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## **Investigating the Relative Toxicity of Nitrogen Dioxide in a Human Respiratory Tract Lining Fluid Model**

Katsaiti, Irene Natali

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# **Investigating the Relative Toxicity of Nitrogen Dioxide in a Human Respiratory Tract Lining Fluid Model**

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Thesis is submitted in partial fulfilment of the requirements for the degree  
of Doctor of Philosophy in Environmental Toxicology.

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## **Dedication**

**This thesis is dedicated to my parents and brother;**

**Drs Lilly, Odysseus & Alexander Katsaitis**

## Abstract

Epidemiological studies have shown that long term exposure to ozone (O<sub>3</sub>), nitrogen dioxide (NO<sub>2</sub>) and particulate matter (PM) are associated with adverse effects on lung function. However, disentangling the independent effects of each pollutant has proved difficult, if not an impossible task. Toxicological evidence is necessary, but no experimental study so far has compared the oxidative effects of these three air pollutants under the same controlled conditions. Air pollutants are believed to cause their pathophysiological actions in the respiratory system, at least in part, through oxidative stress. The principal objective of this study was to assess their order of significance by measuring their oxidative potential in our respiratory tract lining fluid model.

Synthetic respiratory tract lining fluid (synRTLF) was exposed to varying O<sub>3</sub> and NO<sub>2</sub> concentrations (0, 50, 150, 400, 1000 ppb) for 120 minutes. Aliquots of synRTLF were removed from the exposure chamber every 30 minutes and the concentrations of ascorbate, urate, and glutathione (reduced and oxidised) were measured. Similarly, filter disc cuts of PM collected daily from roadside and background locations in London, were incubated in synRTLF. The quantification methods used were the same as those used for the gaseous exposures.

These experimental findings constitute the first to compare the effects of O<sub>3</sub> and NO<sub>2</sub> in a lung lining fluid model under the same experimental conditions. O<sub>3</sub> caused significantly greater antioxidant depletion than NO<sub>2</sub> *in vitro*. Direct comparison of the antioxidant depletion actions of the three air pollutants was achieved by applying the experimentally calculated rates on the pollutant's daily mean concentrations. The results revealed that (on the days considered) PM was responsible for higher antioxidant depletion across all tested days and sites when compared with the gases. The higher levels of NO<sub>2</sub> at roadside compared with O<sub>3</sub> at the same location resulted in a significantly higher overall oxidative capacity of NO<sub>2</sub> compared with O<sub>3</sub> at roadside, while the opposite effect was observed at the background London site.

All three air pollutants considered resulted in antioxidant depletion in our lung lining fluid model, and thus our findings offer further support that the antioxidants in the respiratory system are playing a protective role. Also, these results highlighted the oxidative potential of PM and the significance of NO<sub>2</sub> exposure at roadside locations.

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## Abbreviations

|                               |                                                                    |
|-------------------------------|--------------------------------------------------------------------|
| AA                            | ascorbate                                                          |
| AH <sub>2</sub>               | ascorbic acid (ascorbate)                                          |
| AP-1                          | activator protein-1                                                |
| AQEG                          | air quality expert group                                           |
| BAL                           | bronchial alveolar lavage                                          |
| BW                            | bronchial wash                                                     |
| CO <sub>2</sub>               | carbon dioxide                                                     |
| COPD                          | chronic obstructive pulmonary disease                              |
| DEFRA                         | department for environment, food and rural affairs                 |
| DEP                           | diesel exhaust particulate                                         |
| DFO                           | desferrioxamine                                                    |
| DHA                           | dehydroascorbic acid                                               |
| DNA                           | deoxyribonucleic acid                                              |
| DTNB                          | 5,5'-Dithiobis(2-nitrobenzoic acid)                                |
| DTT                           | dithiothreitol                                                     |
| EC                            | European Commission                                                |
| EC-SOD                        | extracellular superoxide dismutase                                 |
| EDTA                          | ethylenediaminetetraacetic acid                                    |
| ERG                           | Environmental Research Group                                       |
| ESR                           | electron spin resonance                                            |
| EU                            | European Union                                                     |
| g                             | Gram                                                               |
| GM-CSF                        | granulocyte-macrophage colony-stimulating factor                   |
| GSSG                          | glutathione disulphide                                             |
| GSx                           | total glutathione (GSH + GSSG)                                     |
| GSH                           | reduced glutathione                                                |
| h                             | hour                                                               |
| HCL                           | hydrochloric acid                                                  |
| HPLC                          | high performance liquid chromatography                             |
| H <sub>2</sub> O <sub>2</sub> | hydrogen peroxide                                                  |
| <i>hν</i>                     | sunlight                                                           |
| ICP-MS                        | inductively coupled plasma mass spectrometry                       |
| IL                            | interleukin                                                        |
| L                             | litre                                                              |
| LDH                           | lactate dehydrogenase                                              |
| MAPKS                         | mitogen activated protein kinases                                  |
| MDA                           | malondialdehyde                                                    |
| mg                            | milligram                                                          |
| µg                            | microgram                                                          |
| min                           | minute                                                             |
| MIP-2                         | macrophage inflammatory protein 2                                  |
| ml                            | millilitre                                                         |
| mmol                          | millimole                                                          |
| Mo                            | molybdenum                                                         |
| MOUDI                         | micro-orifice uniform deposited impactor                           |
| MPA                           | metaphosphoric acid                                                |
| MPO                           | myeloperoxidase                                                    |
| MTT                           | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay |

