each sample was calculated. Error bars represent the standard deviation (SD) of three independent experiments.

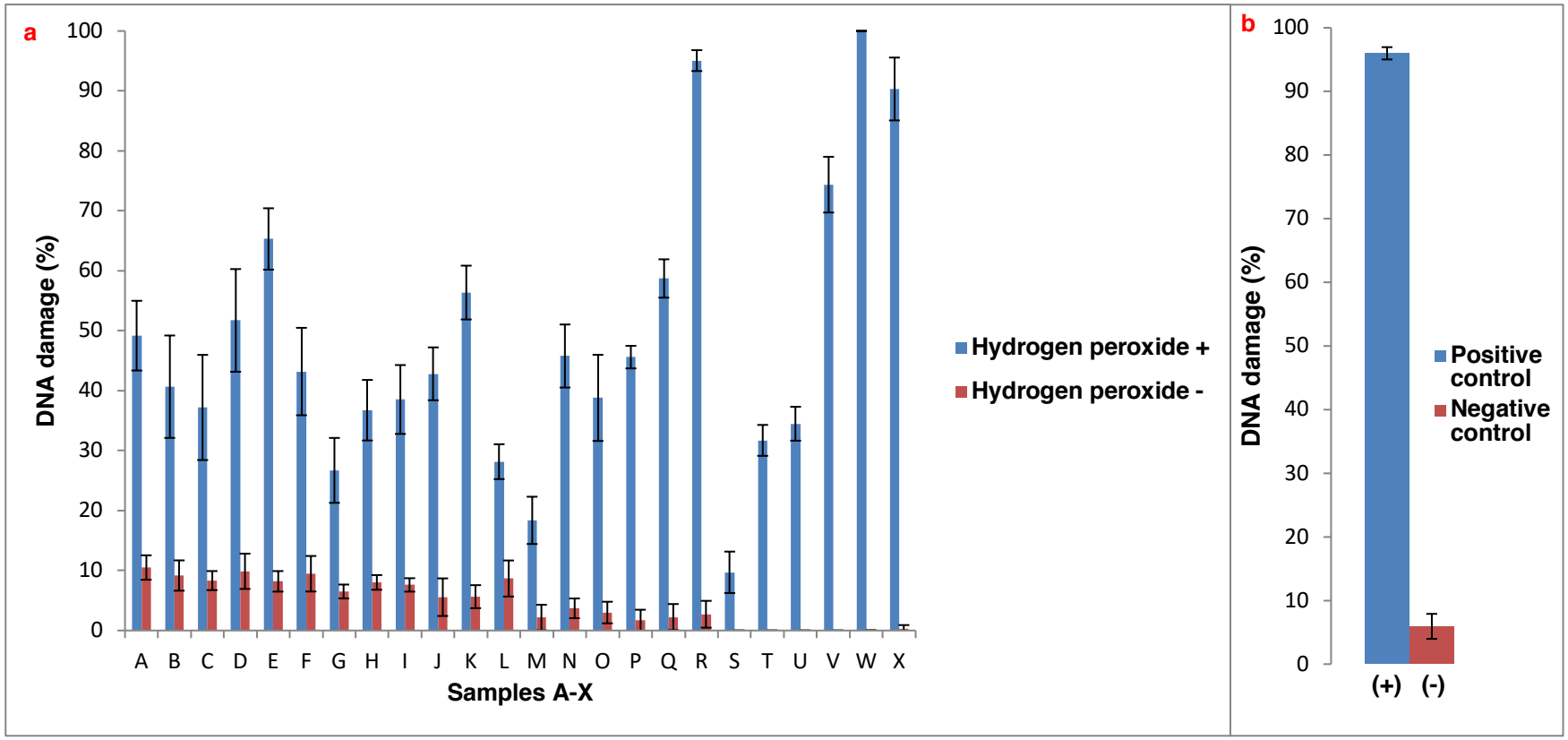

Figure 3.3, Plasmid Scission assay of Panasequeira CRPs, DNA strand breaks caused by CRPs (A-X) at 1250 µg/ml

(a - % DNA damage induced by 1250 µg/ml of all CRPs in the presence of H_2O_2) Samples were suspended in distilled water (5 mg/ml) by sonication for a total of 3 mins. Sonication was performed at 80 % amplitude. The samples were used directly after being suspended without centrifugation. pchAT Plasmid DNA was incubated at 37 °C for 5 hours with and without H_2O_2 at the highest concentration of the sample. (b - % DNA damage induced by negative and positive controls) positive ($FeSO_4$ 25 µM, Plasmid DNA (5 ng), H_2O_2 3.5mM, and Elution Buffer (10 mM Tris-HCl pH 8.5)) and negative (Plasmid DNA (5 ng), H_2O_2 3.5mM, and Elution Buffer (10 mM Tris-HCl pH 8.5)) controls were also added. The reaction was stopped by the addition of loading buffer. The samples were separated on 0.6 % TBE-agarose gel at 100 V for 45 mins. Six independent experiments were carried out and the mean for each sample was calculated. Error bars represent the standard deviation (SD) of six independent experiments.

Table 3.8, A comparison of the level of DNA strand breaks (Mean / Standard deviation of six independent experiments) between powdered rock samples from Panasqueira (n=24) incubated with and without H_2O_2 using a paired samples t-test.

Sample	With H_2O_2 Mean / Standard deviation	Without H_2O_2 Mean / Standard deviation	Ratio for with/without H_2O_2
A	49.18 / 5.82	10.51 / 2.04	4.7 *
B	40.67 / 8.54	9.17 / 2.53	4.4 *
C	37.20 / 8.77	8.33 / 1.59	4.5 *
D	51.73 / 8.56	9.88 / 2.94	5.2 *
E	65.31 / 5.12	8.20 / 1.71	8.0 *
F	43.19 / 7.29	9.47 / 2.96	4.6 *
G	26.71 / 5.41	6.51 / 1.16	4.1 *
H	36.74 / 5.05	8.02 / 1.22	4.6 *
I	38.54 / 5.74	7.61 / 1.12	5.1 *
J	42.82 / 4.41	5.56 / 3.13	7.7 *
K	56.37 / 4.49	5.65 / 1.92	10.0 *
L	28.16 / 2.91	8.67 / 3.01	3.2 *
M	18.37 / 3.94	2.16 / 2.13	8.5 *
N	45.79 / 5.26	3.70 / 1.65	12.4 *
O	38.80 / 7.18	3.00 / 1.80	12.9 *
P	45.60 / 1.88	1.70 / 1.77	26.8 *
Q	58.72 / 3.20	2.24 / 2.17	26.2 *
R	95.06 / 1.74	2.71 /2.24	35.1 *
S	9.71 / 3.45	0.00 / 0.00	*(-)
T	31.71 / 2.58	0.00 / 0.00	*(-)
U	34.49 / 2.83	0.00 / 0.00	*(-)
V	74.37 / 4.65	0.00 / 0.00	*(-)
W	100.00 / 0.00	0.02 / 0.04	5567 *
X	90.32 / 5.24	0.28 / 0.63	323 *

*There was a significant difference for all samples when incubated with and without H_2O_2. All *p* values for the t-test were < 0.001.
*(-); values could not be calculated

The percentage of plasmid DNA damage in the presence of H_2O_2 was correlated with MnO, P_2O_5, Rb, S, Cu, Zn, Mo, Sn, and W as well as Sn, Ni, Bi, and dravite tourmaline but to a lesser extent (*Figure 3.4*). None of the particle size distribution parameters were correlated with the percentage of plasmid DNA damage. In a multiple linear regression model, MnO ($\beta = 0.541$, $p < 0.001$), S ($\beta = 1.170$, $p < 0.002$), Cu (β = -

0.761, $p < 0.031$), and Zn ($\beta = 0.253$, $p < 0.035$) were significant predictors of the percentage of DNA damage. These elements combined explained 83% of the variance ($R2 = 0.834$, $p < 0.001$). In the absence of H_2O_2, no correlation was found between the percentage of DNA damage and the physiochemical composition of the samples.

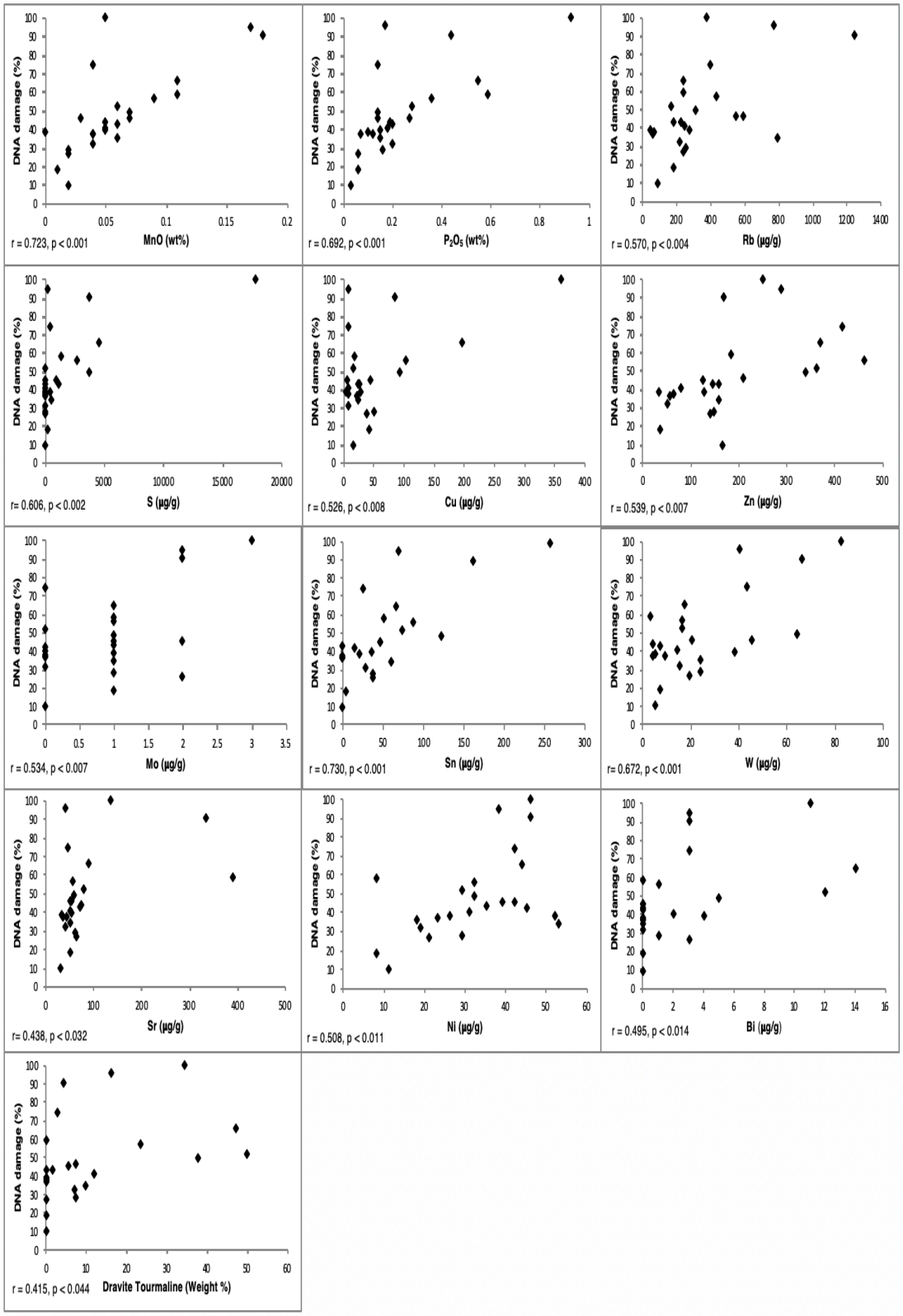

Figure 3.4, Correlation plots between DNA damage observed at 1250 µg/ml of CRPs in a plasmid scission assay and content of significantly correlated components (r and p values shown were obtained from Bivariate Correlation Analysis)

3.4 Discussion

All samples collected from inside and around the Panasqueira mine area were able to induce DNA damage when incubated as a CRP with plasmid DNA in the presence of H_2O_2. The percentage of plasmid DNA damage varied significantly with MnO, S, Cu, and Zn, these chemical components also being significant predictors of DNA damage in a multivariate model. To the best of knowledge, this is the first study to determine direct apparent effects MnO, S, Cu, and Zn in CRPs in a cell-free DNA scission assay, although it is noted that the large degree of covariance of these compositional parameters with other compositional parameters means that the DNA damage cannot be uniquely ascribed to each of these components and this represents a fundamental limitation of such toxicological studies involving real multicomponent geological materials.

It is hypothesised that the Plasmid DNA damage observed in the presence of H_2O_2 is caused by ROS generation from MnO, S, Cu, and Zn's participation in the Fenton chemistry/Haber-Weiss reaction (Jaishankar et al. 2014; Manoj and Padhy 2013; Szivák et al. 2009). Ions of the aforementioned components could have reacted with H_2O_2 and yielded hydroxyl radicals that are extremely reactive and toxic to biological molecules such as DNA (Thannickal and Fanburg 2000; Manke et al. 2013).

Previous studies have reported associations between these chemical components and DNA damage in cell-based studies (Alarifi et al. 2017; Frick et al. 2011; Hoffman et al. 2012; Linder 2012; Cervantes-Cervantes et al. 2005; Arciello et al. 2005; Zyba et al. 2016; Sharif et al. 2012; Ho and Ames 2002; Ho et al. 2003; Wysokinski et al. 2012). Manganese oxide nanoparticles have been associated with DNA strand breaks

in human neuronal cells (Alarifi et al. 2017) and type-II alveolar epithelial cells (Frick et al. 2011). Sulphur (Hoffman et al. 2012) and copper (Linder 2012; Cervantes-Cervantes et al. 2005; Arciello et al. 2005) have been associated with superoxide and hydroxyl radials which can results in oxidative stress and can lead to DNA damage. An increase in dietary zinc is actually known to reduce DNA damage (Zyba et al. 2016). Zinc deficiency on the other hand induces oxidative stress which leads to DNA damage (Sharif et al. 2012; Ho et al. 2003; Ho and Ames 2002). However, it has been reported before that zinc behaves differently in normal cells and cancer cells. Wysokinski et al. (2012) found that cancer cells exhibited higher levels of DNA damage in the presence of zinc while in normal lymphocytes such an effect was not found (Wysokinski et al. 2012). In this study, zinc levels were associated with an increase in DNA damage.

It has been previously reported that arsenic causes DNA strand breaks in mouse lungs (Yamanaka and Okada 1994), human fibroblasts (Mourón et al. 2006), and human HeLa S3 cells (Schwerdtle et al. 2003). However, in our study we found no significant linear association between arsenic concentrations and DNA strand breaks, notwithstanding that the CRPs contained up to 3000 μg/g As. The lack of association between arsenic and DNA damage could have been attributed to its insolubility in a wide range of pH conditions (Flora 2014). Moreover, certain contaminants need to be converted from their original form by enzymes first in the human body to show any adverse effects. That especially applies for arsenic as its metabolism is a critical determinant of its toxic effects (Navas-Acien and Guallar 2008; Hughes et al. 2011; Jomova et al. 2011). This could be why arsenic was not found in this study to be

associated with DNA damage as the assay was cell-free while in the other previously mentioned studies the assays were cell-based.

3.5 Conclusion

Crushed rock powders of known chemical composition have been shown to induce variable levels of DNA damage in a cell-free assay. MnO, S, Cu, and Zn were significant predictors of this DNA damage. Further work is required to characterise the mechanism of DNA damage formation and to determine to what extent these cell-free studies correlate with cellular studies. In particular, the perhaps surprising lack of association of DNA damage with arsenic concentration in the crushed rock powders highlights how cell-free assays may not be representative of toxicity behaviour in human cells, but the assays nevertheless confirm that the toxicity of μm-scale particles may be strongly dependent upon their chemical and mineralogical composition.

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Chapter 4: Geochemical compositional controls on DNA damage and cell viability induced in *in vitro* cell-based assays by crushed rock powders from the Panasqueira mine area, Portugal: A comparison with a cell-free assay

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Keywords: particles, heavy metals, DNA damage, cells death, cell-based assays, toxicity, Panasqueira

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Abstract

Exposure to heavy metals and metalloids can have toxic effects on cells. The aim of this study was to assess the dependence of the cytotoxic and genotoxic effects caused by μm-scale solid particles on chemical compositions, notably of heavy metals and arsenic, of those particles, using the cell-based Neutral Comet and MTT assays on A549 lung cells and compare the effects found with those observed in a cell-free Plasmid Scission assay. In regards to the Comet assay, the percentage of DNA in the tail varied between the samples ranging from 24 – 38%. Using bivariate correlation analysis and simple linear regression, MnO was found to be the only significant predictor of DNA damage ($\beta = 466$, $p < 0.022$). In regards to the MTT assay, the percentage of cell viability varied between the samples ranging from 43 – 73%. Using bivariate correlation analysis and multiple linear regression, MnO ($\beta = 0.452$, $p = 0.009$) and Clinochlore IIb2 ($\beta = -0.450$, $p < 0.010$) were found to be associated with loss of viable cells. In comparison, DNA damage in a cell-free Plasmid Scission assay ranged from 10 – 100 % with MnO, S, Cu, and Zn being significant predictors of the observed damage. In all assays, MnO appeared as a significant predictor; it was found to be positively correlated with DNA damage in the cell-free Plasmid Scission assay and in the cell-based Neutral Comet assay, however to a much lesser extent. Also, it was found to be negatively correlated with cell viability in the cell-based MTT assay. It is hypothesised that some proportion of MnO was present in the large size fraction of the particles. Given that in a cell-free assay size isn't important since DNA is exposed to particles of various sizes and components, MnO was strongly correlated with DNA damage. In contrast, since in a cell-based assay size is extremely important as some particles might penetrate into cells and other might not due to their large size,

MnO was weakly correlated with DNA damage and wasn't causing the loss of viable cells. It is speculated that compositional properties were important in determining cell viability and DNA damage. However, physical properties are thought to have a bigger role in being controlling factors for the aforementioned endpoints. Smaller sized particles were possibly able to penetrate and reach more cellular components and their chemical components inflicted cellular damage through the release of reactive oxygen species. Further work is required to assess the extent of contribution of reactive oxygen species and particles' physical structures to the DNA damage and loss of viable cells observed. Nevertheless, this study highlights the potential role of chemistry, physical structure, and mineralogy to the extent and/or nature of DNA damage and cell death caused by particulates.

4.1 Introduction

The physicochemical and mineralogical composition of particles are known to be controlling factors for toxicity (Schmidt et al. 2019; Potthoff et al. 2015). Even though the exact mechanism of inducing toxic effects remains to be determined (Øvrevik et al. 2015), the most dominant theory is that particles exert their toxicity through their release of Reactive Oxygen Species (ROS) which could result in oxidative stress leading to genotoxic (i.e. DNA strand breaks) and cytotoxic (i.e. cell death) effects (Øvrevik 2019; Jaeger et al. 2012; Chen and Lippmann 2009; Priftis et al. 2017; Shang et al. 2014). These effects form the basis for categorising assays used for *in vitro* assessment of particles' toxicity (Schmidt et al. 2019).

A variety of acellular and cellular *in vitro* systems are available for investigating toxic effects of particles. An acellular system enables the study of reactions that might occur within cells however, without the full cell system (Swartz 2006). Although this leads to a reduction in complex interactions associated with working on cellular systems and is considered to be an advantage sometimes, it might lead to false negative findings (Ayres et al. 2008) by limiting the ability of certain component (e.g. arsenic) to induce toxic effects due to the lack of biological interactions (Navas-Acien and Guallar 2008; Hughes et al. 2011; Jomova et al. 2011). This limits the use of acellular systems as they only allow for investigating inherent toxic potential of particles. For the assessment of total toxic potential, cellular systems are used as they enable the study of reactions taking place between specific agents, such as particles and their constituents, and the various components of living cells (Ayres et al. 2008). Still, a cellular system has its disadvantages as it can lead to understating the toxicity of an entire particle mixture and/or certain particle components as a result of defence and/or

repair mechanisms (Kawaguchi et al. 2008; Kawaguchi et al. 2010). Ideally, toxic effects of particles should be investigated in acellular and cellular systems in order to reach a better understanding of the component or group of components responsible for the observed effects (Ayres et al. 2008).

Therefore, given the known effects of exposure to particles of complex physiochemical and mineralogical compositions, the overall objectives of this study were to (i) determine the effect of the samples of different physical, chemical and mineralogical properties on DNA damage and cell viability using the cell-based Neutral Comet and MTT assays (ii) identify the main determinants of DNA damage formation and cell viability using bivariate correlation analysis and linear regression and (iii) compare results obtained here with those observed in a cell-free Plasmid Scission assay previously presented in (Badri et al. 2020a) - *Chapter 3,* this volume. The samples selected for this study were Crushed Rock Powders (CRPs), collected from inside and around the Panasqueira mine area in Portugal and used because of their known and wide variation in the concentration of arsenic, heavy metals and other traces major oxide concentrations.

4.2 Materials and methods

Detailed descriptions of the samples, their respective physicochemical and mineralogical analyses, and their effect on DNA strand breaks in a cell-free Plasmid Scission assay are presented in (Badri et al. 2020a) – *Chapter 3,* this volume. In brief, rock samples were collected from in and around the Panasqueira mine, Portugal. After crushing, the resultant crushed rock powders (CRP) were analysed by means of X-ray Diffraction (XRD), X-ray Fluorescence (XRF), and a Beckman Coulter LS 13 320

particle size analyser (analysis was conducted at the British Geological Survey (BGS) in Keyworth) to investigate their mineralogical, chemical, and particle size characteristics respectively. The crystalline minerals identified by XRD in the samples were mostly silicates with minor sulphides. These included quartz, muscovite 2M1, dravite tourmaline, and albite amongst others. A variety of components were identified by XRF but the most dominant were SiO_2, Al_2O_3, and Fe_2O_3 with notable traces including S, Ba, and As. The particle size analysis was used to calculate three parameters for describing particle size distributions; D90 (the 90th percentile particle size), D50 (the median particle size), and D10 (the 10th percentile particle size). pchAT Plasmid DNA was exposed to different levels of CRPs and H_2O_2 in an Elution Buffer and incubated for 5 hours at 37 ^{o}C. The reaction was stopped by adding loading buffer and the whole mixture was loaded to TBE-agarose gel. Electrophoresis was conducted in TBE buffer and the different forms of plasmid were visualised on a variable mode imager. The intensity of the different forms of plasmid in each lane was analysed and the level of damaged plasmid in each sample was calculated.

4.2.1 Cell culture

Adenocarcinomic human alveolar basal epithelial cells (A549 cells) kindly provided by Dr. Caroline Ridley were used. Cells were cultured in Minimum Essential Medium (MEM - Gibco, UK) supplemented with 10 % Fetal Bovine Serum (FBS – Gibco, UK), L-glutamine (Sigma, UK), sodium bicarbonate (Sigma, UK), and penicillin-streptomycin (Sigma, Israel). Cells were grown in Corning 75 cm^2 (T75) culture flasks at 37 °C, 3 % O_2 and 5 % CO_2 in 10 ml MEM medium for 24 hrs. Upon reaching confluence, the MEM medium was removed, and cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS; Lonza, USA). After washing, cells were detached

enzymatically using 750 µl of Trypsin-EDTA (Sigma, USA) for 5 mins at 37 °C. The reaction was stopped by adding 6 ml MEM medium. The cell suspension was centrifuged at 1000 rfc for 5 mins at 4 °C. The supernatant was discarded and 1 ml of MEM medium was added to re-suspend the cells. Cell concentration was determined using a haemocytometer.

4.2.2 Neutral Comet assay

The ability of CRPs to cause cellular DNA damage was investigated using the Neutral Comet assay as described previously (Dumax-Vorzet et al. 2015) with minor modifications. Cells were prepared at a concentration of 5000 cells/500 µL media and add to a 24 well plate and allowed to settle for 24 hrs. On the second day, the medium was replaced with medium containing CRPs or H_2O_2 (positive control) (500 µL per well) and incubated for 3 hours in the cell incubator. After that, the medium was discarded, and cells detached using trypsinization. Cells were then re-suspended in 0.7 % LMP (Agarose, low gelling temperature (LGT) (Sigma)) and subsequently dispensed on NMP agarose (Agarose, normal gelling temperature (NGT) (Bioline)) coated glass microscope slides and kept at 4 °C for 10 mins. Cells were lysed for 90 mins at 4 °C in lysis buffer (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris-base, 10% Dimethyl Sulfoxide (DMSO) and 1% Triton X-100, pH 10) and DNA was unwound for 20 min in electrophoresis buffer (300mM of sodium acetate and 100 mM of Tris-base, pH 9) prior to electrophoresis for 30 min at 25 V/300 mA. Following neutralisation with neutralisation buffer (0.4 M Tris-base in ddH_2O, pH 7.5), the slides were stained with SYBR gold (SYBR gold (Invitrogen, UK) solution in 10 mM Tris-HCl, 1mM EDTA, pH 7.5) in the dark. Dry slides were stored in the dark until image capture using a fluorescent microscope and analysis with CometScore.

4.2.3 MTT assay

The effect of CRPs on cell viability was investigated using the MTT assay. A549 cells were seeded in a 96-well plate in MEM culture medium and incubated at 37 ºC, 5 % CO_2 and 3 % O_2 for 24 hrs. The following day, the medium was replaced with medium containing increasing concentration of powders (samples), negative (100 µL of medium with untreated cells) and positive (medium containing H_2O_2) controls and incubated for 48 hours. Then, the medium was removed and replaced with 100 µL of fresh medium and 10 µL of MTT solution previously prepared and incubated at 37 ºC for 4 hrs. After that, 85 µL was removed from the medium and 50 µL of DMSO was added to each well and mixed and incubated for 10 mins at 37 ºC. Absorbance was read at 595 nm using MTT program of TECAN GENios plate reader. The percentage of cell viability was calculated as shown in Equation 1.

$$CV\ (\%) = \frac{At}{Ac}\ x\ 100 \qquad (1)$$

Where;

CV is the percentage of viable cells

At is the absorbance of cells treated with samples or positive control

Ac is the absorbance of control cells

4.2.4 Statistical analysis

Data obtained from each Neutral Comet and MTT assay were described using the mean and standard deviation. Data was analysed using a one-way analysis of variance (ANOVA) to determine significance relative to the negative control. A bivariate correlation analysis was then conducted to examine possible associations between

DNA damage and cell viability and the physicochemical composition of the samples. All the significant variables from this analysis were then plotted against the percentage of DNA in the tail and the percentage of cell viability to examine the correlations and entered into a backward stepwise multiple linear regression models or a simple linear regression. The least significant variable was eliminated step by step and all the models were compared using Bayesian information criterion (BIC) to choose the best explanatory model. Statistical analyses were performed using SPSS Statistics version 22. Graphs and scatterplots were created using Microsoft Excel 2010.

4.3 Results

4.3.1 Plasmid Scission assay

As previously reported in (Badri et al. 2020a) – *Chapter 3,* this volume, the percentage of DNA damage at 1250 µg/ml of CRPs varied between 10 – 100 % depending on the sample. In a multiple linear regression model, MnO ($\beta = 0.541$, $p < 0.000$), S ($\beta = 1.170$, $p < 0.002$), Cu ($\beta = -0.761$, $p < 0.031$), and Zn ($\beta = 0.253$, $p < 0.035$) were significant predictors of the percentage of DNA damage. These elements combined explained 83 % of the variance ($R^2 = 0.834$, $p < 0.001$) (Badri et al. 2020a).

The relationship between the significant predictors of DNA damage, MnO, S, Cu, and Zn, was examined (*Figure 4.1*). A positive correlation was found between S and Cu ($r = 0.954$, $p < 0.001$), MnO and Zn ($r = 0.406$, $p < 0.049$), Cu and Zn ($r = 0.363$, $p < 0.081$), S and Zn ($r = 0.294$, $p < 0.163$), MnO and S ($r = 0.17$, $p < 0.427$), and MnO and Cu ($r = 0.156$, $p < 0.466$).

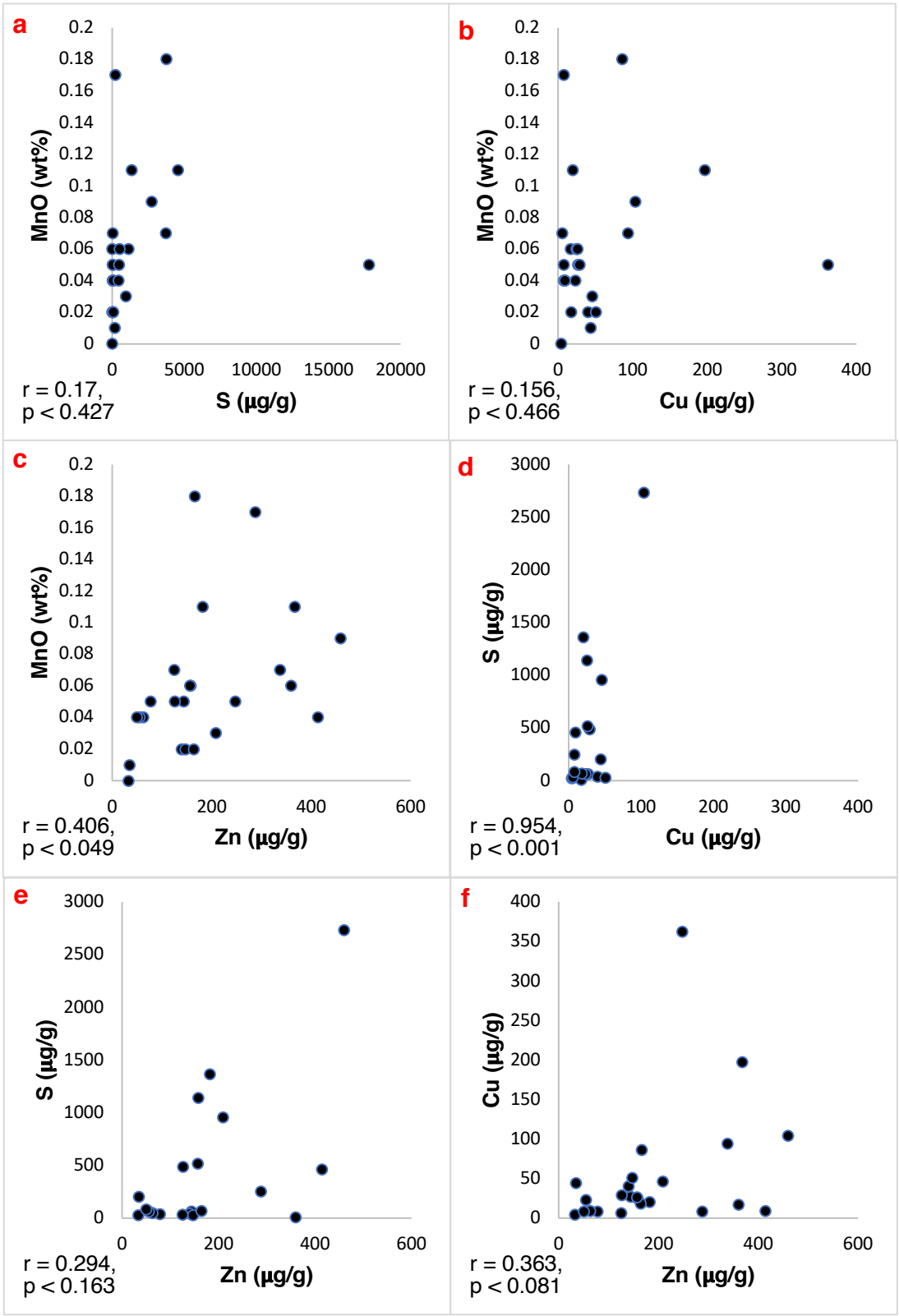

Figure 4.1, Correlation between: (a) MnO and S, (b) MnO and Cu, (c) MnO and Zn, (d) S and Cu, (e) S and Zn, and (f) Cu and Zn content of Panasqueira crushed rock powders (r values shown were obtained from Bivariate Correlation Analysis)

4.3.2 Neutral Comet assay

Increasing concentrations of H_2O_2 (0 - 100 μM) resulted in a dose-dependent increase in the percentage of DNA in the tail (*Figure 4.2*). Therefore, at 100 μM, it was considered our positive control.

The addition of CRPs to the reactions mix resulted in a dose-dependent increase in the percentage of DNA in the tail as shown in *Figure 4.3*.

After the establishing the existence of a dose-response relationship, 24 samples (A-X) have been used at the same concentration (100 μg/ml) to compare their effects and identify an attribute or group of attributes that are causing the damage (*Figure 4.4*)

The percentage of DNA damage at 100 μg/ml of the samples was found to be correlated with MnO only using bivariate correlation analysis (*Figure 4.5*). In a simple linear regression, MnO ($\beta = -0.466$, $p < 0.022$) was a significant predictor of DNA damage. This element explained 21% of the variance only ($R^2 = 0.218$, $p < 0.022$).

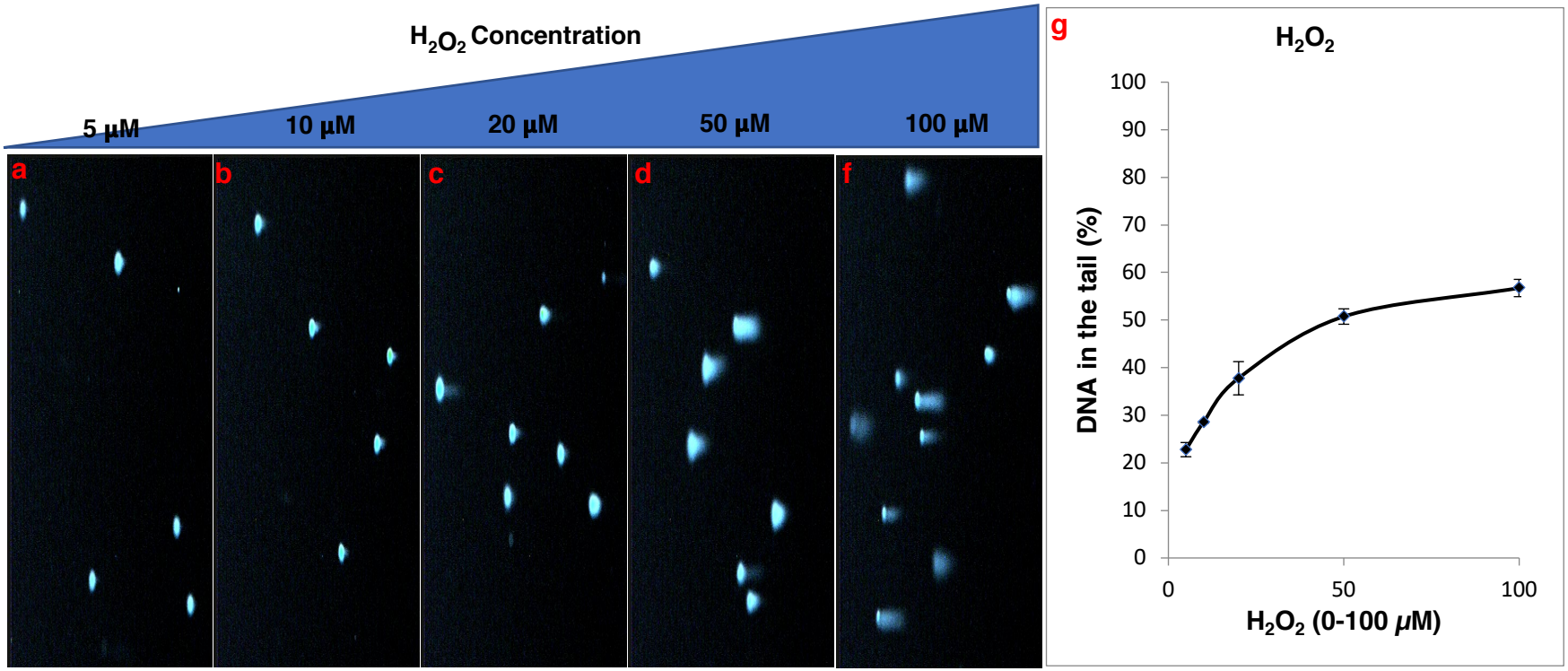

Figure 4.2, Neutral Comet assay of CRPs (positive control), Dose dependent DNA damage arising from H_2O_2

(a – f, microscopic images) / (g, % DNA damage induced by increasing amounts of H_2O_2). Cells were prepared at a concentration of 5000 cells/500 µL media and add to a 24 well plate and allowed to settle for 24 hrs. On the second day, the medium was replaced with medium containing increasing concentrations of H_2O_2 (0 - 100 µM) and incubated for 3 hours in the cell incubator. After that, the medium was discarded and cells detached using trypsinization. Cells were then resuspended in 0.7 % LMP and subsequently dispensed on agarose-coated glass microscope slides and kept at 4 °C for 10 mins. Cells were lysed for 90 mins at 4 °C in lysis buffer and DNA was unwound for 20 min in electrophoresis buffer (pH > 9) prior to electrophoresis for 30 min at 25 V/300 mA. Following neutralisation, the slides were stained with SYBR gold in the dark. Dry slides were stored in the dark until image capture using a fluorescent microscope and analysis with CometScore. Results represent the mean and standard deviation (SD) of two experiments.

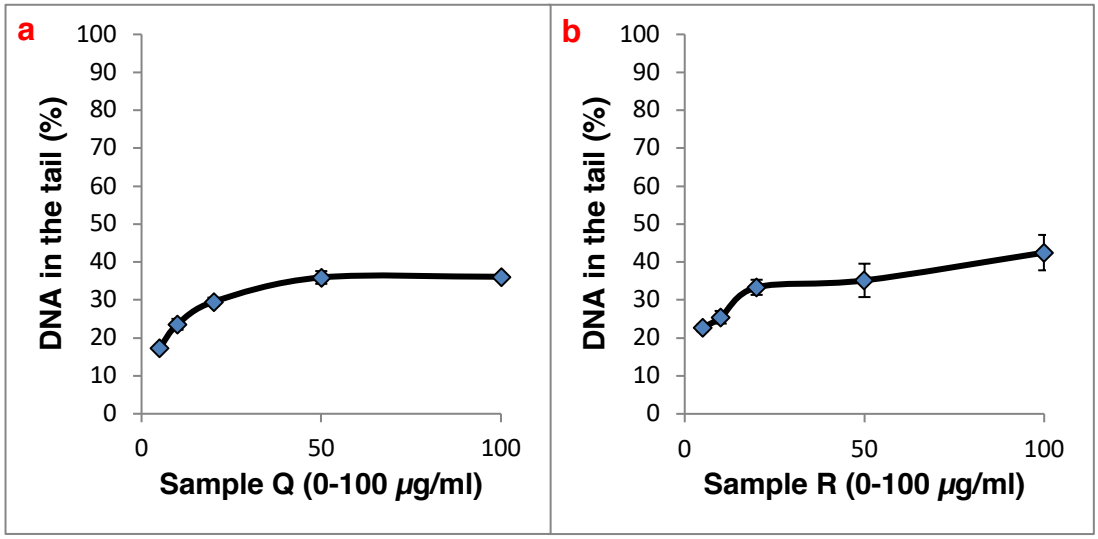

Figure 4.3, Neutral Comet assay of CRPs, Dose dependent DNA damage arising from samples (a) Q and (b) R

Cells were prepared at a concentration of 5000 cells/500 µL media and add to a 24 well plate and allowed to settle for 24 hrs. On the second day, the medium was replaced with medium containing increasing concentrations of samples (0 - 100 µg/ml) and incubated for 3 hours in the cell incubator. After that, the medium was discarded and cells detached using trypsinization. Cells were then re-suspended in 0.7 % LMP and subsequently dispensed on agarose-coated glass microscope slides and kept at 4 °C for 10 mins. Cells were lysed for 90 mins at 4 °C in lysis buffer and DNA was unwound for 20 min in electrophoresis buffer (pH > 9) prior to electrophoresis for 30 min at 25 V/300 mA. Following neutralisation, the slides were stained with SYBR gold in the dark. Dry slides were stored in the dark until image capture using a fluorescent microscope and analysis with CometScore. Results represent the mean and standard deviation (SD) of two experiments.

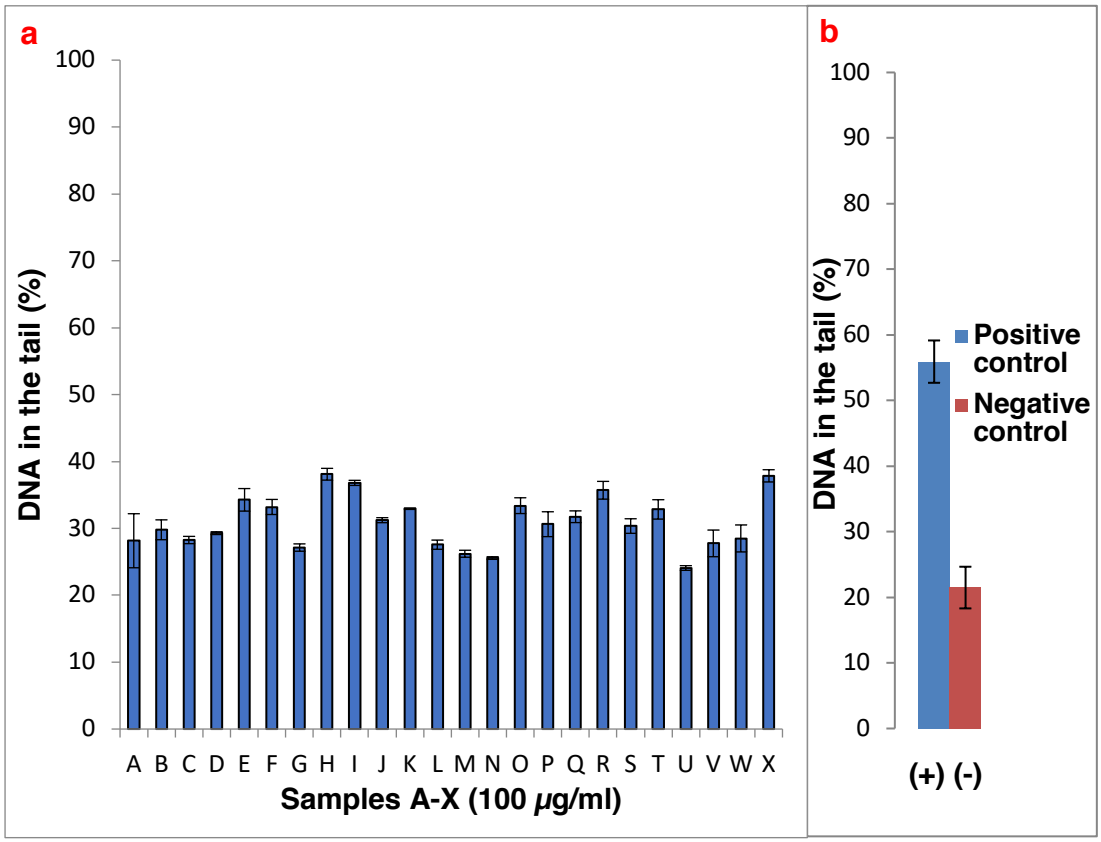

Figure 4.4, Neutral Comet assay of CRPs, Dose dependent DNA damage arising from; (a) samples A-X and (b) positive (H_2O_2) and negative (untreated cells) controls

Cells were prepared at a concentration of 5000 cells/500 µL media and add to a 24 well plate and allowed to settle for 24 hrs. On the second day, the medium was replaced with medium containing samples A-X (100 µg/ml) and H_2O_2 (100 µM) and incubated for 3 hours in the cell incubator. After that, the medium was discarded and cells detached using trypsinization. Cells were then re-suspended in 0.7 % LMP and subsequently dispensed on agarose-coated glass microscope slides and kept at 4 °C for 10 mins. Cells were lysed for 90 mins at 4 °C in lysis buffer and DNA was unwound for 20 min in electrophoresis buffer (pH > 9) prior to electrophoresis for 30 min at 25 V/300 mA. Following neutralisation, the slides were stained with SYBR gold in the dark. Dry slides were stored in the dark until image capture using a fluorescent microscope and analysis with CometScore. Results represent the mean and standard deviation (SD) of two experiments for CRPs and six experiments for controls. All samples (A-X) were found to be significantly different from the negative control using ANOVA ($p < 0.05$)

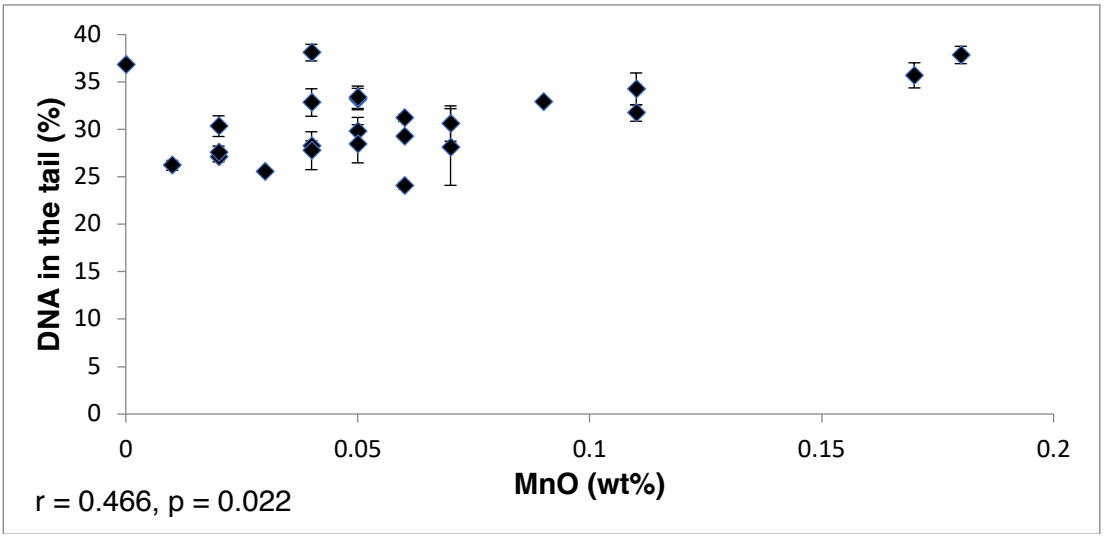

Figure 4.5, Correlation between DNA damage in a cell-based Neutral Comet assay and MnO content of Panasqueira crushed rock powders (r and p values shown were obtained from Bivariate Correlation Analysis)

4.3.3 MTT assay

To optimize the assay, the effect of cell number on absorbance levels was investigated by using different number of cells (1000-20000 cells/well) (*Figure 4.6)*. According to the figure below, 5000 cells is the best number to use because after that the curve starts to flatten from the addition of more cells which could have been caused by the depletion of nutrients in the cell culture medium.

Increasing concentrations of H_2O_2 (0 – 100 μM) in the culture medium resulted in a dose-dependent decrease in cell viability (*Figure 4.7*). Therefore, at 100 μM, it was considered our positive control.

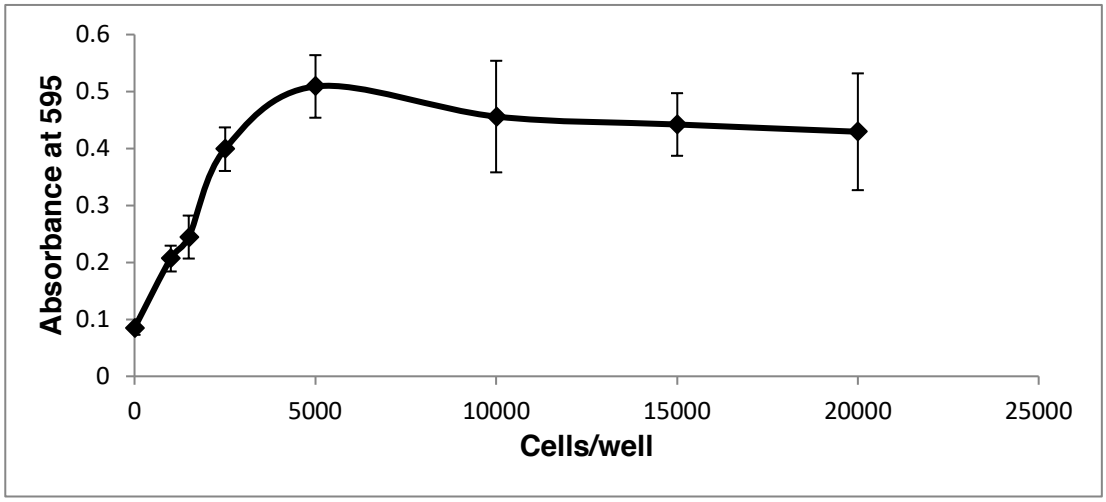

Figure 4.6, Correlation between MTT reduction and cell seeding density

A549 lung cells were seeded at densities from 1000 cells per well to 20000 cells per well in 100 µl culture media in a 96-well microplate at 37 ºC, 5 % CO_2 and 3 % O_2 for 72 hours. Following incubation, the medium was removed and replaced with 100 µL of fresh culture medium and 10 µl of MTT solution was added. The plate was incubated at 37 °C for 4 hours. After that, all but 25 µL of medium was removed and 50 µL of DMSO was added to each well. This was incubated for 10 minutes. The absorbance was read at 595 nm and plotted as a function of cell density. Each value represents the mean of and standard deviation (SD) of three experiments.

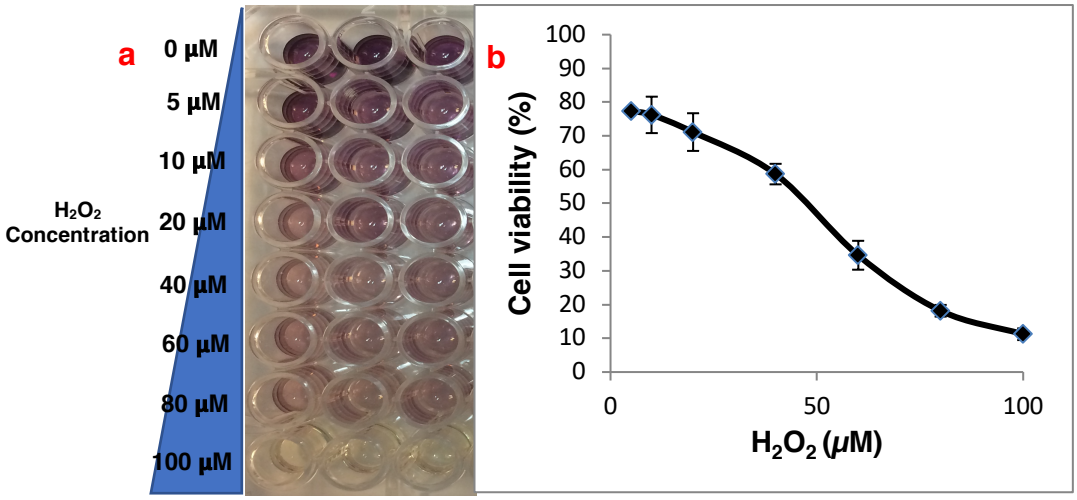

Figure 4.7, MTT assay of CRPs, Cytotoxic effects arising from H_2O_2

(a, MTT 96 well plate photo) / (b, % cell viability caused by increasing amounts of H_2O_2) A549 cells were seeded in a 96-well plate at a density of 5,000 cells/well/100 µL MEM culture medium and incubated at 37 ºC, 5 % CO_2 and 3 % O_2 for 24 hrs. The following day, the medium was replaced with medium containing increasing concentration of H_2O_2 and incubated for 48 hours. Then, the medium was removed and replaced with 100 µL of fresh medium and 10 µL of MTT solution previously prepared and incubated at 37 ºC for 4 hrs. After that, 85 µL was removed from the medium and 50 µL of DMSO was added to each well and mixed and incubated for 10 min at 37ºC. Absorbance was read at 595 nm. The percentage of cell viability was calculated as shown in Equation 1. Results represent the mean and standard deviation (SD) of three experiments.

As for the samples, 24 have been used and in each run positive and negative controls were added. The addition of different CRPs to the reactions mix resulted in a dose-dependent decrease in cell viability as shown in Figure 4.8.

The percentage of cell viability at 50 μg/ml of the samples was found to be positively correlated (i.e. not causing loss of viable cells) with MnO, CaO, P_2O_5, Zn, Sn, Dravite Tourmaline, and D50 (μm) and negatively correlated with Clinochlore IIb2 (i.e. causing loss of viable cells) using bivariate correlation analysis (*Figure 4.9*). In a multiple linear regression model, only MnO ($\beta = 0.452$, $p < 0.009$) and Clinochlore IIb2 ($\beta = -0.450$, $p < 0.010$) were found to be significant predictors of cell viability. These variables combined explained 50 % of the variance ($R^2 = 0.508$, $p < 0.001$).

The relationship between the significant predictors of cell viability, clinochlore IIb2 and MnO, was examined (*Figure 4.10*). A negative correlation between the two components was found, albeit week ($r = -0.249$, $p < 0.241$).