|                               |                                                                |
|-------------------------------|----------------------------------------------------------------|
| NaOH                          | sodium hydroxide                                               |
| NADPH                         | $\beta$ -nicotinamide adenine dinucleotide phosphate           |
| NF- $\kappa$ B                | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NO                            | nitric oxide                                                   |
| NO <sub>2</sub>               | nitrogen dioxide                                               |
| NO <sub>x</sub>               | nitrogen oxides                                                |
| N <sub>2</sub> O <sub>5</sub> | dinitrogen pentoxide                                           |
| OP                            | oxidative potential                                            |
| O <sub>2</sub>                | oxygen                                                         |
| O <sub>3</sub>                | ozone                                                          |
| OH                            | hydroxyl                                                       |
| PAH                           | polyaromatic hydrocarbon                                       |
| PM                            | particulate matter                                             |
| PM <sub>0.1</sub>             | PM with an aerodynamic diameter less than 0.1 micron           |
| PM <sub>0.1-2.5</sub>         | PM with an aerodynamic diameter ranging from 0.1 to 2.5 micron |
| PM <sub>2.5</sub>             | PM with an aerodynamic diameter less than 2.5 micron           |
| PM <sub>2.5-10</sub>          | PM with an aerodynamic diameter ranging from 2.5 to 10 micron  |
| PM <sub>10</sub>              | PM with an aerodynamic diameter less than 10 micron            |
| PMNS                          | polymorphonuclear leukocytes                                   |
| pmol                          | picomol                                                        |
| ppb                           | parts per billion                                              |
| ppm                           | parts per million                                              |
| PTFE                          | polytetrafluorethylene                                         |
| RANTES                        | regulated on activation, normal T cell expressed and secreted  |
| RTL                           | respiratory tract lining fluid                                 |
| s                             | second                                                         |
| synRTL                        | synthetic respiratory tract lining fluid                       |
| TEOM                          | tapered element oscillating micro-balance                      |
| TNF- $\alpha$                 | tumour necrosis factor- $\alpha$                               |
| TNB                           | nitrobenzoic acid                                              |
| $\alpha$ -Toc                 | $\alpha$ -tocopherol                                           |
| UA                            | urate                                                          |
| VCAM-1                        | vascular cell adhesion molecule 1                              |
| VOC                           | volatile organic compound                                      |
| WHO                           | World Health Organisation                                      |
| 8-OHdG                        | 8-hydroxy-2' -deoxyguanosine                                   |
| °C                            | degree centigrade                                              |
| *                             | radical                                                        |

## **1 Introduction**

Air pollution is considered the world's top environmental health risk [1] and there is stronger epidemiological evidence than ever before supporting that it can result in respiratory morbidity and mortality [2]. However, disentangling the independent effects of each pollutant has proved difficult, if not an impossible task. In addition, laboratory studies comparing the toxicity of single air pollutants while utilising the same model and conditions are scarce. This study aims to contribute to this body of evidence by comparing the effects of three major air pollutants in a respiratory model under carefully matched controlled conditions.