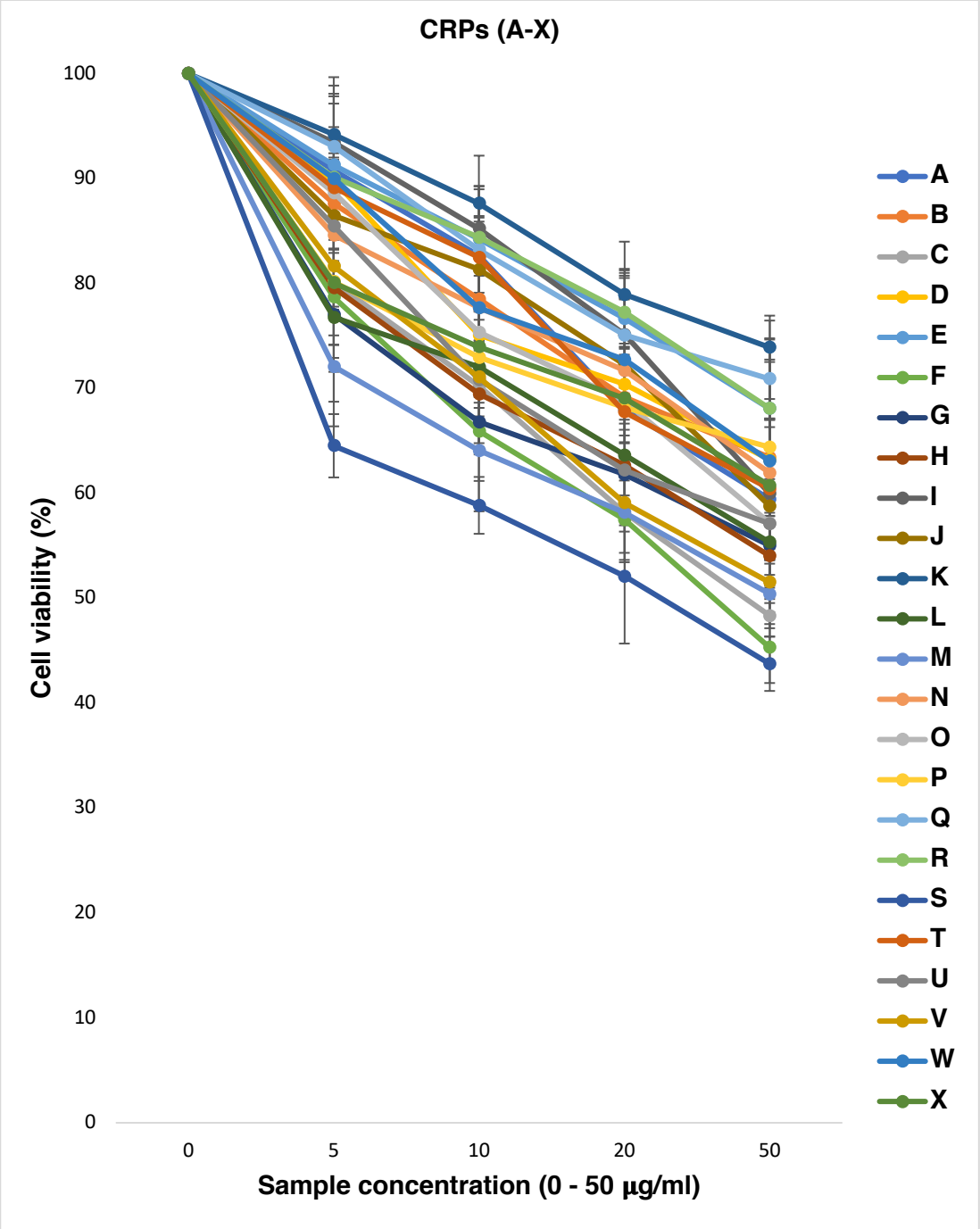

Figure 4.8, MTT assay of CRPs, Cytotoxic effects arising from CRPs (A-X) (0 – 50 µg/ml)

A549 cells were seeded in a 96-well plate at a density of 5,000 cells/well/100 µL MEM culture medium and incubated at 37 ºC, 5 % CO_2 and 3 % O_2 for 24 hrs. The following day, the medium was replaced with medium containing increasing concentration of powders (samples), negative (100 µL of medium with untreated cells) and positive (medium containing H_2O_2) controls and incubated for 48 hours. Then, the medium was removed and replaced with 100 µL of fresh medium and 10 µL of MTT solution previously prepared and incubated at 37 ºC for 4 hrs. After that, 85 µL was removed from the medium and 50 µL of DMSO was added to each well and mixed and incubated for 10 min at 37 ºC. Absorbance was read at 595 nm. The percentage of cell viability was calculated as shown in Equation 1. Results represent the mean and standard deviation (SD) of three experiments.

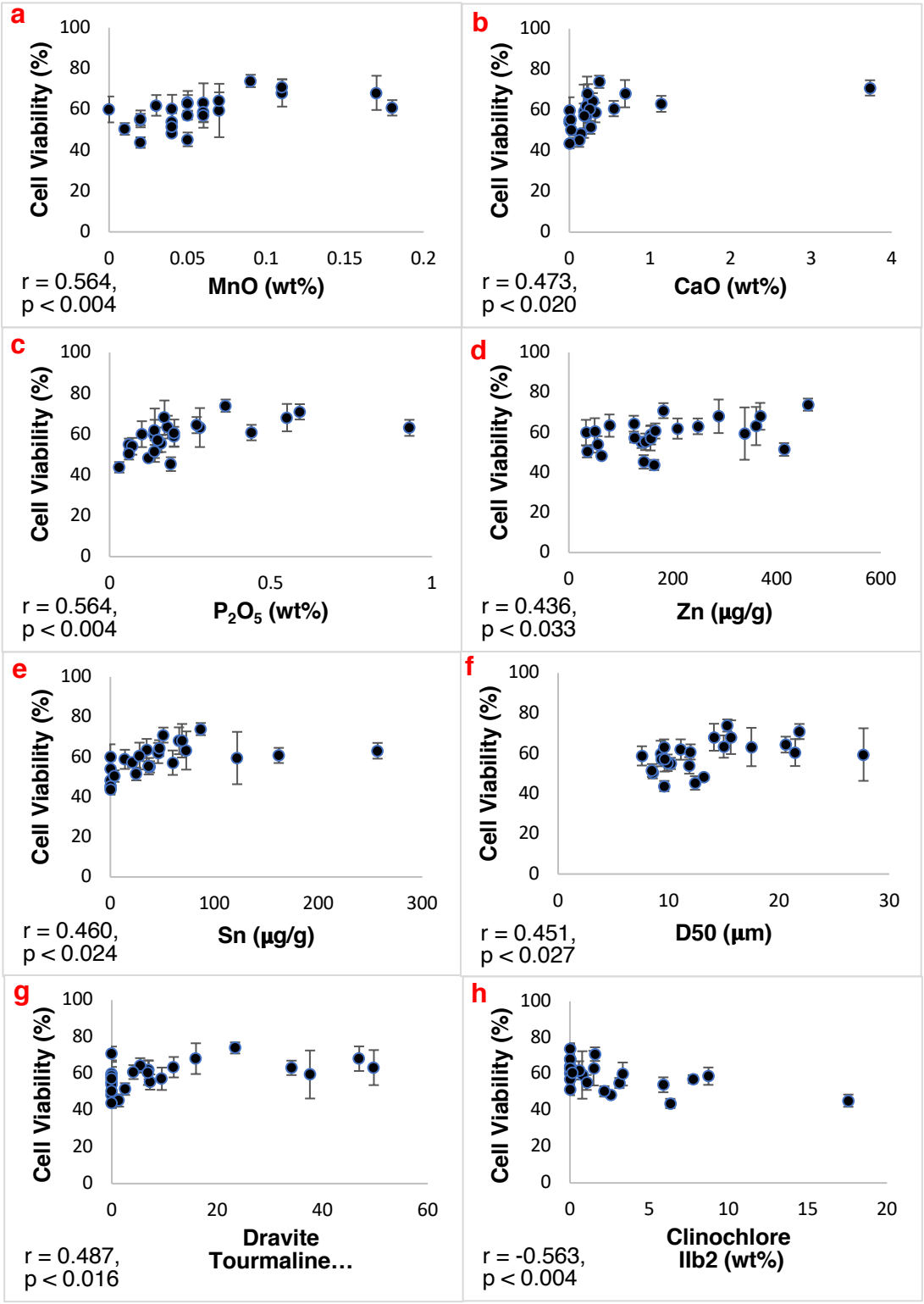

Figure 4.9, Correlation between cell viability in a cell-based MTT assay and: (a) MnO, (b) CaO, (c) P_2O_5, (d) Zn, (e) Sn, (f) D50, (g) dravite tourmaline, and (h) clinochlore IIb2 content of Panasqueira crushed rock powders - (r and p values shown were obtained from Bivariate Correlation Analysis)

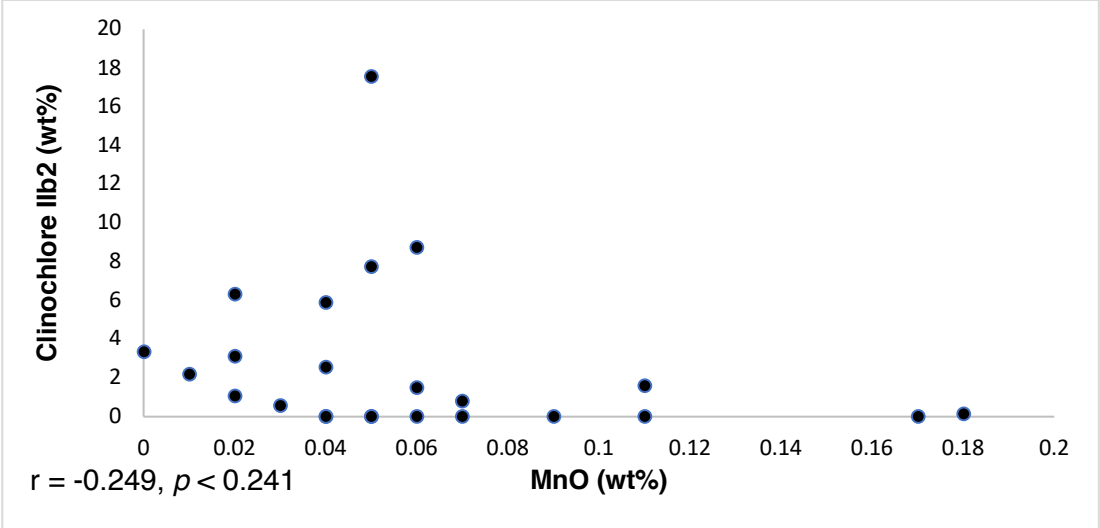

Figure 4.10, Correlation between Clinochlore IIb2 and MnO content of Panasqueira crushed rock powders (r values shown were obtained from Bivariate Correlation Analysis)

4.3.4 Relationship between cell-based and cell-free assays

Using bivariate correlation analysis, a significant correlation was found between the results of the cell-free Plasmid Scission and cell-based MTT assays only (Table 4.1 - Figure 4.11). The correlation shows that as the percentage of DNA damage increased in the Plasmid Scission assay, the percentage of cell viability increased (i.e. less loss of viable cells caused by CRPs) in the MTT assay.

Table 4.1, Correlations between assays used for the assessment of CRPs toxic effects

		Plasmid Scission assay[1]	Neutral Comet assay[2]	MTT assay[3]
Plasmid Scission assay[1]	Pearson Correlation	1	0.308	**0.528**
	Significance. (2-tailed)		0.142	**0.008**
Neutral Comet assay[2]	Pearson Correlation	0.308	1	0.239
	Significance. (2-tailed)	0.142		0.26
MTT assay[3]	Pearson Correlation	**0.528**	0.239	1
	Significance. (2-tailed)	**0.008**	0.26	

[1]The percentage of DNA strand breaks caused by 1250 µg/ml of CRPs
[2]The percentage of DNA damage caused by 100 µg/ml of CRPs
[3]The percentage of cell viability caused by 50 µg/ml of CRPs

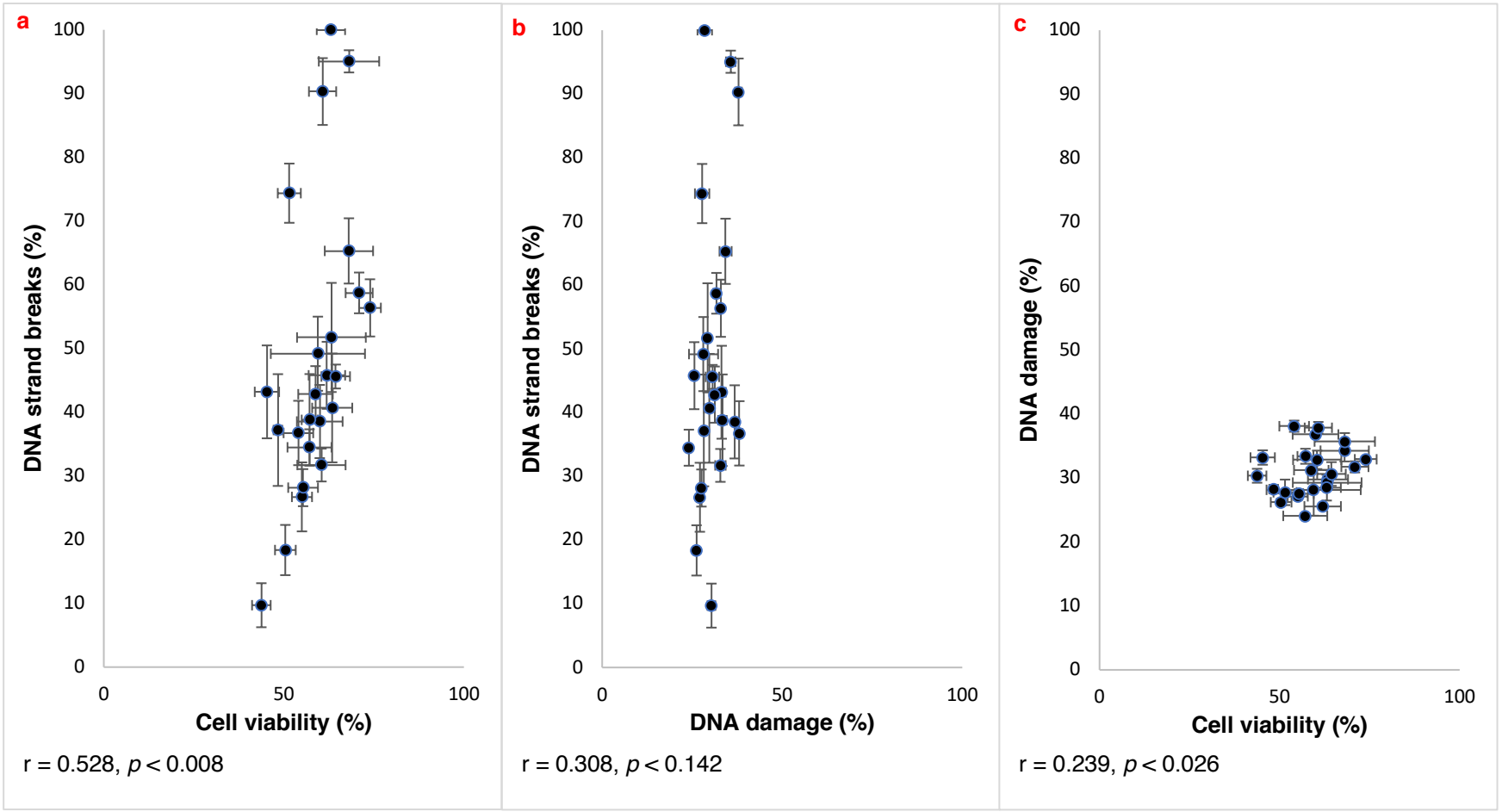

Figure 4.11, Correlation plots of Panasqueira crushed rock powders induced effects; (a) DNA strand breaks in the cell-free Plasmid Scission assay and cell viability in the cell-based MTT assay, (b) DNA strand breaks in the cell-free Plasmid Scission assay and DNA damage in the cell-based Neutral Comet assay, (c) DNA damage in the cell-based Neutral Comet assay and cell viability in the cell-based MTT assay

4.4 Discussion

CRPs were able to induce toxic effects in cell-free and cell-based assays. Variations in the toxic effects observed were found to be correlated with structural and compositional properties of CRPs. In the cell-based Neutral Comet assay, the only component found to be a significant predictor of DNA damage was MnO and it only explained 21 % of the variance. That is very different compared to toxic effects observed in the cell-based MTT assay and cell-free Plasmid Scission assay where MnO and Clinochlore IIb2 were significant predictors of cell viability, explaining 50 % of the variance and where MnO, S, Cu, and Zn were significant predictors of DNA damage, explaining 83 % of the variance respectively. Even though MnO appeared to be a significant predictor for effects observed in all assays, only the results of the cell-free Plasmid Scission and cell-based MTT assays were found to be significantly correlated. Although it is noted that findings from the bivariate correlation analysis and linear regression cannot be used to confirm the presence of a cause and effect relationship as it is not certain that the components highlighted were responsible for the toxic effects observed, it was extremely useful in highlighting the possible presence or absence of a relationship between the toxicological endpoints investigated and the physicochemical and mineralogical composition of CRPs.

The inconsistency in effects and correlations with the physicochemical and mineralogical composition of CRPs could be attributed to several factors: (i) cellular defence and repair mechanisms that could have led to limiting the effects of certain components; (ii) size limitations as it is possible that only fine and ultra-fine particles out of the overall CRP mixture were able to penetrate into cells and cause DNA damage and loss of viable cells in the cell-based assays while others couldn't due to

their large size; (iii) and the complex relationship between structure and composition as some components are mainly present in fine and ultra-fine particles while others are mainly present in coarse particles. To reach a better understanding of CRPs' toxic effects, the impact of the three aforementioned factors will be further explored.

4.4.1 Impact of cellular defence and repair mechanisms on CRPs' toxicity

Upon exposure to particles, cellular DNA damage occurs from oxidative stress as a result of ROS release from particles (Øvrevik 2019; Jaeger et al. 2012; Chen and Lippmann 2009; Priftis et al. 2017; Shang et al. 2014). Therefore, cells utilise their defence mechanisms in an attempt to stop further damage and balance the oxidative stress caused while activating repair enzymes to repair damaged DNA and keep the cell alive instead of undergoing cell death (Li et al. 2013; Kampa and Castanas 2008; Jarvis et al. 2014; Jarvis et al. 2013; Wohak et al. 2016; Peixoto et al. 2017). However, if cells were incapable of repairing the incurred damage, the apoptotic pathway is activated and cell death occurs as a result (Peixoto et al. 2017). This suggests that in the Neutral Comet assay, DNA damage could have occurred and then disappeared following a repair event and therefore the toxicity of certain components could have been understated because of this. Also, it suggests that in the MTT assay, cellular damage incurred could have been repaired and the cell survived in certain instances while otherwise it could have been so sever to the point where it was irreparable that cell death occurred. In comparison with a cell-free Plasmid Scission assay, DNA damage occurs without the interference of complex interactions which made its relationship with physicochemical and mineralogical properties of the CRPs much easier to detect.

4.4.2 Toxicity of fine particles compared to coarse particles present in the CRPs

Previous studies in the field of nanoparticles have reported the dependency of DNA damage and cell viability on the size of particles (Kong et al. 2011; Lewinski et al. 2008; Murphy et al. 2008; Nan et al. 2008; Wang et al. 2008; Kawanishi et al. 2020; Karlsson et al. 2009). Similarly, previous studies on airborne particles have reported $PM_{1.0}$ causing greater DNA damage compared to $PM_{2.5}$ in the Comet assay using A549 lung cells (Zou et al. 2017) and $PM_{2.5}$ being much more cytotoxic than PM_{10} in the MTT assay using Rat 6 rodent fibroblast (Hsiao et al. 2000) rat lung epithelial cell (Choi et al. 2004). Smaller particles tend to be more toxic than larger ones due to their size and large surface areas. Their small size enables them to penetrate and reach more cellular components (Lewinski et al. 2008; Dumax Vorzet 2010; Li et al. 2003) while their larger surface areas enable them to carry various toxic components (Xing et al. 2016). Both attributes allow for causing more damage depending on the endpoint under investigations. This suggests that DNA damage and loss of viable cells observed in the cell-based Neutral Comet and MTT assays respectively, was dependent on fine particles present in the CRPs. In comparison with DNA damage in a cell-free Plasmid Scission assay, such dependence didn't exist as plasmid DNA was exposed to all particle sizes.

4.4.3 The relationship between size and composition of particles present in the CRPs

All the significant predictors of DNA damage in the cell-free Plasmid Scission assay, MnO, S, Cu, and Zn, were found to be positively correlated with each other which means that there is a possibility that they were all present in the same size fraction. None except MnO was found to be positively correlated with DNA damage in the cell-

based Neutral Comet assay, albeit weakly. This suggests that some proportions of MnO only was present in the small size fraction while the rest were present in the large size faction. The presence of MnO in small and large size factions is further confirmed in the negative correlation found between the significant predictors of cell viability, clinochlore IIb2 and MnO, which is suggestive of a possibility that some of their particles were present in opposite particle size fractions. The percentage of cell viability decreased as clinochlore IIb2, which is thought to be present in the small size fraction, concentration increased. In contrast, cell viability increased as MnO, which some proportion of its particles are thought to be present in the large size fraction, concentrations increased. Finally, the presence of MnO in small and large size fractions is again further confirmed in the significant positive correlation between results of the cell-free Plasmid Scission assay and cell-based MTT assay which suggest that whatever was causing acellular DNA damage wasn't able to cause the loss of viable cells.

4.5 Conclusion

Crushed rock powders of known chemical composition have been shown to have variable effects on DNA damage and cell viability in cell-based assays. Even though compositional properties were found to be important, cell viability and cellular DNA damage were found to be more controlled by physical properties. That was very different to DNA damage induced by the same crushed rock powders in a cell-free assay where compositional properties were the main determinants of the damage observed rather than physical properties. Further work is required to characterise the mechanism of loss of viable cells and DNA damage formation and the bioavailability of particles of different sizes and compositions inside the cells. The assays used in this

study nevertheless confirm that the toxicity of μm-scale particles may be strongly dependent upon their physical, chemical, and mineralogical characteristics.

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Chapter 5: DNA strand breaks induced by PM_{10} from Makkah, Saudi Arabia: Association with meteorological proxies for source and composition

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Abstract

Exposure to PM_{10} is known to cause adverse health effects in humans. The aims of this study were to investigate the effects of PM_{10} on DNA strand breaks and its association with source and composition as indicated by meteorological parameters used as proxies. Samples of PM_{10} were collected before and during Ramadan (April / May) in 2018 from Makkah, Saudi Arabia. PM_{10} concentrations and DNA damage were found to be; (i) independent on the type of day (i.e. weekday or weekend) or period of sample collection (i.e. before or during Ramadan) suggesting that variable traffic volumes had limited impact on them; (ii) dependent on weather conditions however, differently. PM_{10} concentrations were correlated with weather conditions typical of windblown dust and sand particles while DNA damage was correlated with weather conditions typical of the formation of anthropogenically-sourced secondary particles. It is possible that such particles released reactive oxygen species and as a consequence, oxidative stress occurred and led to DNA damage. Further work is required to assess the extent of contribution of reactive oxygen to the formation of this damage. Nevertheless, irrespective of the mechanisms involved, this study highlights how PM_{10} toxicities might be usefully predicted from widely available meteorological data.

5.1 Introduction

Particulate matter (PM) pollution refers to a complex mixture of solid particles, liquid droplets, and semi-volatile components with different morphologies (e.g. rod shaped, spheroidal, and regular shaped), sizes (from 0.1 μm to 100 μm), chemical compositions (e.g. metals, ions, and carbonaceous material), and origins (natural such as from the Earth's crust and sea spray and anthropogenic such as from power plants and combustion engines) (*Figure 5.1*) (Tsai et al. 2000; Hime et al. 2015). In general, particles of natural origin have irregular shapes and rough surfaces while particles of anthropogenic origin have regular shapes with well-defined edges and/or spherical and rounded shapes with smooth and/or porous surfaces (Zeb et al. 2018; González et al. 2016; Li and Shao 2009; Saitoh et al. 2008; González et al. 2018; Aragon-Pina et al. 2006).

Even though natural and anthropogenic emission sources significantly impact the concentrations and compositions of PM, they are not the only controlling factor (Yáñez et al. 2017). PM concentrations and compositions are also influenced by a largely uncontrollable key factor, meteorological parameters, that contribute heavily to the variation in PM. In fact, it may overcome the influence of certain emission sources (Karagiannidis et al. 2015; Gietl and Klemm 2009). The most important meteorological parameters affecting PM are temperature, relative humidity, wind speed, and wind direction. Heat and humidity are the main determinants of the type and rate of chemical reactions that change PM's structure and composition (Hernandez et al. 2017). Wind speed and wind direction are the main determinants of a source's contribution to the overall PM mixture (Kim et al. 2015). Such changes to PM results in a variety of adverse health effects (Jia et al. 2017; Hime et al. 2018).

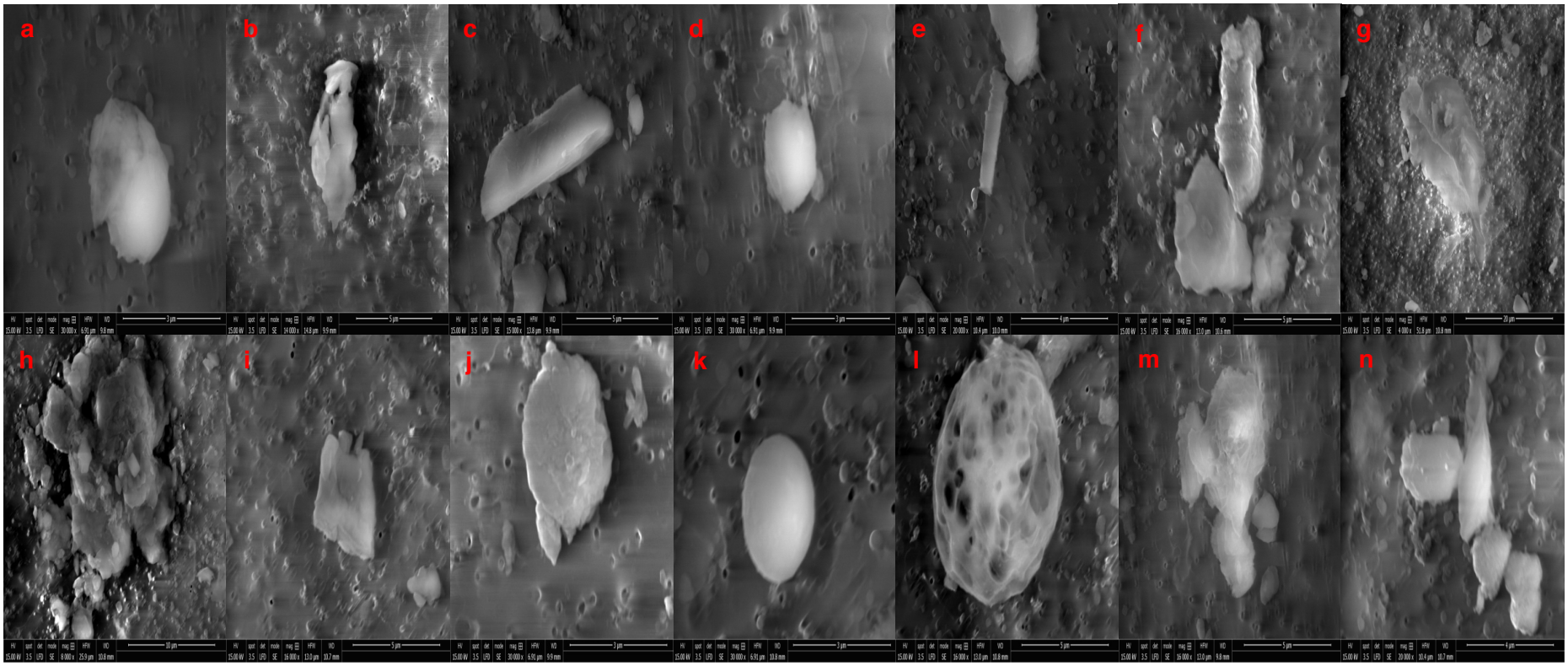

Figure 5.1, Representative scanning electron microscope images of particulate materials from Makkah, Saudi Arabia obtained during testing the effectiveness of a high-volume sampler in collecting PM_{10} samples on filters

Si-rich particles; spheroidal (a), irregular (b), and regular (c). Ca-rich particles (d). Fe-rich particles; rod-shaped (e) and irregularly shaped (f). Cl-rich particles (g, h). Ba-rich particles (j, i). C-rich particles; smooth surface (k), surface porosity (l). Zn-rich particle (m). Zr-rich particle (n). Characterisation of single particles collected on Polycarbonate (PC) membrane filters 0.2 µm, 8 inch x 10 inch was performed using the scanning electron microscope FEI Quanta 650 equipped with a Burker system for EDS analysis, at the Department of Earth and Environmental Sciences, University of Manchester, UK. The filters were cut into small pieces (1cmx1cm) and each cut piece was placed on a stud inside the chamber of the microscope without processing at low vacuum. The stud was located at a working distance of 10 mm from the window of the gaseous secondary electron detector (GSED). The level of magnification varied between 2500-40000 depending on the particle analysed. The accelerating voltage was set to 15 kV.

Numerous studies have found that exposure to PM is associated with an increase in mortality and hospital admissions (Brunekreef and Holgate 2002; Achilleos et al. 2019; Samoli et al. 2008; Dockery et al. 1992; Castillejos et al. 2000; Thalib and Al-Taiar 2012; Bell et al. 2008; Phosri et al. 2019). The impact PM has on human health varies depending on the dose, duration of exposure, composition of PM, and individual susceptibility. The severity of effects ranges from eye irritation to death (Kampa and Castanas 2008; Cohen et al. 2005). Even though the exact mechanism of inducing adverse health effects is still unknown, it is suggested that the effects are associated with Reactive Oxygen and Nitrogen Species produced by PM (Sánchez-Pérez et al. 2009). Reactive Oxygen and Nitrogen Species are known to cause a variety of adverse health effects by causing oxidative stress. Oxidative stress can negatively affect a variety of cellular structures including proteins, lipids, and DNA (Schieber and Chandel 2014; Barrera 2012; Rehman et al. 2018; Engwa et al. 2019).

Researches usually face challenges in investigating the relationship between PM toxic effects and its components. This is mainly due the material obtained via PM samplers often being insufficient for conducting both physicochemical and toxicological analyses of PM (Vuong et al. 2017; Dumax Vorzet 2010). Given that PM concentration and composition is heavily influenced by weather conditions, widely available meteorological parameters can be used as a proxy for predicting concentrations and sources/composition of PM components and hence its toxic effects.

The Holy City of Makkah in the kingdom of Saudi Arabia is considered unique to all Muslims due to its Holy Mosque, which attracts millions of visitors annually from around the world, particularly during the month of Ramadan – accordingly the population of Makkah can quadruple during Ramadan (e.g. 7.8 million visitors during

Ramadan in 2018) (GASTAT 2018)). As a result, the city becomes very busy especially in terms of road traffic. Under such conditions, air quality is expected to deteriorate (Habeebullah 2016; Munir et al. 2017a).

Particulate matter pollution has been investigated in several studies in Makkah (Munir et al. 2013a; Munir et al. 2013b; Habeebullah 2014, 2016; Shaltout et al. 2013) with some focusing specifically on PM pollution during Ramadan (Nasralla and Seroji 2008; Habeebullah 2013b). Unfortunately, however, only one study (El-Assouli 2011) investigated the genotoxicity of PM's organic extractable matter using the *salmonella* TA98 test (PM was mutagenic) and comet assay (PM damaged DNA in human blood cells).

No study in Makkah has examined the relationship between PM components and its induced toxic effects nor the relationship between PM toxic effects and meteorological parameters. Only the relationship between PM_{10} and meteorological parameters have been investigated. The relationships reported were inconsistent throughout the studies due to differences in sampling locations and seasons. Habeebullah (2013), analysed the effect of meteorological parameters on PM_{10} concentration and its precursors, NO_x, CO, and SO_2, with data from an air monitoring station during the month of Ramadan (20 July to 18 August, 2012). The author reported temperature being negatively correlated with PM_{10}, NO_x, CO, and SO_2. Wind speed was positively correlated with PM_{10} only and negatively correlated with its precursors. Similar to wind speed, humidity was positively correlated with PM_{10} only and negatively correlated with NO_x, CO, and SO_2 (Habeebullah 2013b). Munir et al. (2013) modelled PM_{10} concentrations data from an air monitoring station with wind speed, wind direction, temperature, and relative humidity from November 2011 to July 2012 in

Makkah. The authors reported strong positive correlations between PM_{10} and temperature and wind speed. Relative humidity and wind direction were found to be not correlated with PM_{10} (Munir et al. 2013b). Habeebullah et al. (2015), investigated the association of PM_{10} and its precursors NO_x, CO, and SO_2 with wind speed, wind direction, relative humidity, and temperature in Makkah using data from an air monitoring station for the year 2012. The authors reported temperature being positively correlated with PM_{10} and CO and negatively correlated with SO_2. There was no correlation between temperature and NO_x. Wind speed was positively correlated with PM_{10} and negatively correlated with its precursors. Wind direction was positively correlated with SO_2 and negatively correlated with NO_x, PM_{10}, and CO. Humidity was negatively correlated with PM_{10}, NO_x, CO, and SO_2 (Habeebullah et al. 2015). Munir et al. (2017), analysed PM_{10} and its association with meteorological parameters using an air quality monitoring station from January 2014 to September 2015 in Makkah. The authors found a negative correlation between PM_{10} and humidity and a positive correlation with temperature and wind speed (Munir et al. 2017a).

Therefore, given the known health effects of exposure to PM and the lack of data from Makkah in specific, the goal of this study was to contribute to existing knowledge of PM from Makkah and elsewhere by investigating the toxic effects of PM and their association with composition in order to understand the most likely contributor to the toxic effects found. To reach this goal, the first aim of this study was to investigate the toxic effects of particulate matter (PM_{10}) collected from Makkah, Saudi Arabia before and during Ramadan (April / May) in 2018 on DNA strand breaks using a cell-free Plasmid Scission Assay. The second aim was to investigate the association between

the variations in DNA damage and PM_{10} composition and source as indicated by meteorological parameters used as proxies.

5.2 Methodology

5.2.1 Study area

The Holy City of Makkah is located southwestern Saudi Arabia. At an elevation of 277 m above sea level and about 80 km inland towards the east from the coast, the city is located in a desert valley between mountains (Habeebullah 2014). The city's climate is characterised by warm winters with temperatures ranging from 25 °C during the day and 17 °C during the night and extremely hot summers with temperatures ranging from 40 °C during the day and 30 °C during the night. Rainfall is rare with monthly means of 10 - 33 mm and humidity ratios of 45 - 53 %. Winds are mostly north eastern throughout the year. Dust-storms originating from the Arabian Peninsula's deserts or from North Africa occur frequently throughout the year (Al-Jeelani 2009).

5.2.2 Sample collection

A High-Volume Sampler equipped with a PM_{10} size selective inlet was used to collect 24-hour samples of PM_{10} on Polytetrafluoroethylene Membrane Filters (PTFE), 1.0 µm, 8 inch x 10 inch. The sampler was installed in Aziziyah district, a suburb of Makkah that has transformed from being a highly populated residential area to a prime target for the hospitality sector due to its close proximity to religious sites in the city and accordingly it is characterised by high traffic volumes and various types of activities, inside the campus of the General Directorate of Education in Makkah Al-Mukkaramah Region (*Figure 5.2*). The sampler was installed away from obstacles

such as buildings and wall fences by around 5 meters to get proper airflow. Filters were placed inside the sampler at 10 am the first day and then replaced within 15 minutes at 10 am for the following days. Sampling was carried out for 14 days, Monday to Friday for each period before and during Ramadan (April – May / 2018). For each sampling day (24-hour), collected samples were immediately transported and stored at -20 °C. Samples were then shipped on dry ice to the University of Manchester for analysis.

A Certified Reference Material (CRM), ERMCZ120 - Fine dust (PM10-like) - trace elements, was purchased as a reference for subsequent DNA damage measurements.

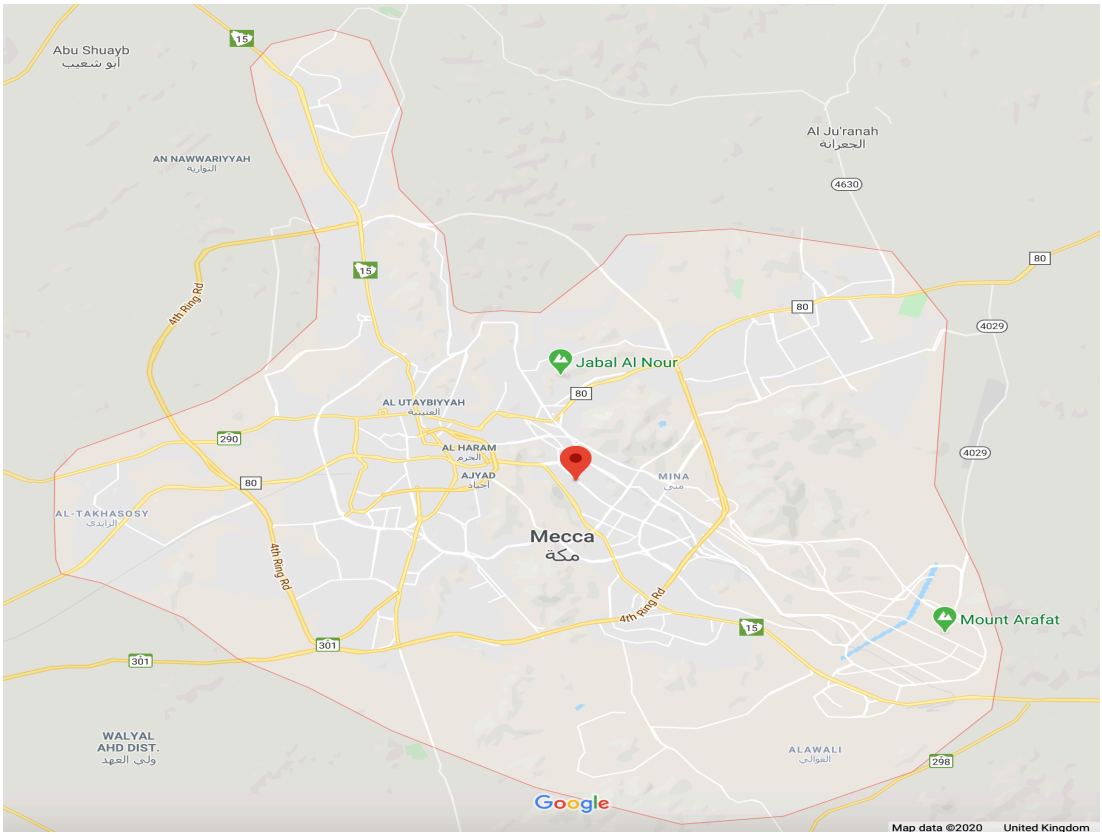

Figure 5.2, Map showing the sampling location of PM_{10} in Aziziyah district, Makkah, Saudi Arabia (From Google Maps (2020))

5.2.2.1 Complementary data

Hourly data on temperature, dew point, humidity, wind speed, wind direction, gust, and pressure for Makkah during 2018, 2019, and 2020 were obtained from www.wunderground.com and www.timeanddate.com.

Daily average air quality data of PM_{10}, NO_2, SO_2, CO, and O_3 from Saudi Arabia's Presidency of Meteorology and Environment (PME) via www.aqicn.org for the years 2019 / 2020 only was acquired as no data set that dates back to the days of sample collection (April / May - 2018) was found for Makkah. The observational data obtained does not represent raw concentrations but Air Quality Index (AQI) values converted using the US EPA standard and it has not been verified nor validated and in no way official (PME 2020; Aqicn 2020). Also, it contains large gaps probably because unlike data collected for this study, it was possibly collected over an extended period, under different conditions, using different instruments and procedures, and with different operators. Hence, for the aforementioned reasons, its quality is questionable. In this paper, we used the AQI values to comment on the condition of air quality in relation to health in Makkah and even though averaging periods of collection were not specified by the data providers, we converted the values back to raw concentrations using EPA guidelines assuming an averaging period of 24 hours for PM_{10}, 8 hours for O_3, 1 hour for NO_2, 1 hour for SO_2, and 8 hours for CO in order to present the aforementioned parameters and their relationships with individual meteorological parameters more accurately (EPA 2018).

5.2.3 Determination of daily PM_{10} concentrations

The method used for calculating daily PM_{10} concentrations was adapted from previously published methods (RTI 2008). Individual PTFE filters were weighed before and after sample collection and the difference in weight was used to calculate PM_{10} concentrations as shown in Equation (1).

$$C_{PM} = \frac{M_{PM}}{V_A} = \frac{(M_F - M_I) \times 10^3}{Q_{AVE} \times T \times 10^{-3}} \quad (1)$$

Where;

C_{PM} is the concentration of PM ($\mu g/m^3$)

M_{PM} is the mass on the filter (μg)

V_A is the filter volume (m^3)

M_F is the weight of filter after collection (mg)

[1]M_I is the weight of filter before collection (mg)

[2]Q_{AVE} is the average flow rate of the sampler (L/min)

T is the total elapsed time of sample collection (min)

[1]The weights of two out of fourteen filter were extrapolated using the known values of M_F and M_{PM} due to a transcriptional error associated with their values.

[2]The equipment required to measure sampler flow rate was not available at the time of sampling. Therefore, sampler average flow rate was obtained by calculating the mean of typical flow rates of a PM_{10} high volume sampler reported elsewhere (U.S. EPA 1999; TISCH 2019).

5.2.4 Sample extraction

Prior to sample extraction, used PTFE filters were left to equilibrate at room temperature for 10 min in the dark. Each filter was processed individually. The extraction method used was adapted from previously published methods (Dumax Vorzet 2010) with minor adjustments. Briefly, the filters were cut into 8 pieces with a clean scalpel blade. Each piece was then weighed before being placed in a clean 500 ml beaker and pre-wetted with 100 µl 100 % ethanol. Double distilled water (45 ml / cut piece) was then added. This solution was ultra-sonicated for 45 min and then transferred to a pre-weighed tube and frozen at -80 °C overnight. The tubes stored were then placed in a freeze-dryer for lyophilisation to dryness. When finished, the tubes were weighed again and the PM_{10} available was diluted to 20 mg/ml with distilled water and kept at -20 °C in the dark. Procedural blanks were prepared in a similar way using blank filters.

5.2.5 Plasmid Scission Assay

A detailed description of the cell-free Plasmid Scission assay used for the assessment of DNA damage is presented in ((Badri et al. 2020a) – *Chapter 3,* this volume). In brief, pchAT Plasmid DNA (5 ng) was diluted to 20 µl in an Elution Buffer (10 mM Tris-HCl pH 8.5) with different levels of PM_{10} samples and H_2O_2. Samples were incubated for 5 hours at 37 ^oC. The reaction was stopped by adding loading buffer (Promega blue/orange 6X loading dye). The whole reaction mixture was loaded to a 0.6 % TBE-agarose gel. Electrophoresis was conducted at 90-100V for 45 min in 1x TBE buffer. The different forms of plasmid were visualised on a Typhoon 9200 variable mode imager. The intensity of the different forms of plasmid in each lane was

analysed using ImageQuantTM (GE Healthcare Life Sciences) and the level of damaged plasmid in each sample was calculated as shown in the Equation (2).

$$DP\ (\%) = \frac{R+L}{R+L+S}\ x\ 100 \qquad (2)$$

Where;

DP is the percentage of DNA damage

R is the intensity of the relaxed form of plasmid DNA

L is the intensity of the linear form of plasmid DNA

S is the intensity of the supercoiled form of plasmid DNA

In each experiment, positive and negative controls were added. The positive control was H_2O_2 (3.5 mM), pchAT plasmid DNA (5 ng), and $FeSO_4$ (25 μM) in elution buffer. The negative control was; H_2O_2 (3.5 mM) and pchAT plasmid DNA (5 ng) diluted in elution buffer.

5.2.6 Statistical analysis

Data obtained from each Plasmid Scission Assay were described using the mean, standard deviation, minimum, and maximum values. A paired samples t-test was conducted to check if there was a significant difference between weekdays and weekends and between periods of sample collection before and during Ramadan. Pearson's correlation was used to check for correlations between daily PM_{10} concentrations, meteorological parameters, and DNA damage. Multiple Linear Regression was used to determining the most likely predictors daily of PM_{10} concentrations and DNA damage. Statistical analyses were performed using SPSS

Statistics version 22. Graphs and scatterplots were created using Microsoft Excel 2010.

5.3 Results

5.3.1 Weather conditions during the days of sample collection

Data on temperature, dew point, humidity, wind speed, wind direction and pressure were obtained for Makkah for the days of sample collection during 2018 (*Table 5.1*) and for the years 2019 / 2020. The relationships between individual meteorological parameters showed similar patterns throughout the years 2018, 2019, and 2020 (*Figure 5.3*).

Meteorological parameters were compared during the two periods of sample collection, before and during Ramadan. A significant difference was found for some parameters only, namely, temperature, dew point, and pressure using a paired samples t-test *(Table 5.2)*. Hourly changes in the parameters are shown in *Figure 5.4* for the period before Ramadan and *Figure 5.5* for the period during Ramadan.

Table 5.1, Meteorological parameters (Means of hourly values over 24 hours), for Makkah region during the days of sample collection before and during Ramadan. Adapted from Wunderground (2020) and timeanddate (2020).

Period	Day of the week	Temperature (°C)	Dew Point (°C)	Humidity (%)	Wind direction (°)	Wind Speed (m/s)	Gust (m/s)	Pressure (mb)
Before Ramadan	Monday	28	18	58	179	4	3	1006
	Tuesday	29	16	47	200	3	3	1005
	Wednesday	32	13	32	157	3	3	1005
	Thursday	29	16	48	224	5	4	1005
	Friday	27	11	38	131	6	5	1008
	Saturday	27	14	43	164	5	4	1009
	Sunday	28	17	52	163	4	4	1008
	Mean	29	15	46	174	4	4	1006
	Standard deviation	2	3	9	30	1	1	1
During Ramadan	Monday	32	19	47	140	4	3	1005
	Tuesday	34	17	36	181	3	4	1006
	Wednesday	29	22	67	258	3	3	1003
	Thursday	30	21	60	245	3	2	1003
	Friday	32	16	41	203	3	3	1002
	Saturday	32	18	44	198	4	3	1003
	Sunday	31	21	56	181	3	3	1003
	Mean	32	19	50	201	3	3	1003
	Standard deviation	2	2	11	40	0	0	1
Periods combined	Mean	30	17	48	187	4	3	1005
	Standard deviation	2	3	10	37	1	1	2

Table 5.2, Comparison of meteorological parameters (%; mean ± SD of seven recordings representing days of the week, Monday - Sunday) recorded during the days of sample collection before and during Ramadan using a paired samples t-test.

Meteorological parameter	Before Ramadan mean ± SD	During Ramadan mean ± SD	*p* value
Temperature	29 ± 2	31 ± 2	**0.044**
Dew point	15 ± 3	19 ± 2	**0.007**
Humidity	45 ± 9	50 ± 11	0.458
Wind direction	174 ± 30	201 ± 40	0.194
Wind speed	4 ± 1	3 ± 0	0.062
Gust	4 ± 1	3 ± 0	0.140
Pressure	1007 ± 1	1004 ± 1	**0.062**

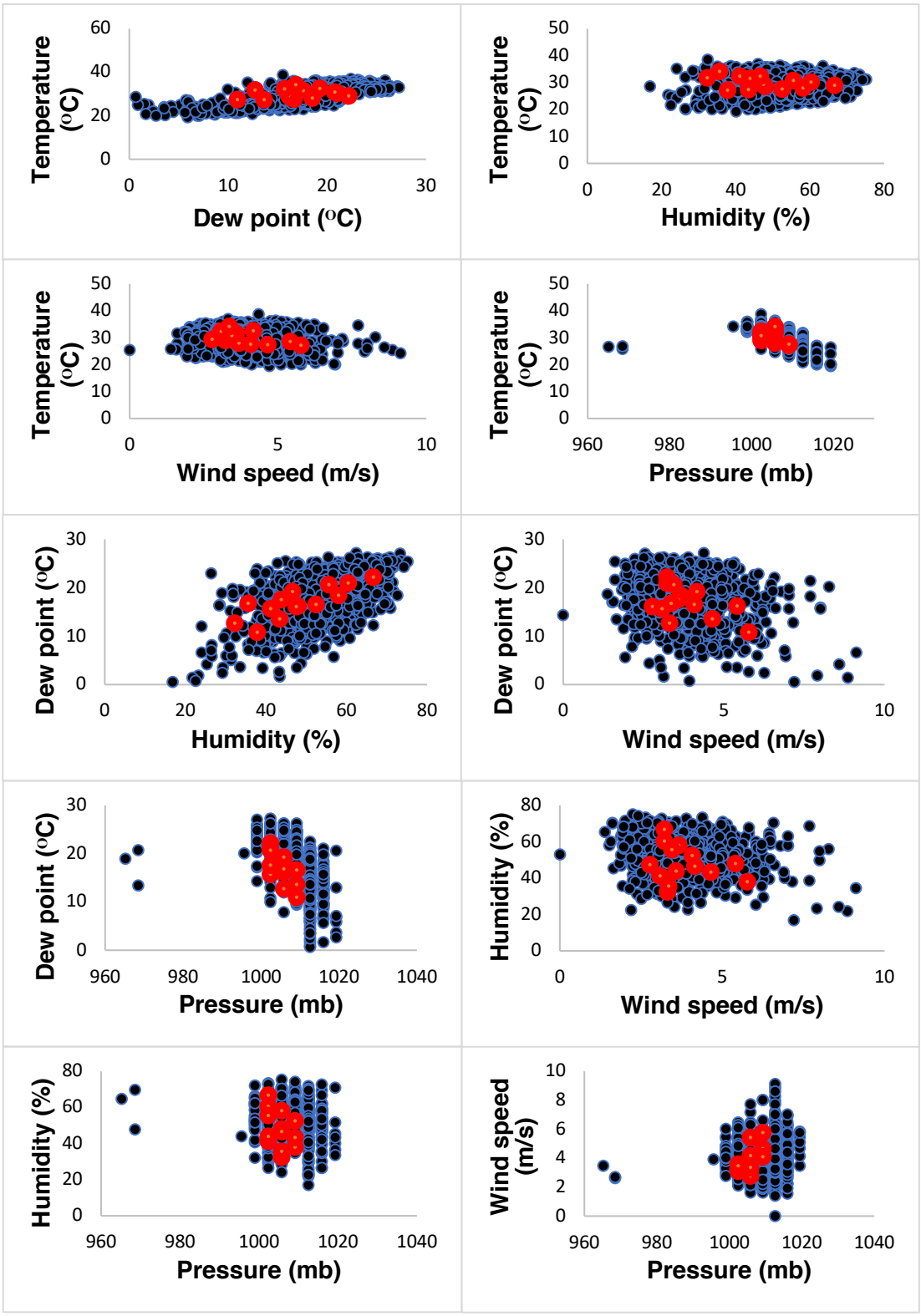

Figure 5.3, Correlation plots for the relationship between individual meteorological parameters for the years 2018, 2019, 2020 – black data points represent the years 2018, 2019, and 2020 in general while red data points represent the periods of sample collections during 2018 in particular (From Wunderground (2020))

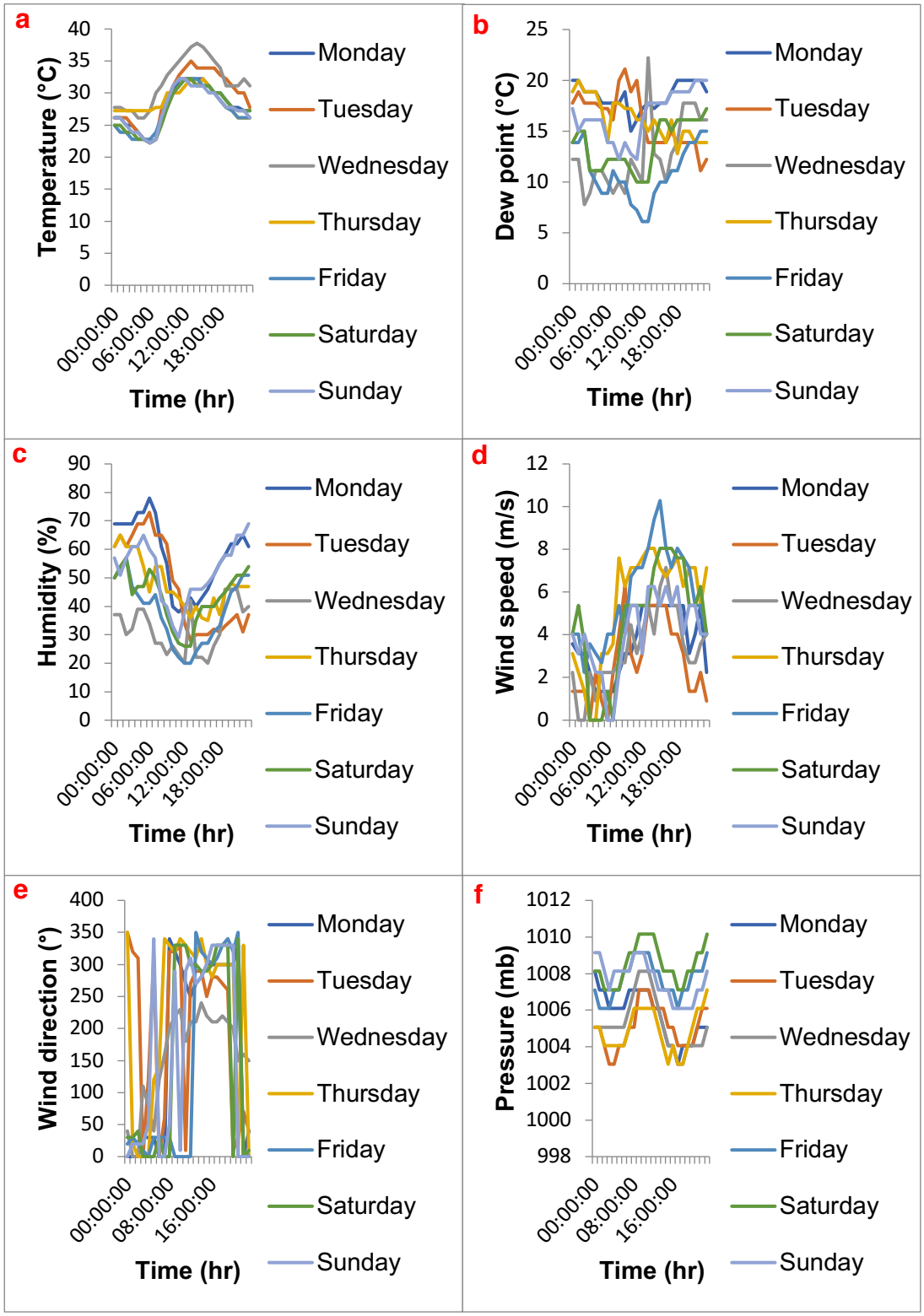

Figure 5.4, Hourly changes in; (a) temperature, (b) dew point, (c) humidity, (d) wind speed, (e) wind direction, and (f) pressure for Makkah in sample collection days before Ramadan (From Wunderground (2020) and timeanddate (2020))