### **1.1 Nitrogen Dioxide, Ozone and Particulate Matter**

Three major air pollutants were considered in this project; nitrogen dioxide ( $\text{NO}_2$ ), ozone ( $\text{O}_3$ ) and particulate matter ( $\text{PM}_{10}$ ).  $\text{NO}_2$  is both a primary and secondary pollutant, that is, it can be directly emitted from sources but mainly can be formed by reactions of primary pollutants and other pollutants ( $\text{O}_3$ ).  $\text{NO}_2$  is an oxidizing, corrosive, reddish-brown gas with a characteristic pungent odour. It has a low solubility in water (Henry constant =  $1.0 \times 10^{-2} \text{ M} \cdot \text{atm}^{-1}$ ), a molar mass of  $46.01 \text{ g mol}^{-1}$  and contributes to the colour and odour of smog [3]. The current research investigation was centred around this air pollutant given the toxicological uncertainty that surrounds its health effects [4]. Its actions were compared against those of another gaseous pollutant;  $\text{O}_3$ . This is a secondary pollutant formed in ambient air through a complex set of sunlight initiated reactions of its precursors: primary emissions of nitrogen oxides ( $\text{NO}_x$ ) and volatile organic compounds (VOCs) from stationary and mobile sources.  $\text{O}_3$  is a tri-atomic, pale-blue, highly oxidising gas with a molar mass of  $48.00 \text{ g mol}^{-1}$ . It has a lower solubility than  $\text{NO}_2$  in water (Henry constant =  $1.3 \times 10^{-3} \text{ M} \cdot \text{atm}^{-1}$ ) and its aroma is associated with rain [3]. It should be noted that the adverse health effects of  $\text{O}_3$  are associated with tropospheric  $\text{O}_3$ , as this reaches the ground, while stratospheric  $\text{O}_3$  has a protective role (absorbs harmful UV radiation) and a different formation mechanism (photolysis of  $\text{O}_2$ ).  $\text{PM}_{10}$  is the third air pollutant that was considered, this is a complex mixture of chemical components (fine solids and/or liquids) with composition dependent on location and emission sources. Further information about the formation mechanisms of the aforementioned pollutants is presented in 1.1.1.

### 1.1.1 Formation

The current project focuses on the actions of exogenous (i.e. inhaled) NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub>. However, it should be acknowledged that the gaseous pollutants can also be formed endogenously, sources of endogenous NO<sub>2</sub> formation include myeloperoxidase and eosinophil peroxidase (released by activated phagocytes) via oxidation of nitrite [5]. In addition, there is evidence that O<sub>3</sub> can be generated throughout the evolution of atherosclerotic plaques and during the antibody catalyzed water oxidation pathway [6].

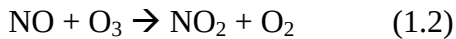
In the environment, the process of NO<sub>2</sub> and O<sub>3</sub> formation is highly connected; most tropospheric reactions are initiated by O<sub>3</sub>'s photolysis in Huggins bands which results in excited oxygen atoms O(1D) that rapidly undergo hydrolysis with water vapour to form OH· [7], on the same time, O<sub>3</sub> production is dependent on NO<sub>x</sub> levels (**Figure 1**).

Natural sources of NO<sub>x</sub> include forest fires, lightning, organic decay, and volcanic action [8]. In addition, anthropogenic sources include fossil fuel and biomass combustion and can occur outdoors but also indoors [9]. Undoubtedly though, the main sources are transportation, combustion and industrial functioning [10].

During combustion processes a small percentage of NO<sub>2</sub> is directly emitted, while NO is the main NO<sub>x</sub> formed (NO<sub>x</sub> = NO + NO<sub>2</sub>) [11]. The fraction of NO<sub>x</sub> emitted as NO<sub>2</sub> can be denoted as  $p$  in the following equation:

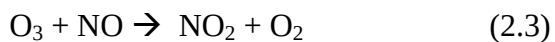
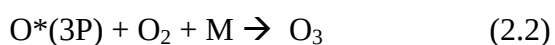
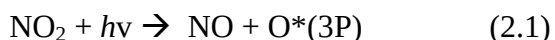
$$p = \frac{e_{NO_2}}{e_{NO_x}}$$

Typical values of  $p$  can range between 10-20% and are expected to rise in the future due to an increase in diesel vehicles [12]. Thus, NO<sub>2</sub> is mainly a secondary air pollutant as it is formed by the oxidation of NO via a number of reactions, such as [13]:



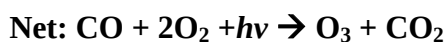
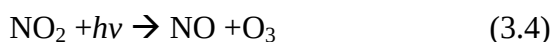
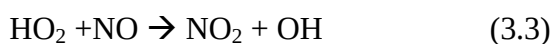
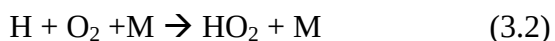
Reaction (1.1) is not significant in ambient air (it is only important when NO > 1ppm). Reaction (1.2) results in conservation of the NO<sub>2</sub> and O<sub>3</sub> formed, while reactions (1.3) and (1.4) involve volatile organic hydrocarbons and NO<sub>x</sub> and result in a net increase of NO<sub>2</sub> and O<sub>3</sub> [13]. The presence of VOCs have a critical role in the formation of O<sub>3</sub>, specifically, in the presence of sunlight (hν), NO<sub>2</sub> decomposes, yielding O<sub>3</sub> which then

is destroyed again to form NO<sub>2</sub>, (2.1-2.3) these reactions known as the ‘Leighton equations’ and were described by Professor Philip Albert Leighton in 1961 [14] .



(M can be any molecule that stabilises the O<sub>3</sub> molecule by absorbing the excess vibrational energy, it is often N<sub>2</sub>)

Under these steady state conditions the concentration of O<sub>3</sub> cannot increase until NO has been oxidised to NO<sub>2</sub>. This steady state is disrupted by the introduction of VOCs [15]. In general, an increase in concentration of the latter leads in an increase in O<sub>3</sub> production under non-NO<sub>x</sub> constrained conditions [16]. An exhaustive description of the VOC reactions is not possible as it has been estimated that the photochemical complex involved in the formation of O<sub>3</sub> contains 384 chemical species and 684 chemical reactions [17]. However the simplest example is given below using carbon monoxide (reaction 3) and a diagrammatic summary of the pathways is provided in **Figure 1**.



An important feature of these VOC related reactions is that there is a net gain of O<sub>3</sub> rather than scrubbing, thus there is fostering of increased ambient concentrations.

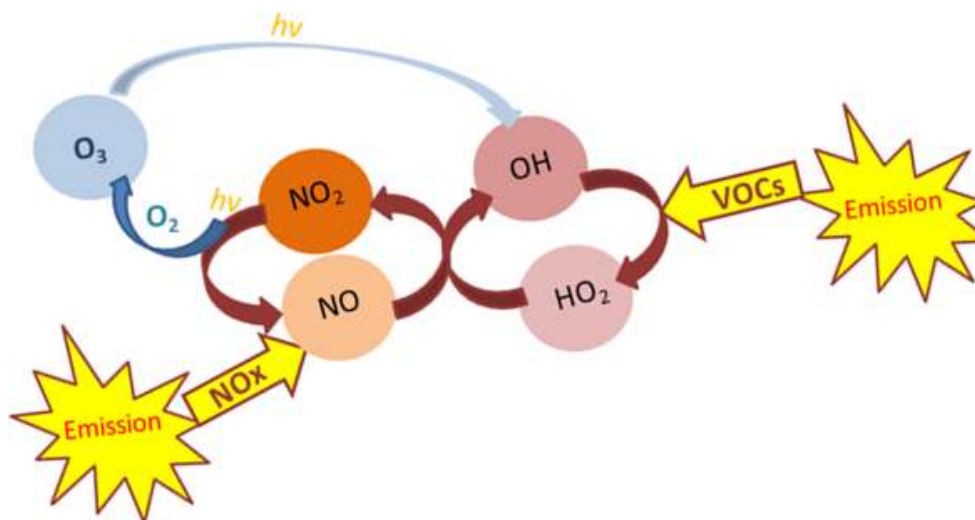


**Figure 1: Simplified Diagrammatic Representation of Ozone and Nitrogen Dioxide Formation.**

In the troposphere  $O_3$  formation is dependent on  $NO_x$ , sunlight and VOCs. In the presence of UV light  $O_3$  undergoes photolysis and hydroxyl radicals can form hydroperoxyl radicals which then can react with  $O_3$  to form  $NO_2$ . Also,  $NO_2$  can form  $O_3$  and the latter can react with  $NO$  to regenerate  $NO_2$ .