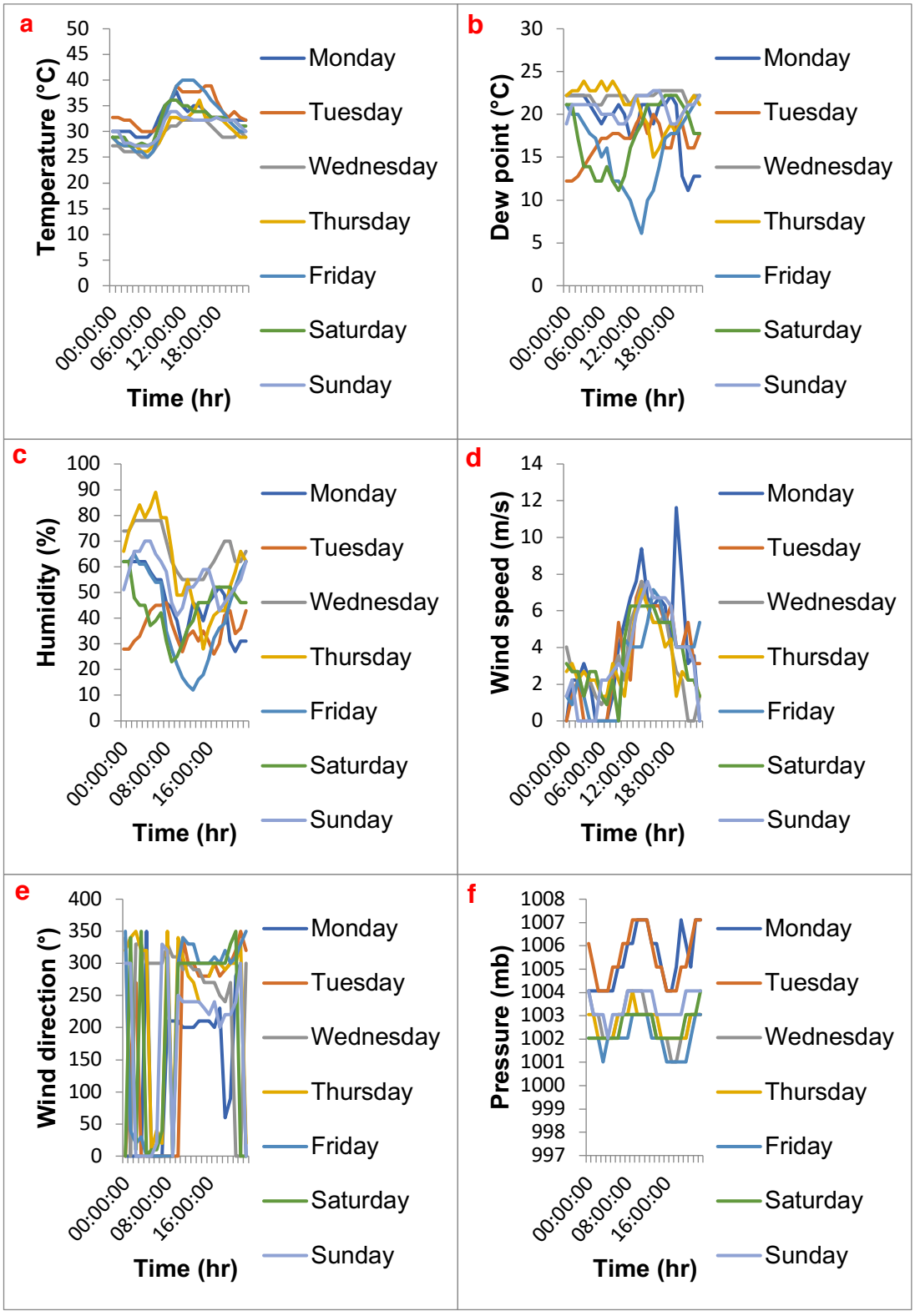

Figure 5.5, Hourly changes in; (a) temperature, (b) dew point, (c) humidity, (d) wind speed, (e) wind direction, and (f) pressure for Makkah in sample collection days during Ramadan (From Wunderground (2020) and timeanddate (2020))

5.3.2 Daily AQI values and concentrations of PM_{10}, NO_2, SO_2, CO, and O_3

Throughout 2019 and 2020, AQI values for PM_{10} were mostly "good" (90.9 %) for health with the exception of some "moderate" (8.2 %) and "unhealthy" (0.9 %) conditions. As for O_3, the majority were "good" (91.2 %) with some "moderate" (8.3 %) and "very unhealthy" (0.5 %) conditions. In regards to NO_2, SO_2, and CO, all values were categorised as "good" (100 %) for health.

No significant difference was found in mean daily (i.e. Monday to Sunday – *Figure 5.6*) concentrations of PM_{10} ($F(6, 224) = 0.43$, $p < 0.862$), NO_2 ($F(6, 212) = 0.30$, $p < 0.936$), SO_2 ($F(6, 207) = 0.61$, $p < 0.722$), CO ($F(6, 224) = 0.71$, $p < 0.646$), O_3 ($F(6, 208) = 0.31$, $p < 0.933$). Also, no significant difference between weekdays and weekends was found in the mean concentrations of PM_{10} (weekdays ($M = 30.27$ $\mu g/m^3$, $SD = 12.17$) / weekends ($M = 30.12$ $\mu g/m^3$, $SD = 10.14$) conditions $t(8) = -0.06$, $p < 0.9655$), NO_2 (weekdays ($M = 5.10$ ppb, $SD = 1.69$) / weekends ($M = 4.53$ ppb, $SD = 1.85$) conditions $t(8) = -1.49$, $p < 0.174$), SO_2 (weekdays ($M = 6.46$ ppb, $SD = 2.36$) / weekends ($M = 6.00$ ppb, $SD = 2.18$) conditions $t(9) = -0.65$, $p < 0.534$), CO (weekdays ($M = 0.84$ ppm, $SD = 0.21$) / weekends ($M = 0.79$ ppm, $SD = 0.18$) conditions $t(9) = -1.46$, $p < 0.179$), and O_3 (weekdays ($M = 0.03$ ppm, $SD = 0.01$) / weekends ($M = 0.03$ ppm, $SD = 0.01$) conditions $t(9) = -0.56$, $p < 0.591$) using a paired samples t-test.

No significant correlation ($r < 0.5$) was found between daily concentrations of PM_{10} and NO_2, SO_2, CO, and O_3 (*Figure 5.7*) nor between PM_{10} and individual meteorological parameters (*Figure 5.8*).

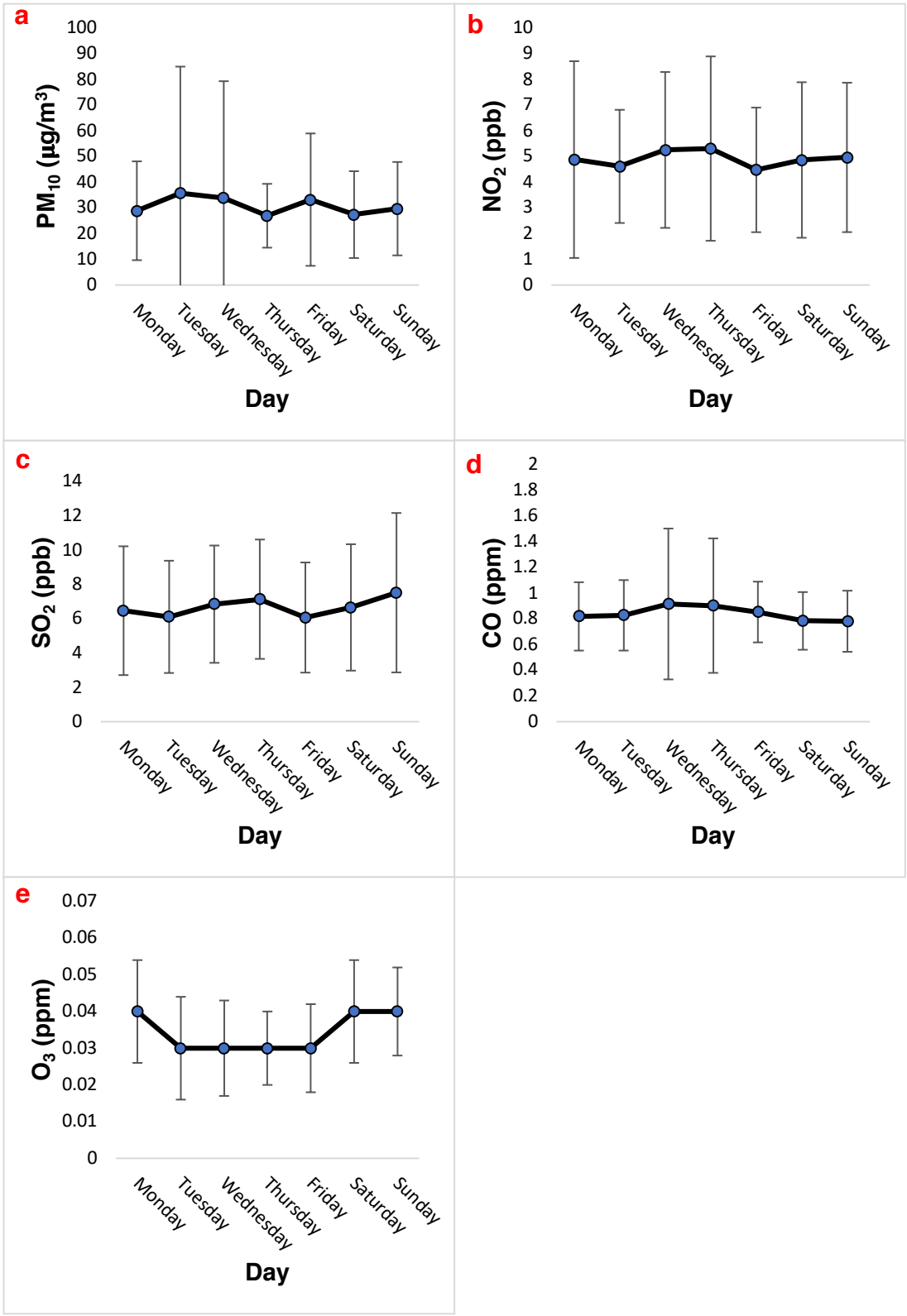

Figure 5.6, Mean daily concentrations of; (a) PM_{10}, (b) NO_2, (c) SO_2, (d) CO, and (e) O_3 - error bars represent the standard deviation (SD) of recordings made during 2019 / 2020 (From PME (2020) and Aqicn (2020))

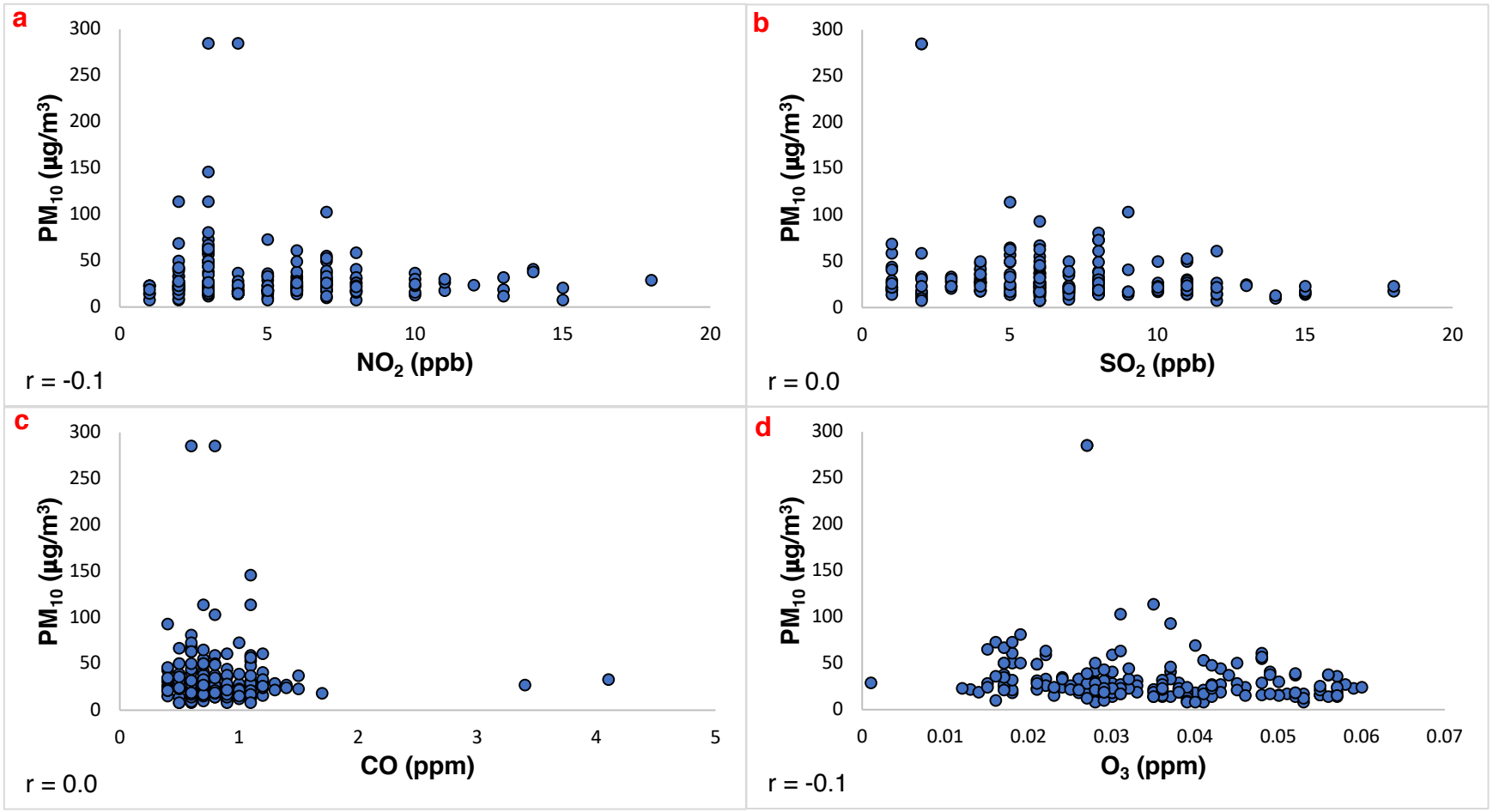

Figure 5.7, Correlation plots for mean daily concentrations of PM_{10} with mean daily concentrations of: (a) O_3, (b) NO_2, (c) SO_2, and (d) CO during 2019 / 2020 - (r values represent Pearson's correlation coefficient) - (From Aqicn (2020) and PME (2020))

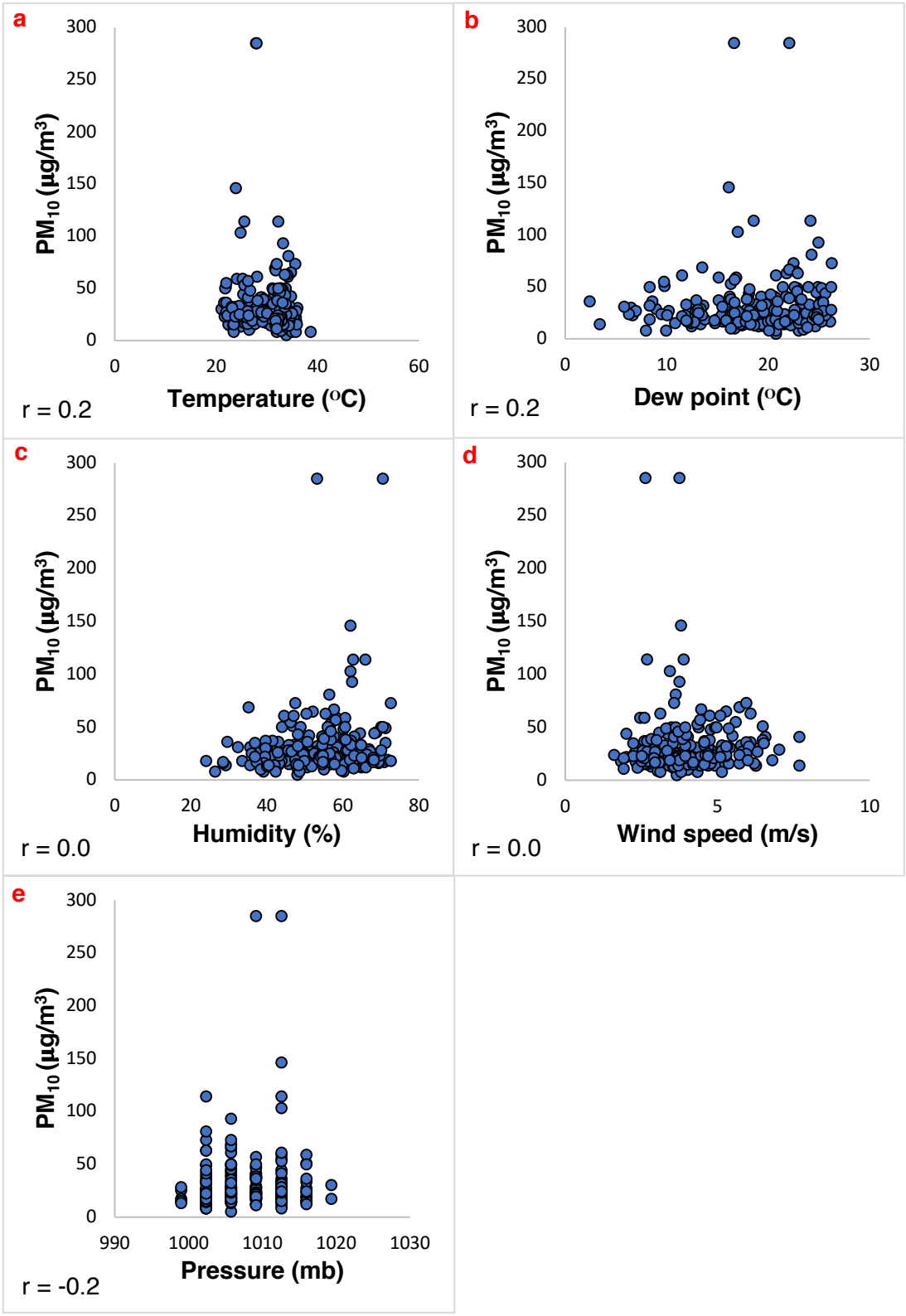

Figure 5.8, Correlation plots for mean daily concentrations of PM_{10} with daily mean recordings of: (a) temperature, (b) dew point, (c) humidity, (d) wind speed and (e) pressure - (r values represent Pearson's correlation coefficient) - (From Aqicn (2020), PME (2020), Wunderground (2020), and timeanddate (2020))

5.3.3 PM_{10} collected and extracted

5.3.3.1 Daily concentrations of PM_{10}

Daily PM_{10} concentrations ranged from 63 – 101 µg/m^3 before Ramadan and from 51 – 175 µg/m^3 during (*Figure 5.9*). No significant difference was found that suggests that PM_{10} concentrations were higher in weekdays compared to weekends (weekdays (M = 92.52 µg/m^3, SD = 11.21) / weekends (M = 99.98 µg/m^3, SD = 31.45) conditions t(1) = 0.521, $p < 0.694$) nor that PM_{10} concentrations were higher before Ramadan compared to during (before Ramadan (M = 82.64 µg/m^3, SD = 14.47) / during Ramadan (M = 106.67 µg/m^3, SD = 44.19) conditions t(6) = -1.49, $p < 0.186$) using a paired samples t-test.

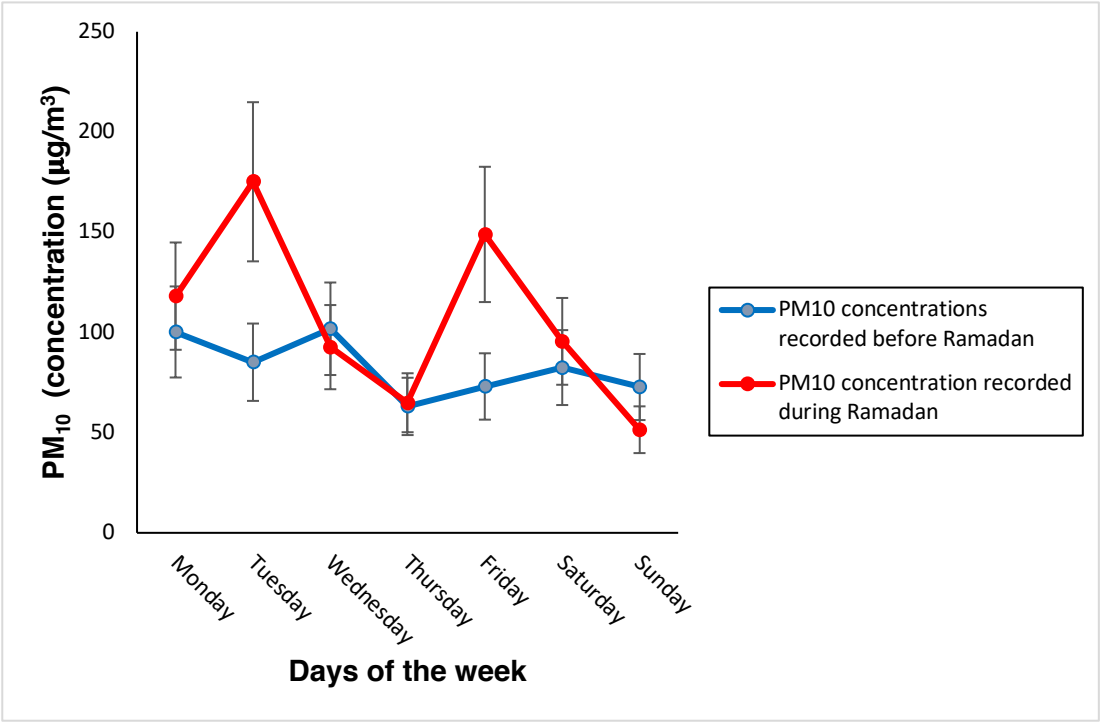

Figure 5.9, Daily concentrations of PM_{10} collected from Makkah before and during Ramadan (April / May – 2018) – (error bars represent the percentage of error associated with variations in the average flow rate of the sampler)

Daily PM_{10} concentrations were found to be positively correlated with temperature (r = 0.7) (*Figure 5.10*). Correlations with other meteorological parameters were too weak to consider (r < 0.5).

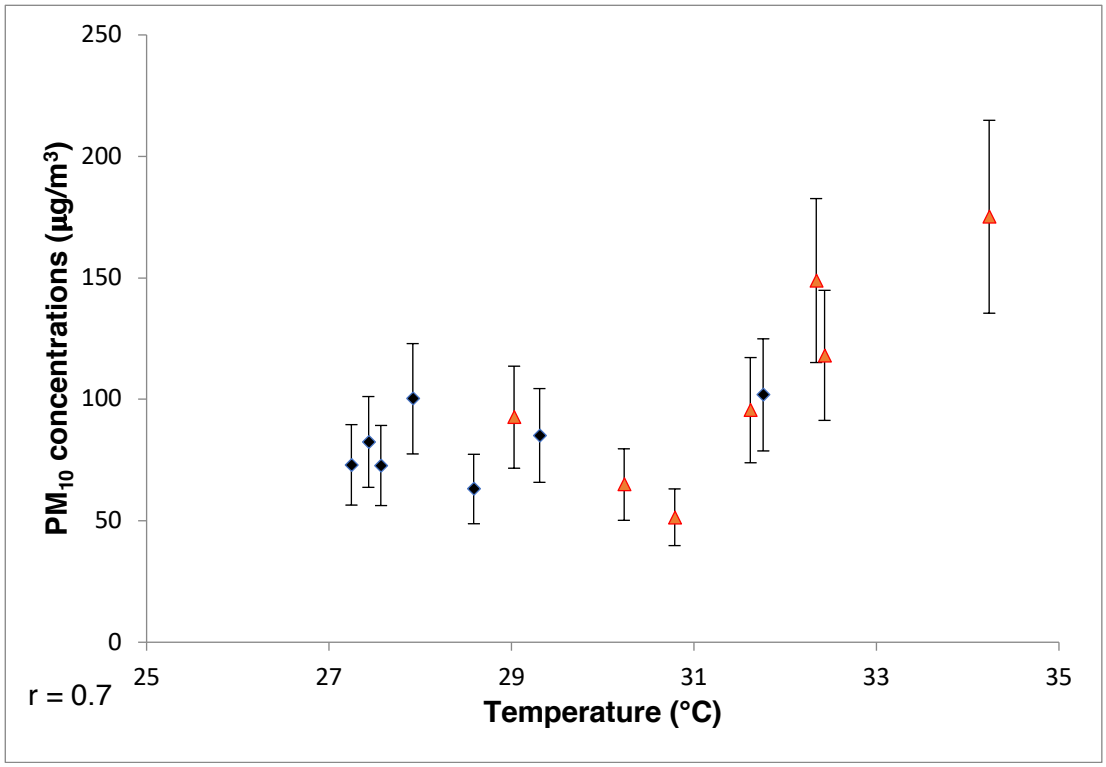

Figure 5.10, Correlation plot between daily PM_{10} concentrations collected from Makkah before (blue data points) and during (red data points) Ramadan (April / May – 2018) and temperature (r value represents Pearson's correlation coefficient) – (error bars represent the percentage of error associated with variations in the average flow rate of the sampler) daily PM_{10} concentrations from this study. Meteorology parameters from Wunderground (2020) and timeanddate (2020).

In a multiple linear regression, several models were created for investigating the dependence of daily PM_{10} concentrations on temperature, dew point, humidity, wind speed, gust, wind direction, and pressure (*Table 5.3*). The models were compared using the Akaike Information Criterion (AIC) and the model with the lowest AIC value had temperature ($\beta = 2.560$, $p < 0.032$), dew point ($\beta = -2.550$, $p < 0.106$), humidity ($\beta = 2.788$, $p < 0.117$), and pressure ($\beta = 0.571$, $p < 0.124$) as predictors of daily PM_{10}

concentrations. These variables combined explained 68 % of the variance ($R^2 = 0.682$, $p < 0.024$). A simpler model that was much easier to interpret only had temperature as a significant predictor ($\beta = 0.700$, $p < 0.005$) of which it explained 49 % of the variance ($R^2 = 0.490$, $p < 0.005$).

Table 5.3, Regression models for daily PM_{10} concentrations and individual meteorological parameters.

Model (daily PM_{10} concentrations)	R Squared	Significance	Akaike Information Criterion
Pressure (millibar), Humidity (%), Wind Speed (m/s), Wind direction (°), Temperature (°C), Gust (m/s), Dew point (°C)	0.713	0.188	96.20
Pressure (millibar), Humidity (%), Wind direction (°), Temperature (°C), Gust (m/s), Dew point (°C)	0.713	0.095	94.20
Pressure (millibar), Humidity (%), Wind direction (°), Temperature (°C), Dew point (°C)	0.705	0.046	92.56
Pressure (millibar), Humidity (%), Temperature (°C), Dew point (°C)	**0.682**	**0.024**	**91.64**
Humidity (%), Temperature (°C), Dew point (°C)	0.580	0.029	93.53
Temperature (°C), Dew point (°C)	0.549	0.012	92.52
Temperature (°C)	**0.490**	**0.005**	**92.23**

5.3.3.2 PM_{10} extracted from collected samples

The extraction process of PM_{10} samples resulted in a mean recovery of 80 % and 75 % for samples collected before and during Ramadan, respectively. No significant difference was found in the percentage of recovery for samples collected before Ramadan compared to during Ramadan (before Ramadan (M = 79.70 %, SD = 5.99) / during Ramadan (M = 75.26 %, SD = 9.47) conditions $t(6) = 0.770$, $p < 0.470$) using a paired samples t-test.

The total amount of extracted PM_{10} from samples collected before Ramadan was 548 mg compared to 704 mg during Ramadan. No significant difference was found in the amount of PM_{10} extracted from samples collected before Ramadan compared to during (before Ramadan (M = 78.29 mg, SD = 20.17) / during Ramadan (M = 100.57 mg, SD = 56.18) conditions t(6) = -0.95, $p < 0.378$) using a paired samples t-test.

Daily PM_{10} concentrations were found to be positively correlated with the amount of PM_{10} extracted from the samples collected (r = 0.9) (*Figure 5.11*).

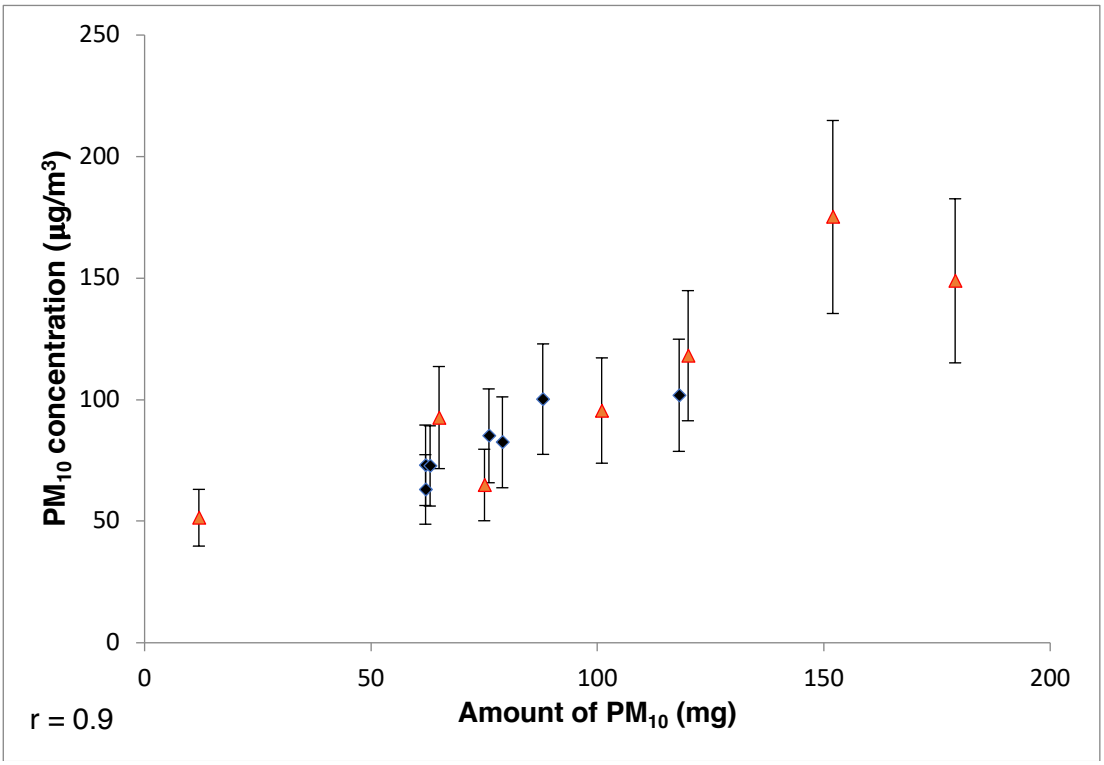

Figure 5.11, Correlation plot between daily PM_{10} concentrations calculated and the amount of PM_{10} extracted from samples collected from Makkah before (blue data points) and during (red data points) Ramadan (April / May – 2018) - (error bars represent the percentage of error associated with variations in the average flow rate of the sampler)

5.3.4 DNA damage induced by PM_{10} extracts

The addition of increasing amounts of PM_{10} extracts to a reaction mix containing pchAT plasmid DNA, H_2O_2 and Elution Buffer resulted in a dose-dependent increase in the proportion of damaged plasmid (*Figure 5.12*).

The extent of DNA damage (%) for samples collected on each day was compared for the assay involving 500 µg/ml of each sample extract. No significant difference in the percentage of DNA damage was found from samples collected in weekdays compared to weekends (weekdays (M = 53.34 %, SD = 2.81) / weekends (M = 37.91 %, SD = 9.01) conditions t(1) = 1.85, $p < 0.316$) nor from samples collected before Ramadan compared to during Ramadan (before Ramadan (M = 49.33 %, SD = 12.41) / during Ramadan (M = 48.53 %, SD = 23.75) conditions t(6) = 0.10, $p < 0.926$) using a paired samples t-test.

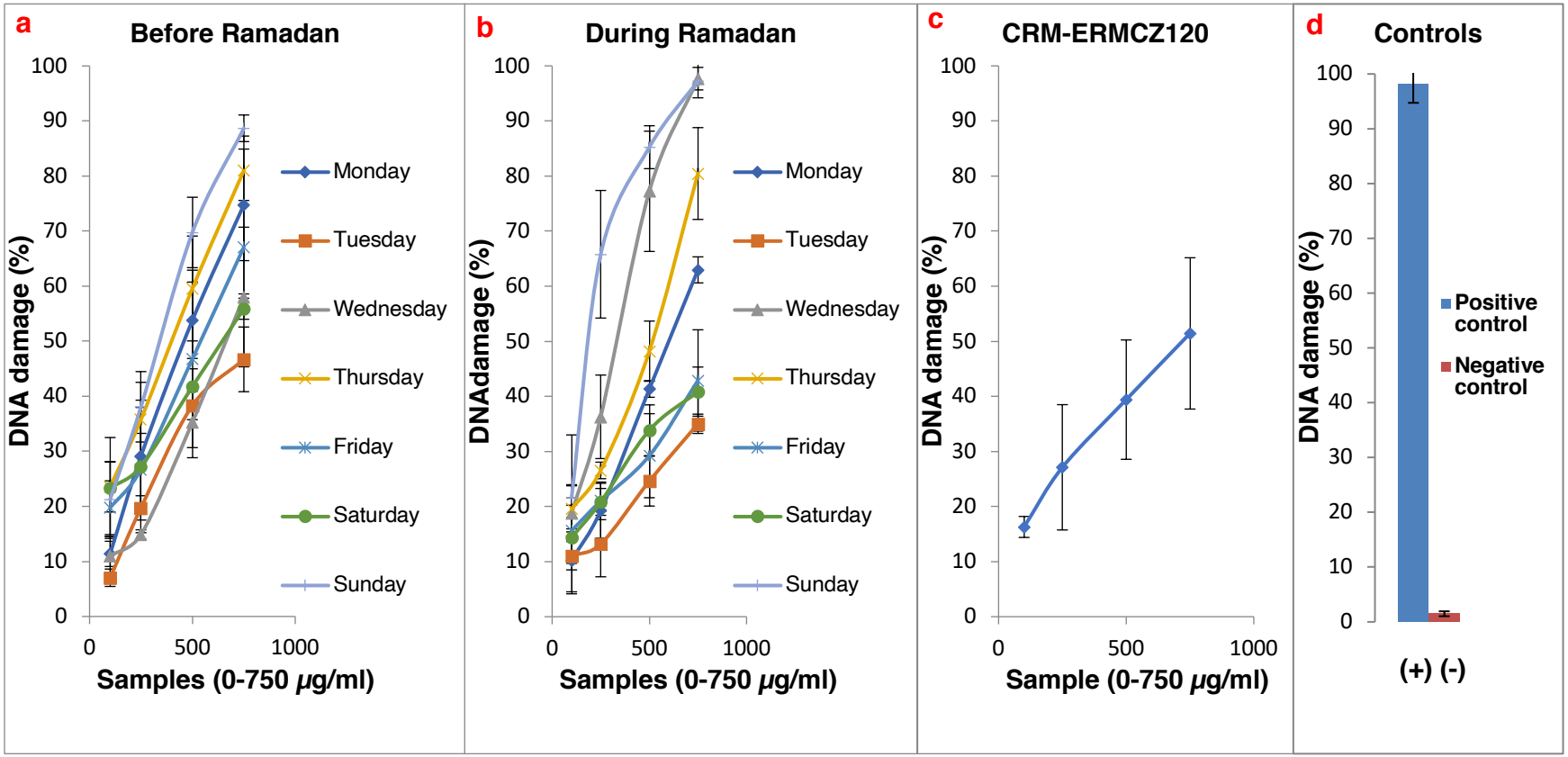

Figure 5.12, Plasmid Scission assay of Makkah PM_{10}, Dose dependent DNA strand breaks arising from samples (a) before Ramadan, (b) during Ramadan, (c) CRM-ERMCZ120, and (d) positive and negative controls

Plasmid DNA (5 ng) was diluted to 20 µl in an Elution Buffer (10 mM Tris-HCl pH 8.5) with different concentrations of PM_{10} (0-750 µg/ml) and $FeSO_4$ (25 µm) with H_2O_2 (3.5 mM). Samples were incubated for 5 hours at 37 °C. The reaction was stopped by adding loading buffer (Bioline 5X DNA loading buffer, blue). The whole reaction mixture was loaded to a 0.6 % TBE-agarose gel. Electrophoresis was conducted at 90 – 100 V for 45 min in 1x TBE buffer. The different forms of plasmid were visualised on a Typhoon 9200 variable mode imager. The intensity of the different forms of plasmid in each lane was analysed using ImageQuantTM (GE Healthcare Life Sciences). Three independent tests were carried out and the mean for each sample was calculated. Error bars represent the standard deviation (SD) of these three independent tests for each sample.

The percentage of DNA damage was found to have a negative correlation with daily concentrations of PM_{10} (r = -0.7) (*Figure 5.13*). Also, the percentage of DNA damage was found to have a positive and negative correlation with humidity (r = 0.7) and temperature (r = -0.5) respectively. Dew point, wind speed, wind direction, and pressure were found to have a weak relationship with the percentage of DNA damage (r < 0.5). (*Figure 5.14*).

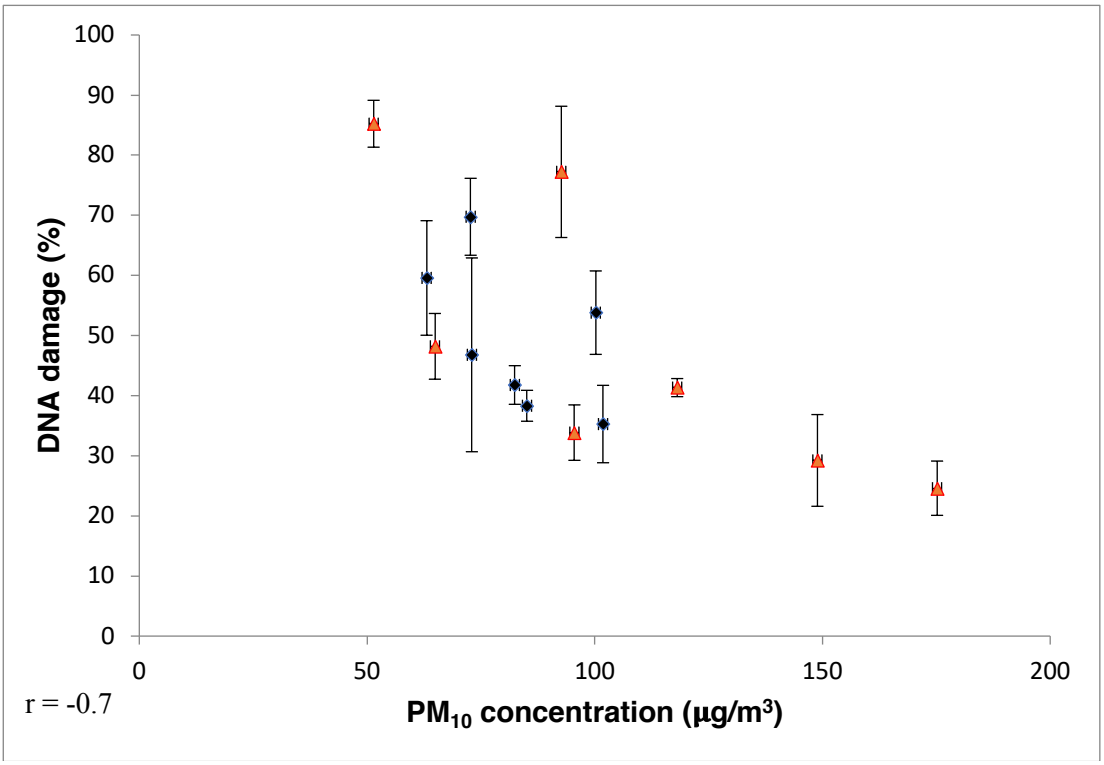

Figure 5.13, Correlation plot between DNA damage induced by Makkah PM_{10} and its daily concentrations before (blue data points) and during (red data points) Ramadan (r values represent Pearson's correlation coefficient - error bars represent the standard deviation (SD) of three independent tests for each sample)

In a multiple linear regression, several models were created for investigating the dependence of DNA damage on temperature, dew point, humidity, wind speed, gust, wind direction, and pressure (*Table 5.4*). Similar to regression for the dependence of the daily PM_{10} concentrations on individual meteorological parameters, models were

compared using the AIC and the model with the lowest value had humidity ($\beta = 0.907$, $p < 0.001$) and gust ($\beta = 0.,405$ $p < 0.056$) as predictors of DNA damage. These variables combined explained 68 % of the variance ($R^2 = 0.676$, $p < 0.002$). A simpler model that was much easier to interpret only had humidity as a significant predictor ($\beta = 0.736$, $p < 0.003$) of which it explained 54 % of the variance ($R^2 = 0.541$, $p < 0.003$).

Table 5.4, Regression models for DNA damage and individual meteorological parameters.

Model (DNA damage)	R Squared	Significance	Akaike Information Criterion
Pressure (millibar), Humidity (%), Wind Speed (m/s), Wind direction (°), Temperature (°C), Gust (m/s), Dew Point (°C)	0.72	0.180	78.48
Pressure (millibar), Humidity (%), Wind direction (°), Temperature (°C), Gust (m/s), Dew Point (°C)	0.72	0.091	76.51
Pressure (millibar), Humidity (%), Wind direction (°), Temperature (°C), Gust (m/s)	0.72	0.040	74.62
Pressure (millibar), Humidity (%), Wind direction (°), Gust (m/s)	0.71	0.016	72.85
Humidity (%), Wind direction (°), Gust (m/s)	0.69	0.007	71.88
Humidity (%), Gust (m/s)	**0.68**	**0.002**	**70.44**
Humidity (%)	**0.54**	**0.003**	**73.32**

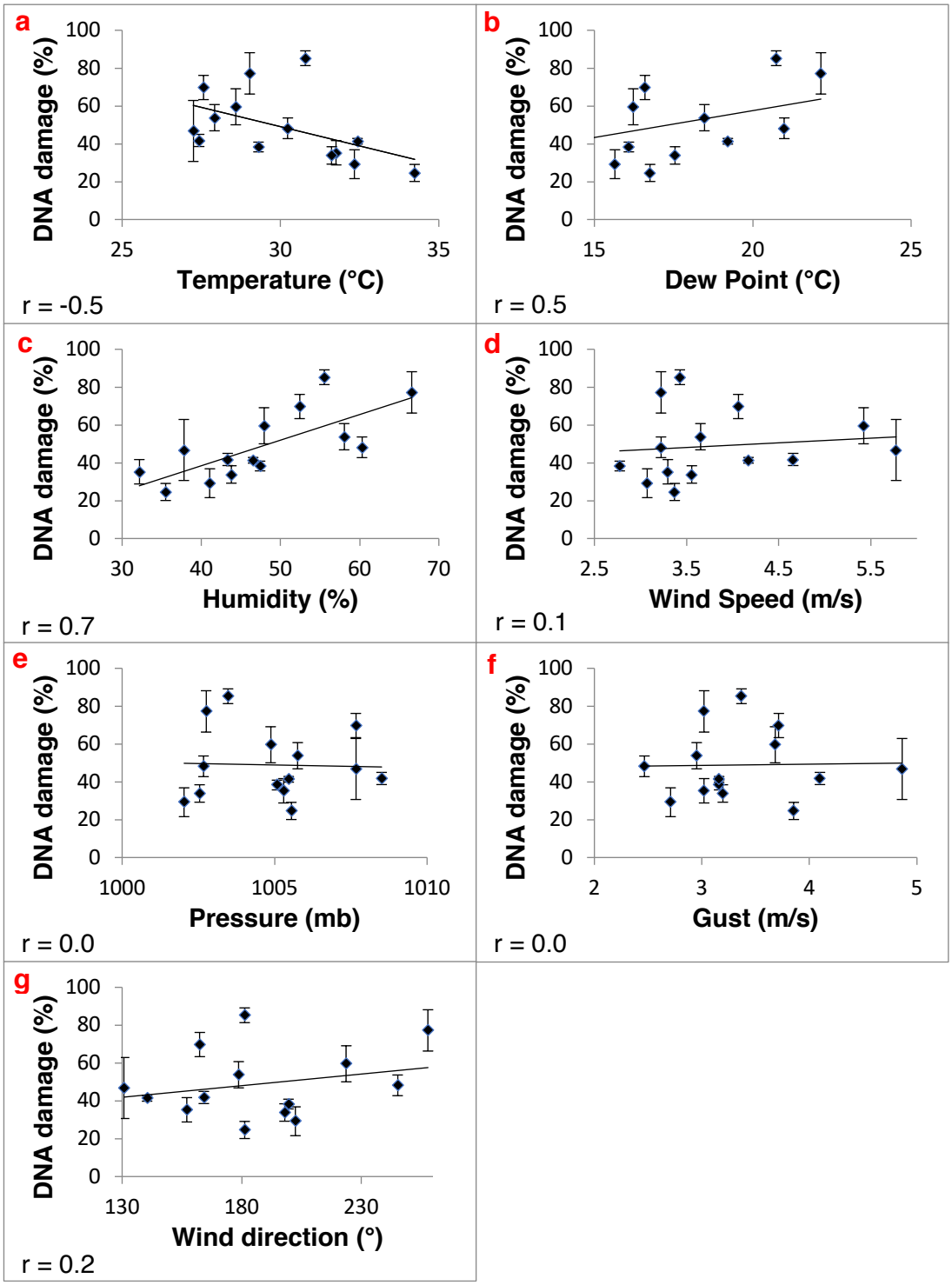

Figure 5.14, Correlation plots between DNA damage observed at 500 µg/ml of PM_{10} extracts from samples collected before and during Ramadan in a plasmid scission assay and; (a) temperature, (b) dew point, (c) humidity, (d) wind speed, (e) pressure, (f) gust, and (g) wind direction (r values represent Pearson's correlation coefficient - error bars represent the standard deviation (SD) of these three independent tests for each sample). DNA damage from this study. Meteorology parameters from Wunderground (2020) and timeanddate (2020).

5.4 Discussion

In this study, it has been determined that variations in daily PM_{10} concentrations in weekdays compared to weekends and before Ramadan compared to during are insignificant. PM_{10} concentrations seemed to vary as a function of meteorology, in general, and temperature, in particular. On the other hand, DNA damage incurred by extracts of the PM_{10}, which had insignificant differences in their recovery and were found to be highly correlated with daily PM_{10} concentrations suggesting that they were representative of what has been collected, seemed to vary as a function of humidity. Information obtained from the aforementioned explorations doesn't depict an overall picture of the driving force behind differences in PM_{10} concentration, composition, and toxic effects. To better understand that, consideration must be given to processes, whether anthropogenic (e.g. traffic) and/or natural (e.g. weather), that could have affected PM_{10} during the days of sample collections and eventually led to variations in toxic effects.

In terms of anthropogenic processes, it is speculated that traffic related pollutants (e.g. diesel particles and NO_X gases) could have impacted the ratio between natural and anthropogenic particles in an area that is otherwise known to be mainly affected by particles of natural origin (e.g. dust and sand particles) due to its arid nature and geographical location that is surrounded by sandy deserts (Habeebullah 2016; Habeebullah 2013a; Munir et al. 2017b; Nayebare et al. 2018).

As for natural processes, there are several ways that PM_{10} concentration and composition could have been affected by weather conditions and it all depends on the state of individual meteorological parameters and the interactions that could have occurred between them during the days of sample collection. We expect that at

conditions of high temperature, low humidity, and irrespective of wind direction, PM_{10} concentrations are more likely to increase and its composition is most likely to be dominated by dust and sand particles originating from local and/or regional deserts (European Environment Agency 2012; Bolles et al. 2019; Bourotte et al. 2011). Also, we expect that at opposite conditions of low temperature, high humidity, and winds blowing from the Red Sea (i.e. west, south-west, and north-west), PM_{10} concentrations are more likely to decrease and its composition is most likely to be dominated by secondary particles, formed through the conversion of anthropogenically emitted gases into particles, and bioaerosols, particles from microbial, plant, and animal origin (Shao and Mao 2015; Cholakian et al. 2019; Hernandez et al. 2017; Jones and Harrison 2004; Rogoff 2014; Qiu et al. 2019; Huffman et al. 2013).

The effect of weather conditions on PM_{10} is not just limited to the interactions listed above but is also evident in atmospheric phenomena such as thermal inversions. Normally, temperatures are warmer near the surface and gets colder as altitude increases. However, cold air can settle at the ground and the warmer air is actually above the surface layered between cooler air both above and below. When this situation occurs, it's called an inversion (Whiteman 2000; Stryhal et al. 2017; Czarnecka et al. 2016; Czarnecka et al. 2019). The importance of inversions in relation to PM_{10} is that the warmer air layer acts as a lid trapping PM_{10} underneath (Wagner and Brandley 2020). Several studies have reported that episodes of PM pollution occur frequently during inversions (Kukkonen et al. 2005; Janhäll et al. 2006; Vecchi et al. 2007; Silva et al. 2007; Olofson et al. 2009; Guzmán-Torres et al. 2009; Zhao et al. 2019; Wagner and Brandley 2020).

The impact of each of the previously mentioned processes on PM_{10} concentration and composition will be investigated in detail to reach a better understanding of the type of differences that occurred and eventually led to variations in its toxic effects.

5.4.1 Impact of traffic related pollutants on PM_{10}

Significant differences between PM_{10} concentrations in weekdays and weekends serve as an indication of the level of contribution of traffic related pollutants to the overall PM_{10} mixture since generally, PM_{10} concentrations tend to b higher in weekdays compared to weekends mainly due to higher traffic volumes during the week (Alharbi et al. 2015; Ancelet et al. 2014). In Makkah, one study (Munir et al. 2017b) reported PM_{10} concentrations being lower on Friday and Saturday, which represent the weekend in Saudi Arabia unlike many other countries around the world where the weekend is Saturday and Sunday, while other studies (Habeebullah 2013a; Habeebullah 2016) reported the opposite where Friday and Saturday had the highest levels of PM_{10}. In our study, we found that PM_{10} concentrations in weekdays did not differ significantly from those found in weekends. That was in agreement with findings obtained from comparing daily concentrations of PM_{10}, NO_2, SO_2, CO, and O_3 from 2019 / 2020 during weekends and weekdays. The inconsistency in findings could be attributed to differences in the sampling equipment used, the sampling location, the sampling period, and the state of individual meteorological parameters during the days of sample collection.

Another possible indicator of the level of contribution of traffic related pollutants to PM_{10} is significant correlations between PM_{10} and NO_2, SO_2, CO, and O_3. Usually, positive correlations between PM_{10} and gaseous pollutants suggest that they enhance

its abundance (Mao et al. 2018). No such correlation was found between daily concentrations of PM_{10} and NO_2, SO_2, CO, and O_3 from 2019 / 2020.

The findings of our study suggest that PM_{10} concentrations and compositions in Makkah are mainly affected by particles of natural origin (i.e. dust and sand particles) rather than traffic related pollutants (i.e. carbonaceous soot, metals, and secondary aerosols formed by chemical reactions with gases such as NO_2, SO_2, CO, and O_3) which is consistent with the overall consensus regarding the main contributors to PM_{10} abundance in the city (Habeebullah 2016; Habeebullah 2013a; Munir et al. 2017b; Nayebare et al. 2018).

5.4.2 Impact of weather conditions on PM_{10}

Certain weather conditions serve as an indication of the type and source of particles mainly affecting PM_{10} concentrations and compositions. As shown in the results section, daily PM_{10} concentrations increased under conditions of high temperature. It has been reported previously that during dust events, generally, temperature levels are high (European Environment Agency 2012; Bolles et al. 2019). However, that cannot be applied everywhere since dust activity differs from one place to the other (Rezazadeh et al. 2013). Nevertheless, a study by Albugami et al. (2019) found that the relationship between high temperatures and dust storms in Saudi Arabia was insignificant for all regions except the western coast, which is where Makkah is located. (Albugami et al. 2019). This serve as an indication that at high temperature, re-suspended and windblown desert dust and sand particles are the most significant contributors to PM_{10} concentrations in Makkah.

The results of the regression further confirm our hypothesis regarding the controlling factors of PM_{10} concentrations and composition in Makkah. Temperature was found to be a significant predictor for daily PM_{10} concentrations. It is hypothesised that the concentrations increased due to significant contributions from particles of natural origin (i.e. dust and sand particles) due to the high temperature levels that were suitable for their formations (European Environment Agency 2012; Bolles et al. 2019; Bourotte et al. 2011) compared to secondary particles of anthropogenic origin formed by reactions with NO_2, SO_2, O_3, and CO due to the lack of proper conditions suitable for their formation (Shao and Mao 2015; Cholakian et al. 2019; Hernandez et al. 2017).

The relationships between the daily PM_{10} concentrations and individual meteorological parameters observed in our data set couldn't be found in daily concentrations of PM_{10} from 2019 / 2020. We suspect that this is mainly because it's much easier to find relationships between PM_{10} and individual meteorological parameters when the data collected is for a short period of time with the weather regime being the same throughout rather than an extended period of time with different weather regimes.

5.4.3 Impact of thermal inversions on PM_{10}

Although thermal inversion might take place in other times of year in Makkah, there is no strong evidence that thermal inversion took place during the period of sample collection or otherwise we would've observed a high contribution from traffic related pollutants to the daily concentrations of PM_{10} irrespective of high or low traffic days. There is no study that either confirms or disproves the occurrence of thermal inversions in Makkah. The possibility of their occurrence has been suggested due to

the high levels of NO_X (Seroji 2010) and heavy metals found in the city (Adly et al. 2017; Adly et al. 2019). Also, their occurrence is possible due to Makkah's location, in a valley surrounded by mountains, which makes it susceptible to such phenomena (Allaby 2014; Seroji 2011). Previous studies have reported that urbanised or city valleys such as Cache Valley (Logan, Utah, USA) (Silva et al. 2007; Malek et al. 2006) and Alpine valleys in the area of Grenoble (Largeron and Staquet 2016) do experience thermal inversions frequently. These inversions could occur when cold air transported by downslope breezes accumulates at the bottom of the valley during the night and then sealed off from above by a layer of warm air (Miró et al. 2020; Feng et al. 2020).

5.4.4 DNA damage and its association with PM_{10} and meteorology

Although it has been established (sections *3.4.1*, *3.4.2*, and *3.4.3*) that traffic related pollutants are not significant contributors to PM_{10} abundance in Makkah while re-suspended and windblown desert dust and sand particles are, our findings suggest that traffic related pollutants are nevertheless controlling factors for toxicity while the latter aren't – indeed DNA damage's independence on re-suspended and windblown desert dust and sand particles is indicated in the negative correlation found between the daily PM_{10} concentrations and DNA damage. DNA damage's dependence on traffic related pollutants and bioaerosols is indicated in its correlation with weather conditions (positively with humidity and negatively with temperature) that are; (i) typical of chemical reactions changing the nature of PM_{10} compositions, in particular the conversion of anthropogenically-sourced and generally more hazardous gaseous emissions into particles (Cholakian et al. 2019; Hernandez et al. 2017); and (ii) favourable for inducing certain emission mechanisms of some biological particles (e.g. active squirting of fungal spores) (Huffman et al. 2013; Qiu et al. 2019) and

suitable for the survival of others (e.g. airborne bacteria and viruses) (Islam et al. 2019).

The results of the regression further confirm our hypothesis regarding the controlling factors of DNA damage. Humidity was found to be a significant predictor for the damage observed. It is hypothesised that high humidity levels, caused by low temperature levels and winds blowing from the west and south-west (i.e. from the Red sea), during certain days of sample collection enabled the conversion of gaseous emissions into secondary particles via condensation and/or nucleation (Clement and Ford 1999). Also, the aforementioned weather conditions could have allowed for the emission, survival, and attachment of bioaerosols, whether bacteria, fungal spores, and/or viruses to primary and/or secondary PM_{10} particles (Lighthart 1997; Meklin et al. 2002; Alghamdi et al. 2014). After the conversion of gaseous emissions into secondary particles and the attachment of bioaerosols to primary and/or secondary PM_{10} particles, particles could have either been scavenged by clouds and removed from the atmosphere in a process called wet deposition (i.e. removal of particles by rain drops as they fall) or fall from the atmosphere by gravity in a process called dry deposition (i.e. via non-precipitation) (Williams et al. 2002; Pöschl 2005; Dolske and Gatz 1985; Chate et al. 2003; Wu et al. 2018). The lack of rain during the days of sample collection suggests that particles were deposited via dry deposition and it is possible that as particles were settling, the sampler was able to collect more toxic material containing biological material and/or anthropogenically sourced emissions usually suspended in the atmosphere. Thus, high levels of DNA damage correlated positively with high levels of humidity.

To sum up, it is noted that, due to the low amount of material obtained, the lack of a complete physicochemical and mineralogical analysis of the PM_{10} samples collected means that the DNA damage observed couldn't be uniquely ascribed to a specific component or group of components from the overall PM_{10} mixtures and this represents a fundamental limitation of toxicological studies involving PM_{10} samples collected via certain PM_{10} samplers. Moreover, it is noted that findings from the bivariate correlation analysis and linear regression cannot be used to confirm the presence of a cause and effect relationship as its not certain that the weather conditions highlighted were responsible for the changes in PM_{10} concentrations and compositions and hence toxic effects observed. However, findings from such analysis were extremely useful in highlighting the possible presence or absence of a relationship between the aforementioned endpoints and meteorology and thus, helpful in predicting the type and source of particles contributing to overall PM_{10} abundance and toxic potential. Keeping that in mind, by investigating the different natural and anthropogenic processes and their effect on PM_{10} concentrations and composition, we found that in Makkah, the impact of traffic related pollutants on PM_{10} is insignificant. This is supported by; (i) the lack of a difference in PM_{10} concentrations on weekdays compared to weekends and in PM_{10} concentrations before Ramadan compared to during; and (ii) the lack of a difference in DNA damage induced by PM_{10} extracts from samples collected on weekdays compared to weekends and by PM_{10} extracts from samples collected before Ramadan compared to during. In contrast, we found that weather in general significantly impacts not only PM_{10} concentrations but also toxicity and hence composition. Significant increases in PM_{10} concentrations were found to be associated with high temperatures, which are typical conditions for the formation of re-suspended and windblown desert dust and sand particles. Significant

increases in toxicity were found to be associated with PM_{10} samples collected during days of high humidity, which are typical conditions for the formation of secondary particles from anthropogenically sourced gases and the presence of biological material attached to primary and/or secondary PM_{10}. Other than that, by investigating the toxic effects of a widely available and reasonably priced PM_{10} like CRM-ERMZC120, we have provided a common reference that can be used for comparative purposes for researchers interested in examining the toxic effects of PM.

5.5 Conclusion

DNA damage in a plasmid scission assay was caused by PM_{10} collected from Makkah, Saudi Arabia during April - May 2018. A significant association was observed for the extent of PM_{10} induced DNA damage with meteorological parameters – notably higher DNA damage was observed from PM_{10} collected on lower temperature, higher humidity days when the predominant wind direction was from the west (i.e. from the Red Sea) compared to those collected on higher temperature, lower humidity days irrespective of the wind direction. The analysis of the interactions between DNA damage and meteorological parameters revealed that secondary particles of anthropogenic origin and bioaerosols were most likely responsible for the damage. Our findings confirm the complexity of the relationship between meteorology and PM_{10} chemical composition and physical structure as the interactions between individual meteorological parameters and PM_{10} can change the state of its mixture from safe to hazardous. Further work is required to characterise the nature of the inferred compositional differences, the mechanism of DNA damage formation and to determine to what extent these cell-free studies correlate with cellular studies.

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Chapter 6: The cytotoxic and genotoxic effects of PM_{10} from Makkah, Saudi Arabia on A549 lung cells: A comparison with a cell-free assay

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Abstract

Exposure to PM_{10} has been linked with a variety of adverse genotoxic and cytotoxic effects. This study aimed to compare previously observed effects of PM_{10} samples collected before and during Ramadan in April / May - 2018 from Makkah, Saudi Arabia on DNA strand breaks in a cell-free Plasmid Scission assay with its effects on DNA damage and cell viability in cell-based Neutral Comet and MTT assays respectively. It was found that the effects of PM_{10} in the aforementioned assays differed significantly depending on the endpoint investigated; (i) DNA strand breaks ranged from 35 - 85 % with significant variations found between the effects of all samples using a One-way analysis of variance ($p < 0.001$) but no significant difference found between the effects of samples collected before Ramadan compared to during using a paired samples t-test ($p < 0.926$); (ii) DNA damage ranged from 28 - 59 % with significant variations found between the effects of all samples using a One-way analysis of variance ($p < 0.001$) and a significant difference found between the effects of samples collected before Ramadan compared to during using a paired samples t-test ($p < 0.005$); and (iii) cell viability ranged from 55 – 64 % with no significant variations found between the effects of all samples using a One-way analysis of variance ($p < 0.723$) and no significant difference found between the effects of samples collected before Ramadan compared to during using a paired samples t-test ($p < 0.507$). No significant correlation was found between effects observed in cell-based Neutral Comet and MTT assays ($r = 0.2$, $p < 0.492$) nor between effects observed in a cell-free Plasmid Scission assay and cell-based assays, MTT ($r = 0.123$, $p < 0.674$) and Neutral Comet ($r = -0.378$, $p < 0.183$) using bivariate correlation analysis. The difference in effects and lack of a correlation was most probably due to the exposure process being completely different between cell-based and cell-free assay. Further

work is required to investigate the physicochemical properties of PM_{10} from Makkah and their relationship with toxic effects found in cell-free and/or cell-based assays. Nevertheless, this study highlights the toxic effects of PM_{10} on A549 lung cells and how it differs from toxic effects observed on an isolated cellular component such as DNA.

6.1 Introduction

The association between PM and adverse health effects in humans have been well documented in both epidemiological (Harrison and Yin 2004; Dockery et al. 1993; Pope et al. 1995; Abbey et al. 1999; Schwartz et al. 1999; Schwartz and Neas 2000; Achilleos et al. 2019; Lelieveld et al. 2015; Ayres 2010; Pope et al. 2014; Perez et al. 2012; Guaita et al. 2011) and toxicological studies (van Berlo et al. 2012; Ghio et al. 2012; de Oliveira Alves et al. 2014; de Oliveira Galvão et al. 2017; Dumax-Vorzet et al. 2015). Unfortunately, even though the association has been scientifically documented, the numbers and forms of air pollution sources are still increasing especially in developing countries. That is certainly the case for Saudi Arabia as the country is still suffering from high air pollution levels. The increase in air pollution levels there is a result of rapid developments in the economy, industry, construction, and urbanization (Nayebare et al. 2016). Moreover, levels that are higher than usual in cities such as Makkah can also result from religious activities such as the Umrah. The Umrah is a religious duty for all Muslims and takes place all year round. In 2018, the total number of Umrah performers for the month of Ramadan only was around 7.8 million (GASTAT 2018).

In Makkah, several studies have investigated PM concentration and characteristics in different seasons including Ramadan in the past (Munir et al. 2013a; Munir et al. 2013b; Habeebullah 2014, 2016; Shaltout et al. 2013; Nasralla and Seroji 2008; Habeebullah 2013b; Seroji 2011). Seroji (2011), found that just in the last ten days of Ramadan, PM_{10} daily mean concentrations exceeded 250 $\mu g/m^3$ (Seroji 2011) compared to 56 – 196 $\mu g/m^3$ in other months (Habeebullah 2014, 2016). The author hypothesised that the increase in PM concentration could be attributed to the high

activities of visitors in the city. That could have been the case in that particular year, however, it is important to note that PM in Makkah is mostly dominated by re-suspended and windblown dust and sand particles rather than traffic related pollutants (Habeebullah 2016; Habeebullah 2013a; Munir et al. 2017; Nayebare et al. 2018). That must be taken into consideration when examining the toxic effects of PM from Makkah.

More is still needed to investigate the cytotoxic and genotoxic effects of PM from Makkah. To the best of our knowledge, only one study (El-Assouli 2011) has investigated PM toxicity and found that the extractable organic matter from PM was mutagenic in the cell-free *salmonella* TA98 test and damaged DNA in the cell-based comet assay. The authors of the study did not use the entire PM mixture and did not compare the effects found in the two assays.

Cell-free assays allow for investigating reactions that might occur within cells between PM and isolated cellular components (e.g. DNA) but without the complete cell system, thus, reducing the amount of complex interactions (e.g. particles getting stuck in cytoplasmic vacuoles and not reaching the cellular component under investigation) that are associated with working with whole cells (Swartz 2006; Li et al. 2003; Dumax Vorzet 2010). However, in terms of actual exposure, they might not be representative given the complexity of the exposure process and the factors involved in protecting important cellular components from damage leading to false positive results due to the lack of protective mechanisms. That is the advantage of using cell-based assays as they allow for investigating reactions occurring between PM and the various components of living cells. Therefore, to better asses the toxic effects of PM, it is

recommended to compare results obtained from cell-free and cell-based assays (Ayres et al. 2008).

Given the lack of data on PM toxicity from Makkah in general, and the difference in its effects in cell-based assays compared to cell-free assays in particular, the goal of this study was to contribute to existing knowledge of PM from Makkah and elsewhere by further investigating its toxic and address gaps in knowledge. To reach this goal, the study's aim was to compare previously observed effects of PM_{10} samples collected before and during Ramadan in April / May - 2018 from Makkah, Saudi Arabia on DNA strand breaks in a cell-free Plasmid Scission assay ((Badri et al. 2020c) – *Chapter 5*, this volume) with its effects on DNA damage and cell viability in cell-based Neutral Comet and MTT assays respectively.

6.2 Materials and methods

Detailed descriptions of the study area, sample collection, weather conditions during the days of sampling, sample extraction, and the effect of PM_{10} extracts on DNA strand breaks in a cell-free Plasmid Scission assay have been presented previously in (Badri et al. 2020c) *Chapter 5*, this volume. In brief, samples were collected from Azizyah, Makkah, Saudi Arabia before and during Ramadan (April / May – 2018) using a High-Volume Sampler equipped with a PM_{10} size selective inlet on Polytetrafluoroethylene (PTFE) filters. Also, a Certified Reference Material (CRM), ERMCZ120 - Fine dust (PM10-like) - trace elements, was purchased as a reference for subsequent DNA strand breaks measurements. Weather data for Makkah during the sample collections days were obtained from www.wunderground.com and www.timeanddate.com. Samples of PM_{10} were extracted using a method adapted from previous publications with minor adjustments (Dumax Vorzet 2010; Dumax-Vorzet et al. 2015). pchAT Plasmid DNA

was exposed to different levels of PM_{10} extracts and H_2O_2 in an Elution Buffer and incubated for 5 hours at 37 ^oC. The reaction was stopped by adding loading buffer and the whole mixture was loaded to TBE-agarose gel. Electrophoresis was conducted in TBE buffer and the different forms of plasmid were visualised on a variable mode imager. The intensity of the different forms of plasmid in each lane was analysed and the level of damaged plasmid in each sample was calculated.

Detailed descriptions of the toxicological techniques utilised for the assessment of PM_{10} genotoxic and cytotoxic effects have been presented previously in (Badri et al. 2020b) *Chapter 4*, this volume. In brief, the effects of PM_{10} on DNA damage and cell viability were investigated using the Neutral Comet and MTT assays respectively. In the Neutral Comet assay, A549 lung cells were exposed to increasing concentration of PM_{10} and incubated for 3 hours at 37 °C. After incubation, cells were detached, resuspended in low gelling temperature agarose, and dispensed on a normal gelling temperature agarose coated microscopic slide. Then, cells were lysed in lysis buffer, DNA unwounded in electrophoresis buffer, stained with a fluorescent stain, and analysed using a fluorescent microscope. In the MTT assay, A549 lung cells were exposed to increasing concentration of PM_{10} and incubated for 48 hours at 37 °C. After that the MTT solution was added and cells were further incubated for 4 hours at 37 °C. Then DMSO was added and cells incubated again for 10 mins at 37 °C. Absorbance was read at 595 nm using MTT program of TECAN GENios plate reader.

Data obtained from each MTT and comet assay were described using the mean and standard deviation. A One-way analysis of variance was used to check for significant variations between the effects of the samples used in each assay. A paired samples t-test was used to check for significant differences between the effects of samples

collected before Ramadan compared to during. Pearson's correlation was used for to check for correlations between the effects observed in each assay. Statistical analyses were performed using SPSS Statistics version 22. Graphs and scatterplots were created using Microsoft Excel 2010.

6.3 Results

6.3.1 Plasmid Scission assay

As previously reported in (Badri et al. 2020c) *Chapter 5*, this volume, at 500 µg/ml, DNA damage caused by the samples collected varied from 35 - 85 % with the CRM-ERMCZ120 causing around 39 %. The presence of a significant variation between the effects of all samples was further confirmed using a One-way analysis of variance ($F(13, 28) = 18.22$, $p < 0.001$). However, the effect of samples collected before Ramadan compared to during did not differ significantly as found using a paired samples t-test (before Ramadan (M = 49.33 %, SD = 12.41) / during Ramadan (M = 48.53 %, SD = 23.75) conditions $t(6) = 0.10$, $p < 0.926$). DNA damage was found to be: (i) correlated negatively with daily PM_{10} concentrations which itself was found to be correlated with weather conditions typical of dust events indicating that the main contributor to the samples was re-suspended and windblown dust and sand particles from nearby deserts; (ii) correlated positively with humidity and negatively with temperature which are conditions typical of chemical reaction involved in converting gaseous emissions from anthropogenic sources into particles. These correlations suggest that the main determinants of the DNA damage observed were most likely particles originating from anthropogenic sources (Badri et al. 2020c).

6.3.2 Neutral Comet assay

The addition of different samples to the cells resulted in a dose-dependent increase in the proportion of damaged DNA as shown in *Figure 6.1*.

At 50 μg/ml, DNA damage caused by the samples collected varied from 24 – 48 % with the CRM-ERMCZ120 causing around 43 %. The presence of a significant variation between the effects of all samples was further confirmed using a One-way analysis of variance ($F(14, 15) = 14.44$, $p < 0.001$). Also, the effect of samples collected before Ramadan compared to during differed significantly as well as found using a paired samples t-test (before Ramadan (M = 40.60 %, SD = 4.99) / during Ramadan (M = 32.68 %, SD = 3.94) conditions $t(6) = 4.08$, $p < 0.007$). No significant correlation was found between DNA damage and daily PM_{10} concentrations nor between DNA damage and individual meteorological parameters ($r < 0.5$).

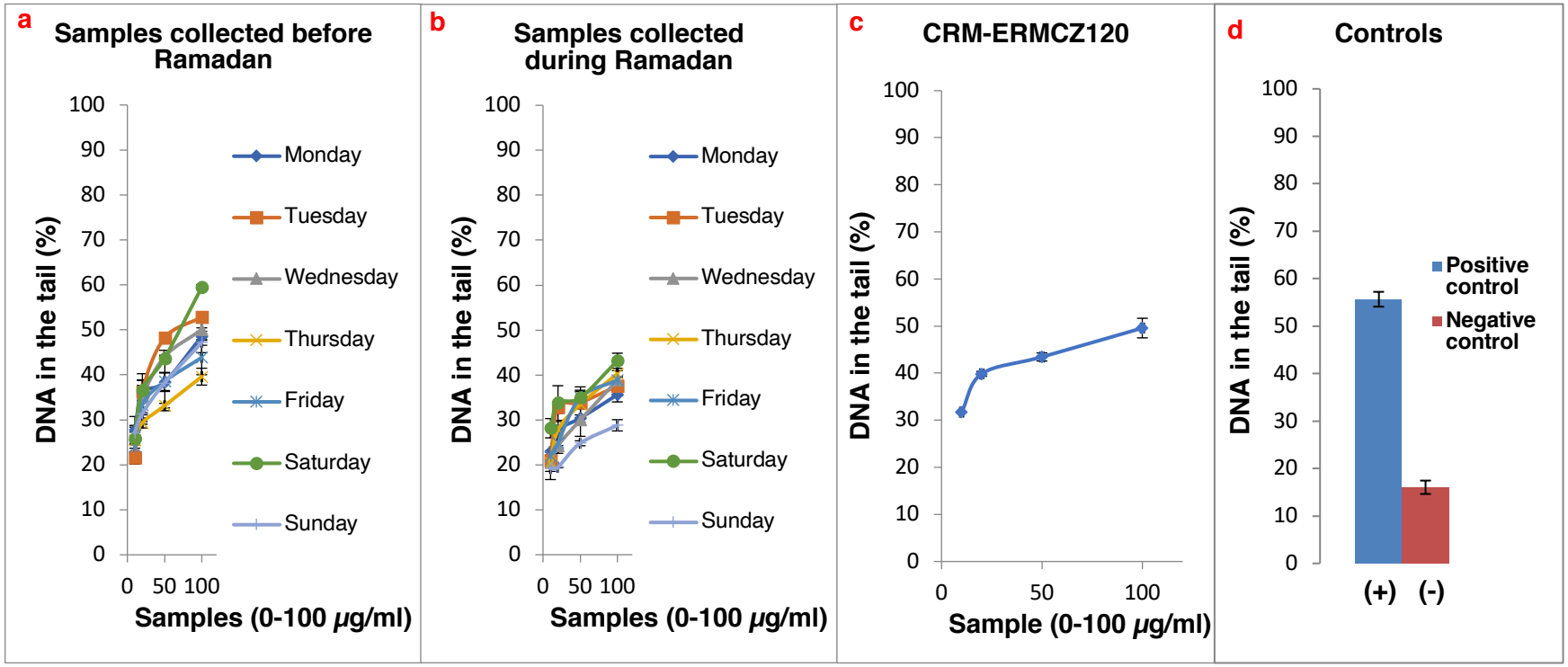

Figure 6.1, Neutral Comet assay of Makkah PM_{10}. Dose dependent DNA damage arising from; samples collected (a) before Ramadan; (b) during Ramadan and (c) from a CRM-ERMCZ120, and (d) positive (H_2O_2) and negative (untreated cells) controls

Cells were prepared at a concentration of 5000 cells/500 µL media and add to a 24 well plate and allowed to settle for 24 hrs. On the second day, the medium was replaced with medium containing increasing concentrations of PM_{10} (0 - 100 µg/ml) and H_2O_2 (100 µM) and incubated for 3 hours in the cell incubator. After that, the medium was discarded and cells detached using trypsinization. Cells were then resuspended in 0.7 % LMP and subsequently dispensed on agarose-coated glass microscope slides and kept at 4 °C for 10 mins. Cells were lysed for 90 mins at 4 °C in lysis buffer and DNA was unwound for 20 min in electrophoresis buffer (pH > 9) prior to electrophoresis for 30 min at 25 V/300 mA. Following neutralisation, the slides were stained with SYBR gold in the dark. Dry slides were stored in the dark until image capture using a fluorescent microscope and analysis with CometScore. Results represent the mean and standard deviation (SD) of two experiments for PM_{10} and six experiments for control.

6.3.3 MTT assay

The addition of different samples to the cells resulted in a dose-dependent decrease in cell viability as shown in *Figure 6.2*.

At 50 µg/ml, all samples collected before and during Ramadan and the CRM-ERMCZ120 had relatively the same effect on cell viability (~ 60 %). The lack of a significant variation in effects between all samples was further confirmed using a One-way analysis of variance ($F(14, 30) = 0.74$, $p < 0.723$). Also, the effect of samples collected before Ramadan compared to during did not differ significantly as well as found using a paired samples t-test (before Ramadan ($M = 59.16$ %, $SD = 1.74$) / during Ramadan ($M = 5.82$ %, $SD = 3.88$) conditions $t(6) = 0.71$, $p < 0.507$). No significant correlation was found between cell viability and daily PM_{10} concentrations nor between cell viability and individual meteorological parameters ($r < 0.5$).

6.3.4 Relationship between cell-based and cell-free assays

Using bivariate correlation analysis, no significant correlation was found between DNA strand breaks in the cell-free Plasmid Scission assay, DNA damage in the cell-based Neutral Comet assay, and cell viability in the cell-based MTT assay *(Table 6.1 – Figure 6.3)*.

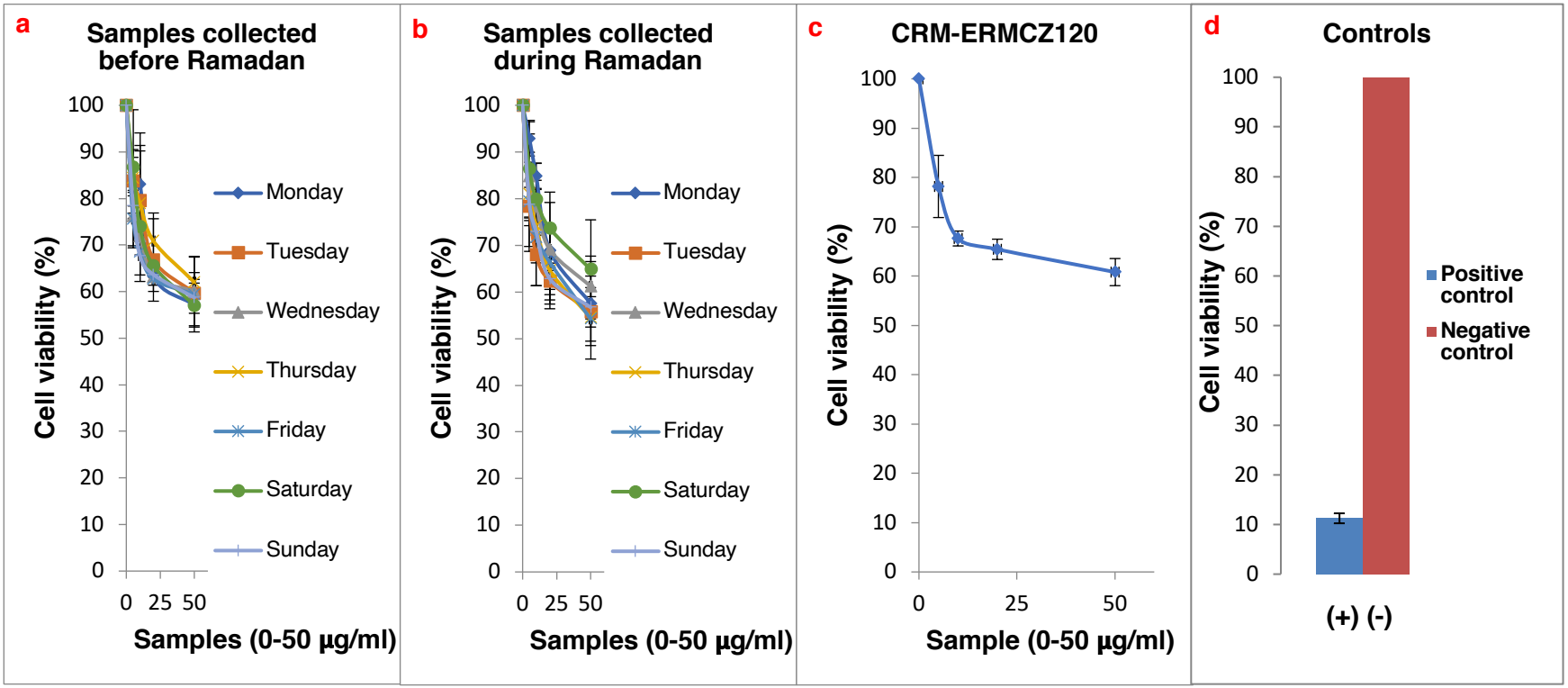

Figure 6.2, MTT assay of Makkah PM_{10}. Cytotoxic effects arising from samples collected (a) before Ramadan; (b) during Ramadan and (c) from a CRM ERMCZ120), and (d) positive (H_2O_2) and negative (untreated cells) controls

A549 cells were seeded in a 96-well plate at a density of 5,000 cells/well/100 µL MEM culture medium and incubated at 37 ºC, 5 % CO_2 and 3 % O_2 for 24 hrs. The following day, the medium was replaced with medium containing increasing concentration of PM_{10} (0 – 50 µg/ml) and H_2O_2 (100 µM) and incubated for 48 hours. Then, the medium was removed and replaced with 100 µL of fresh medium and 10 µL of MTT solution previously prepared and incubated at 37 ºC for 4 hrs. After that, 85 µL was removed from the medium and 50 µL of DMSO was added to each well and mixed and incubated for 10 min at 37 ºC. Absorbance was read at 595 nm. The percentage of cell viability was calculated as shown in Equation 1. Results represent the mean and standard deviation (SD) of three experiments.

Table 6.1, Correlations between assays used for the assessment of PM_{10} toxic effects

		Plasmid Scission assay[1]	Neutral Comet assay[2]	MTT assay[3]
Plasmid Scission assay[1]	Pearson Correlation	1	-0.378	0.123
	Significance. (2-tailed)		0.183	0.674
Neutral Comet assay[2]	Pearson Correlation	-0.378	1	0.2
	Significance. (2-tailed)	0.183		0.492
MTT assay[3]	Pearson Correlation	0.123	0.2	1
	Significance. (2-tailed)	0.674	0.492	

[1]The percentage of DNA strand breaks caused by 500 µg/ml of PM_{10} extracts
[2]The percentage of DNA damage caused by 50 µg/ml of PM_{10} extracts
[3]The percentage of cell viability caused by 50 µg/ml of PM_{10} extracts

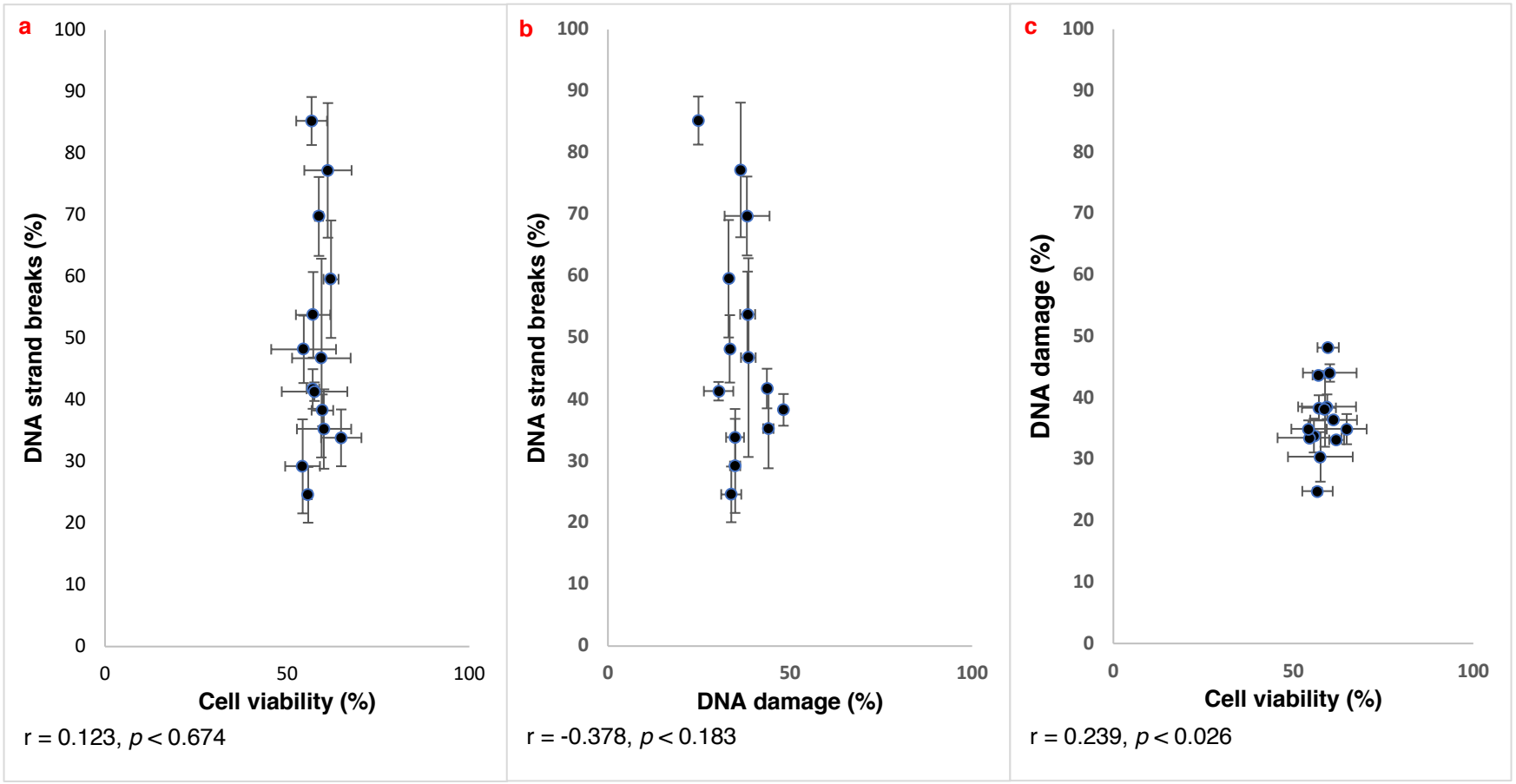

Figure 6.3, Correlation plots of Makkah PM_{10} induced effects; (a) DNA strand breaks in the cell-free Plasmid Scission assay and cell viability in the cell-based MTT assay, (b) DNA strand breaks in the cell-free Plasmid Scission assay and DNA damage in the cell-based Neutral Comet assay, (c) DNA damage in the cell-based Neutral Comet assay and cell viability in the cell-based MTT assay

6.4 Discussion

PM_{10} extracts from samples collected before and during Ramadan from Makkah, Saudi Arabia were able to induce toxic effects in cell-free and cell-based assays. However, there were significant differences in the effects observed depending on the endpoint investigated; (i) in a cell-free Plasmid Scission assay, effects on DNA varied significantly between all samples but were not significantly different between samples collected before Ramadan compared to during; (ii) in a cell-based Neutral Comet assay, effects on DNA varied significantly between all samples and were significantly different between samples collected before Ramadan compared to during; and (iii) effects on cell viability did not vary significantly between all samples and were not significantly different between samples collected before Ramadan compared to during. Hence, no significant correlation was found between the effects observed in both cell-based and cell-free assays. The difference in effects and lack of a correlation could be due to: (i) differences in the endpoints under investigation as each assay measures a different thing; and/or (ii) differences in the exposure process of each assay.

To reach a better understanding of PM_{10} toxic effects, differences found in the effects observed from daily PM_{10} samples and/or from the two periods of PM_{10} samples collection as well as differences in correlations with daily PM_{10} concentrations and individual meteorological parameters in all assays will be further discussed. Also, the differences in exposure processes for cell-based and cell-free assays and how they might've impacted toxicity will be further explored.

6.4.1 A comparison of differences in results between cell-based and cell-free assays

Similar to DNA damage in the cell-free Plasmid Scission assay, DNA damage in the cell-based Neutral Comet assay showed significant variations among daily samples. The differences in DNA damage among daily samples in both assays suggest that the physical and chemical composition of the samples collected varied by day. Such variation could have been caused by contributions from natural and/or anthropogenic sources. Also, such variation could have resulted due to the influence of meteorology on PM_{10} size and composition.

Unlike DNA damage in the cell-free Plasmid Scission assay, DNA damage in the cell-based Neutral Comet assay significantly differed between the two periods of sample collection. The difference in effects between the two assays serves an indication of the importance of PM size. In a cell-free assay, DNA is exposed to the entire PM mixture containing particles of various sizes (below 10 μm) and compositions and toxicity is dependent on particle chemistry and surface reactivity rather than particle size and surface area (Warheit et al. 2007; Karlsson et al. 2009). However, in a cell-based assay, whole cells are exposed to the entire PM mixture but the endpoint under investigation, DNA in this case, is not depending on the size of particles. Fine and ultra-fine particles may penetrate into cells and cause damage while coarse particles might not (Zhang et al. 2010; Yang et al. 2019; Su et al. 2020). It has been previously reported that smaller particles are more genotoxic than larger ones; $PM_{1.0}$ was found to be more genotoxic than $PM_{2.5}$ using the Comet assay on A549 cells (Zou et al. 2017) while $PM_{2.5}$ was found to be more genotoxic than PM_{10} using the Comet assay on human leukocytes (Buschini et al. 2001).

The difference in DNA damage observed in the cell-based Neutral Comet assay between the two periods, higher before Ramadan compared to during, further highlights the role of PM size. DNA damage was actually expected to be higher during Ramadan as the city experiences higher than usual traffic volumes during the holy month (Seroji 2011; Pasha and Alharbi 2015; Habeebullah et al. 2015). Traffic related particles are mostly in the fine and ultra-fine size fractions which are more hazardous compared to particles in the coarse size fractions (Hinds 2012; Kittelson et al. 2004). However, during Ramadan that year, weather conditions were in favour of the formation of windblown dust and sand particles as there were high temperatures and even a dust-storm recorded (Arab news 2018; Alarabiya 2018). Such conditions contributed to the presence of more particles in the coarse size faction in samples collected during Ramadan. Thus, leading to DNA damage being lower during Ramadan compared to before.

Regarding correlations with meteorological parameters and daily PM_{10} concentrations, in contrast to DNA damage observed in a cell-free Plasmid Scission assay where daily PM_{10} concentrations were associated with weather conditions typical of re-suspended and windblown dust and sand particles originating from natural sources and DNA damage was associated with weather conditions typical of anthropogenically sources secondary particles, DNA damage in a cell-based Neutral Comet assay was not correlated with daily PM_{10} concentrations nor with individual meteorological parameters. The lack of a correlation suggest that fine particles of whatever origin were the most likely cause of the damage observed in the cell-based Neutral Comet assay.

Unlike the cell-free Plasmid Scission and cell-based Neutral Comet assays, no significant variation was found in the results of the MTT assay. Our previous findings suggest that there were differences in the chemical composition but size was almost similar across all samples which could have led to relatively equal levels of cell death. That seems likely because all samples, which were collected with a sampler using a PM_{10} size selective inlet, were made up of particles less than 10 μm in diameter. It has been reported previously that particles of the same size cause relatively the same effect on cell viability even if they were collected from different periods (Dumax-Vorzet et al. 2015). Also, it has been reported that smaller particles ($PM_{2.5}$) are more cytotoxic than larger ones (PM_{10}) (Hsiao et al. 2000; Choi et al. 2004). This confirms our assumption that the size of particles was the main determinant of cell viability in this study.

6.4.2 Impact of different exposure processes on PM_{10} toxicity

In the cell-free Plasmid Scission assay, plasmid DNA was exposed directly to PM_{10} for 5 hours at 37 ℃. In comparison, in the cell-based Neutral Comet and MTT assays, whole cells were exposed to a medium containing PM_{10} for 3 and 48 hours respectively at 37 °C. Other than differences in the endpoints investigated by the aforementioned assays, there are two major differences; (i) the lack of protection and/or repair mechanisms that could have limited PM_{10} toxic effects in the cell-free assay and their presence in the cell-based assays; (ii) the short exposure period of the cell-based Neutral Comet assay compared to the long exposure period of the cell-based MTT assay.

Cellular exposure to PM_{10} results in its release of reactive oxygen species which leads to adverse effects depending on the endpoint under investigation. The cells use their defence mechanism to limit the damage incurred by triggering anti-oxidative stress response through gene activation (Tu et al. 2019; Chew et al. 2020). This suggests that DNA strand breaks in the cell-based Neutral Comet assay could have occurred and then disappeared following a repair event (Kawaguchi et al. 2008; Kawaguchi et al. 2010). That is certainly possible due to the short exposure period of the assay that is actually meant to minimise DNA repair events that could lead to underestimating the toxicity of PM as much as possible (Beedanagari et al. 2014) This also suggests that mitochondrial DNA and protein damage in the MTT assay could have occurred and then disappeared as well (Calvo and Mootha 2010; Chew et al. 2020). However, due to the long exposure period, it is possible that the persistence of damage led to the failure of defence and repair mechanisms and eventually cell death occurred through the induction of the apoptotic pathway (Chew et al. 2020; Radi et al. 2014).

6.5 Conclusion

Genotoxicity and cytotoxicity in cell-free and cell-based assays were caused by PM_{10} collected from Makkah, Saudi Arabia before and during Ramadan in April / May 2018. Significant differences were found between the effects observed depending on the assay used and its measured outcome and therefore, no significant correlations was found between outcomes of cell-based and cell-free assays. It is hypothesised that this was most likely due to general differences in the assay's exposure processes and their periods which can lead to; (i) size related complications in cell-based assays resulting in limits on cell exposure to the entire PM mixture compared to cell-free assays; and (ii) defence and repair mechanisms related complications in cell-based assays

resulting in understating the toxicity of PM compared to cell-free assays in the case of short exposure periods or overstating toxicity of PM in the case of long exposure periods. Further work is required to investigate the relationship between physicochemical properties of PM_{10} and toxic effects observed in cell-free and cell-based assays. The assays used in this study nevertheless highlight the possible role of PM_{10} physical and compositional characteristics in inducing toxic effects.

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Chapter 7: Summary, conclusion, and future research

7.1 Summary

This research project aimed to investigate the association between structural and compositional properties of air particulates and ill-health effects in humans. Our rationale behind the project was to add to current knowledge of PM toxicity and address key un-answered questions regarding the role of its physicochemical and mineralogical characteristics in disease causation through the use of cytotoxic and genotoxic effects in cell-based and cell-free assays as endpoint proxies of ill-health outcomes rather than resorting to large scale epidemiological studies that require significant unavailable resources. To maximise our chance of determining the most likely cause of the genotoxic and cytotoxic effects observed in cell-free and cell-based assays, here we focused on three different particle types; (i) Crushed Rock Powders (CRPs) from Panasqueira mine area, chosen for their availability in large amounts and suitability in terms of size and chemical composition; (ii) PM_{10} from Makkah, Saudi Arabia, chosen for being of particular interest to the author's home institution and also due to the extensive gaps in knowledge in PM research there; (iii) and a certified reference material (CRM) - ERMCZ120 fine dust (PM_{10}-like) (trace elements), chosen for its availability, reasonable price, and resemblance of PM_{10} in terms of size and composition. The four manuscripts (Badri et al. 2020a (*Chapter 3*), 2020b (*Chapter 4*); Badri et al. 2020c (*Chapter 5*), 2020d (*Chapter 6*)) contained within this thesis examine not only the cytotoxic and genotoxic effects of different particle types and link a component or group of components from the particle mixture with the observed effects but also compare effects found in cell-free assays with those observed in cell-based assays to determine the significance of PM size compared to its composition and provide useful information that can be used in future PM risk assessment studies.

Based on our investigations on the relationship between toxic effects induced by CRPs and their physicochemical and mineralogical composition (Badri et al. 2020a (*Chapter 3*), 2020b (*Chapter 4*)), it can be concluded that;

i. The magnitude of CRP cytotoxicity and genotoxicity was associated with compositional differences in the CRPs
ii. In these particular samples, (MnO, Zn, S, Cu, and clinochlore IIb2) were associated with differences in cytotoxicity and genotoxicity

These chemical components occur naturally in the environment but can also be released from anthropogenic sources. In the atmospheres of urban areas, Mn-bearing particles are present in combination with natural crustal materials and/or anthropogenic compounds emitted from alloy, steel, and iron production and combustion of fossil fuels and fuel additives (Nayebare et al. 2018; Howe et al. 2004; Moreno et al. 2011; Wang et al. 2020; Lawrence et al. 2013; Miah et al. 2020; Colledge et al. 2015). Excess inhalation of Mn particles can lead to its accumulation in tissues of the human body which results in several symptoms including pneumonia, decreased libido, and sperm damage (Williams et al. 2012; Hobbesland et al. 1997; Yamada et al. 1986; Miah et al. 2020). Zn-bearing particles are also present naturally originating from the Earth's crust (ATSDR 2005). However, in comparison, their release from anthropogenic sources is far greater with emissions originating from the oil refining industry, tyre abrasions, break pad wear, and oil lubricants (Nayebare et al. 2018; Markus and McBratney 1996; Wilcke et al. 1998; Jiries 2003; Al-Khashman 2004; Acosta et al. 2015; Alleman et al. 2010; Choël et al. 2006; Ledoux et al. 2009; Mbengue et al. 2014; Thorpe and Harrison 2008; Mattielli et al. 2009). Acute exposure to Zn particles results in a variety of symptoms including coughing, substernal pain,

and upper respiratory tract irritation (Geiger and Cooper 2010). Similar to Zn, Cu-bearing particles' natural sources are the Earth's crust. Its anthropogenic sources include oil combustion, break wear, and tire tread wear (Nayebare et al. 2018; Becagli et al. 2012; Galindo et al. 2018; Loganathan et al. 2013; Miazgowicz et al. 2020). Inhalation of Cu particles can cause hypersensitivities in the nose, mouth, and eyes (Dorsey and Ingerman 2004). Sulphur in urban areas is mostly anthropogenic and is emitted from the combustion of fossil fuels in the form of hydrogen sulphide, sulphur dioxide, and sulphate aerosols (Cullis and Hirschler 1980; Tricker and Tricker 1999). Inhalation of sulphur compounds can cause respiratory irritations including coughing and sneezing (U.S EPA 2019; Liccione and Little 1998; N.Y DOH 2004; ATSDR 2014). Clinochlore IIb2 is a naturally occurring mineral that is composed of iron, magnesium, aluminium, silicate, and sometimes small amounts of chromium (Welch et al. 2004). Also, such clay mineral has the ability to absorb and desorb substantial amounts of heavy metals as well (Kicińska 2018). Even though mineral particle exposure is an occupational health concern in industries such mining and construction, it could still pose a health threat in urban areas due to the considerable amounts of mineral particles in ambient air from natural processes such as volcano eruptions and sandstorms (Øvrevik et al. 2005). However, it is important to note that we doubt its association with cytotoxic effects observed in this project was due to its composition rather than its presence in fine size fractions.

In summary, chemical composition determines to some extent cytotoxicity and genotoxicity which means that cytotoxicity and genotoxicity might be used as an indicator for compositional variation. Due to difficulties in obtaining enough material for compositional analyses, the studies on PM_{10} from Makkah (Badri et al. 2020c

(*Chapter 5*), 2020d (*Chapter 6*)) relied upon this to infer compositional differences. The studies also noted the association of cytotoxicity and genotoxicity with meteorological parameters – which was of assistance in implied source apportionment of the PM_{10} measured.

By using widely available meteorological parameters as predictors of Makkah PM_{10} concentrations and toxic effects, we have shown that their use provides insight into the possible types and sources of particles that could contribute to overall PM_{10} abundance (re-suspended and windblown desert dust and sand particles) and toxic potential (fine particles of whatever origin and secondary particles formed from the conversion of anthropogenically-sourced gaseous emissions into particles) even though a complete physicochemical and mineralogical analysis was not available for the samples collected. Desert dust and sand particles in the atmosphere depend heavily on meteorology with levels of high temperature and low humidity with winds blowing from desert directions can lead to sever episodes of dust-storms (European Environment Agency 2012; Bolles et al. 2019; Kim et al. 2015). Such particles consist mainly of clays with and without a silicate-based core (Lyles 2018; Richards et al. 1993). Excessive exposure to dust and sand particles has been associated with a variety of health effects including respiratory disorders (asthma, chronic obstructive pulmonary disease, and silicosis) and cardiovascular disorders (stroke, arrhythmia, and ischemic heart disease) as well as dermatological and reproductive disorders, however, to a lesser extent (Thalib and Al-Taiar 2012; Korényi-Both et al. 1992; Vodonos et al. 2014; Krewski et al. 2007; Lee et al. 2008; Bell et al. 2008; Lee and Lee 2014; Yoshida et al. 2009; Zhang et al. 2016). Common secondary particles formed from the conversion of anthropogenically-sourced gaseous emissions into

particles include nitrate, sulphate, and ammonium. These particles are closely related as they are present in the form of ammonium sulphate and ammonium nitrate resulting from the naturalisation of sulfuric acid and nitric acid with ammonia respectively (Stockwell et al. 2003; Squizzato et al. 2013). They can convert from their gaseous phases into a particulate phase under conditions of high humidity via condensation and/or nucleation and vice versa under conditions of high temperature via volatilisation (Wei et al. 2015; Chang et al. 2009; Bassett and Seinfeld 1983; Bassett and Seinfeld 1984). The inhalation of nitrate, sulphate, and ammonium in their gaseous phases has been found to cause respiratory irritations (U.S EPA 2019; Liccione and Little 1998; N.Y DOH 2004). As for inhalation of their particulate phase, although evidence linking the pure form of these particles with adverse health effects is insufficient (Pirani et al. 2015; Grahame and Schlesinger 2005; Weber et al. 2016; Park et al. 2018; COMEAP 2015), it has been suggested that they act as carriers of toxic components such as heavy metals (COMEAP 2015; Guo et al. 2016; Kelly and Fussell 2012).

By investigating the toxic effects of a widely available and reasonably priced PM_{10} like CRM-ERMZC120, we have provided a common reference that can be used for comparative purposes for other researchers investigating the toxic effects of PM.

Based on genotoxic and cytotoxic effects observed in cell-free and cell-based assays from contrasting particulate types (Badri et al. 2020a (*Chapter 3*), 2020b (*Chapter 4*); Badri et al. 2020c (*Chapter 5*), 2020d (*Chapter 6*)), it can be concluded that, in general, toxicity is more dependent on particles' chemistry and surface reactivity in cell-free assays and particle size and surface area in cell-based assays. However, there is always a possibility of some particles exhibiting a "double hazard" effect, small size and a

large surface area with specific chemical components and surface reactivity compared to other particles (Duffin et al. 2007). We found that; (i) in a cell-free Plasmid Scission assay, the level of DNA strand breaks caused by some Makkah PM_{10} and Panasqueira CRPs samples was two folds higher than that caused by the CRM-ERMZC120 even though, in general, Makkah PM_{10} and CRM-ERMZC120 were relatively the same size while Panasqueira CRPs were larger; (ii) in a cell-based Neutral Comet assay, the level of DNA damage caused by some of the smaller sized Makkah PM_{10} and the CRM-ERMZC120 was higher than that caused by the larger sized Panasqueira CRPs; (iii) in a cell-based MTT assay, the effect of some of the larger sized Panasqueira CRPs on cell viability was higher than that of the smaller sized Makkah PM_{10} and CRM-ERMZC120. The findings in (i) confirm the dependence of toxicity on particles' chemistry and surface reactivity in a cell-free assay while the findings in (ii) confirm the dependence of toxicity on particles' size and surface area in a cell-based assay. As for the findings in (iii), even though the assay used was cell-based similar to that used in (ii), particles' size and surface area were not the only determinants of toxicity which suggest that some particles from the overall particle mixture could have exhibited the "double hazard" effect and thus confirming that sometimes the toxicity of particles' could depend on their small size and a large surface area as well as their specific chemical composition and surface reactivity. Our findings suggest that for future PM risk assessment studies, rather than just focusing on a single aspect of PM such as mass concentrations, different size fractions, or chemical compositions, it is best to consider the previous combined along with specific particle properties (size, surface area, and specific chemistry and surface reactivity) and their relationships to better evaluate the toxicity of respirable particles and their effects on human health.

7.2 Conclusion

In this research project, we have investigated the genotoxic and cytotoxic effects of different particle types. While we have used three assays that were either cell-free or cell-based in investigating the aforementioned effects of particles, individually, each assay had its limitation which limited the generalisability of its results; (i) the cell-free Plasmid Scission assay is thought to be un-representative of what might actually happen to DNA in the case of actual exposure to PM due to the lack of cellular defence and repair mechanism and biological interactions between various cellular components and PM; (ii) the cell-based Neutral Comet assay is thought to be understating the toxicity of the overall PM mixture as some of the DNA damage incurred by particles cannot be detected due to the disappearance of strand breaks following a repair event; (iii) and the cell-based MTT assay is thought to have some false-positive or false-negative results that are caused by background interference from the addition of particles. To address limitations in (i), we have used the cell-based Neutral Comet assay for investigating the same endpoint, DNA damage. Unfortunately, we didn't address the limitations in (ii) as measuring DNA repair events was not part of the project. As for limitations in (iii), we addressed that by repeating experiments with abnormal results. Combined, a major limitation of the aforementioned assays is that they don't provide any information on particles' mechanism of inducing toxic effects, albeit current evidence suggest that its oxidative stress caused by the generation of reactive oxygen species by particles. Nevertheless, results obtained from the three assays combined enabled us to examine the role of particles' size and composition by either using available information on their physicochemical and mineralogical properties or by using widely available information on meteorological parameters as proxies for their composition and source

to examine the most likely component or group of components responsible for the toxic effects observed. While we were able to investigate the physicochemical and mineralogical properties of Panasqueira CRPs due to their availability in large amounts using different techniques including X-Ray Fluorescence, X-Ray Diffraction, and Particle Size Distribution, we weren't able to apply the same techniques on Makkah PM_{10} due to their availability in small amounts which limited our ability to identify the significant contributing sources to overall PM_{10} abundance and the exact component or group of components from the overall PM_{10} mixture that was/were associated with observed toxic effects. Having said that, the use of meteorological parameters as proxies for source and composition proved to be useful in highlighting the possible contribution of different emission sources to PM_{10} abundance and toxic potential of a group of components from the PM_{10} mixture. To conclude, this research project has contributed to existing knowledge of PM toxic effects, in general, and the role of size and composition in inducing toxic effects, in particular, and could be useful for future assessment of PM adverse effects on human health.

7.3 Future research

An understanding of the physicochemical and mineralogical properties of PM, its mechanism of inducing *in vitro* toxic effects, and the reliability of laboratory observed toxic effects in relation to actual human exposure is of key concern to policy makers interested in effectively targeting emission sources of the most likely harmful component of PM. Given the variations in toxic effects observed in this research project and elsewhere as well as the anthropogenic and natural processes affecting the structural and compositional properties of PM, the following is recommended for future research:

- Use at least two PM samplers at the same location and dedicate one collected filter for toxicological analysis and the other for physiochemical and mineralogical analysis. This may assist in overcoming the issue of low material obtained during sampling faced by researchers (Vuong et al. 2017; Dumax Vorzet 2010).
- Collect data on traffic volume or density and meteorological parameters during the days of sample collection. PM concentrations and compositions are known to be a function of anthropogenic (i.e. traffic) and natural (i.e. meteorology) processes (Yáñez et al. 2017; Karagiannidis et al. 2015; Gietl and Klemm 2009). Previous studies have reported finding a significant impact of traffic on PM (Munir et al. 2017) while others didn't (Habeebullah 2013; Habeebullah 2016) and attributed changes in PM to meteorology. Therefore, in conjunction with physiochemical and mineralogical analyses of PM, analysis of traffic volumes or density and meteorological parameters and their relationship with PM concentration and composition might assist in understanding the factors affecting PM characteristics and hence, toxic effects.
- Collect data on morbidity and mortality from respiratory and cardiovascular diseases during the days of sample collection. Previous reports have indicated that exposure to PM is associated with an increased risk of morbidity and mortality from respiratory and cardiovascular diseases (Pope III et al. 2015; Brook et al. 2010; Peden 2005; Cançado et al. 2006). Therefore, in conjunction with *in vitro* analysis of PM toxic effects, analysing the association between morbidity and mortality from respiratory and cardiovascular diseases and PM concentrations and compositions might assist in reaching a better

understanding of the most likely harmful component and/or components of the overall PM mixture.

- Examine not only genotoxic and cytotoxic effects of PM but also the extent of contribution of reactive oxygen species to the effects observed. Previous studies (Jovanovic et al. 2019) have found that PM causes an increase in ROS production while others didn't (Dumax-Vorzet et al. 2015). Therefore, this may need to be further investigated.
- Examine the complex interactions between PM and cells and especially DNA repair. It is known that PM inhibits DNA repair and enhances DNA replication errors (Mehta et al. 2008). However, the level of DNA repair that can happen during exposure to PM is relatively unknown. An understanding of such issue will allow for a proper comparison between cell-based and cell-free assays and it would provide a deeper understanding of the ability of cells to handle PM induced toxic effects.
- Examine the bioavailability of the various components of PM inside the cells. Exposure assessment of PM components in cells is based on their known content. However, a bioavailability (what actually penetrates the different components of the cell) based approach may reflect on exposure much more appropriately (Kastury et al. 2017). Therefore, this may need to be further investigated.

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