The formation process of the third pollutant investigated in this thesis is rather different; this is because unlike the aforementioned gaseous pollutants,  $PM_{10}$  is not a single molecular entity but consists of a range of chemical species which in turn are derived from different sources. Therefore, PM composition and size is dependent on primary emissions and subsequent atmospheric processing. PM can originate from natural and anthropogenic sources, primary particles are directly emitted as solids or liquids and examples of sources include burning of fuels, the wear and tear of tyres and brakes (anthropogenic sources) as well as sea salt and Saharan dust (natural sources) [18, 19]. Secondary particles are formed in the atmosphere by chemical reactions of  $NO_x$ , VOCs and  $SO_2$  [18] that lead to new PM formation via condensation and nucleation reactions [19]. However, the formation of secondary PM requires a longer time frame and therefore within street canyons (e.g. Marylebone road due to building structure) direct particle emissions comprise the larger contribution of roadside's increments [12]. PM's secondary formation process is very nicely depicted by Pöschl [19] in **Figure 2**.

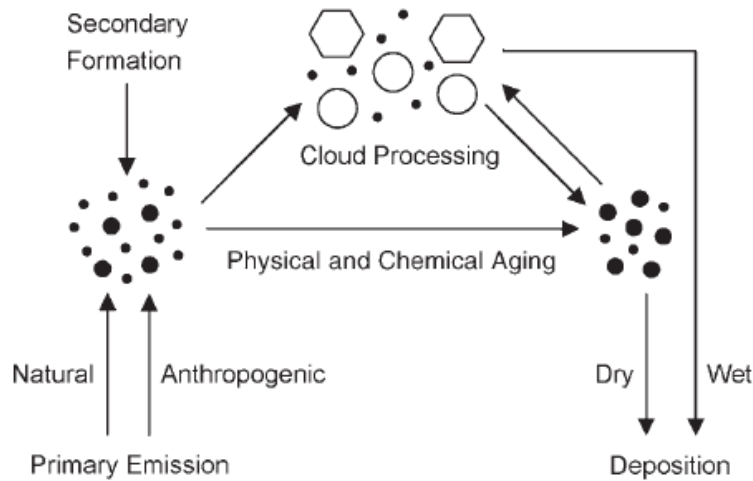


**Figure 2: ‘Atmospheric Cycling of Aerosols’ [19]**

A widely accepted characterization of PM is based on particle size. Particles with a 50% cut-off aerodynamic diameter of  $10\mu\text{m}$  are referred as  $\text{PM}_{10}$ . These particles are also known as thoracic because the inhaled mass fraction penetrates beyond the larynx to the air ways [20].  $\text{PM}_{10}$  can be subdivided into coarse particles ( $\text{PM}_{2.5-10}$ , diameter  $2.5\text{--}10\mu\text{m}$ ); fine particles ( $\text{PM}_{2.5}$ , diameter  $<2.5\mu\text{m}$ ); and ultrafine particles (UFPs, diameter  $<0.1\mu\text{m}$ ) [21].

Each fraction is thought to represent different formation mechanisms, typical urban PM can be conceptually represented by having a trimodal distribution which reflects the size and composition of the particle [22]. The coarse mode particles (diameter  $>1\mu\text{m}$ ) are produced by mechanical and resuspension processes, this size is associated with reduced atmospheric visibility and due to their larger size they are subject to gravitational influence. Coarse particles have been shown to consist almost completely of non exhaust particles [23]. Generally, the larger the particle, the less it is expected to travel. The next size conceptually is the accumulation mode (**Figure 3**) which is representative of the fine particles, these particles are mainly produced by coagulation of smaller particles and gaseous condensation [18, 24]. The nucleation mode (includes ultrafines) has the shortest atmospheric half life and consists of newly formed particles by homogenous nucleation in the atmosphere or within the emissions [18, 24]. These particles have a small mass but actually dominate the total particle number concentrations in urban areas [22].

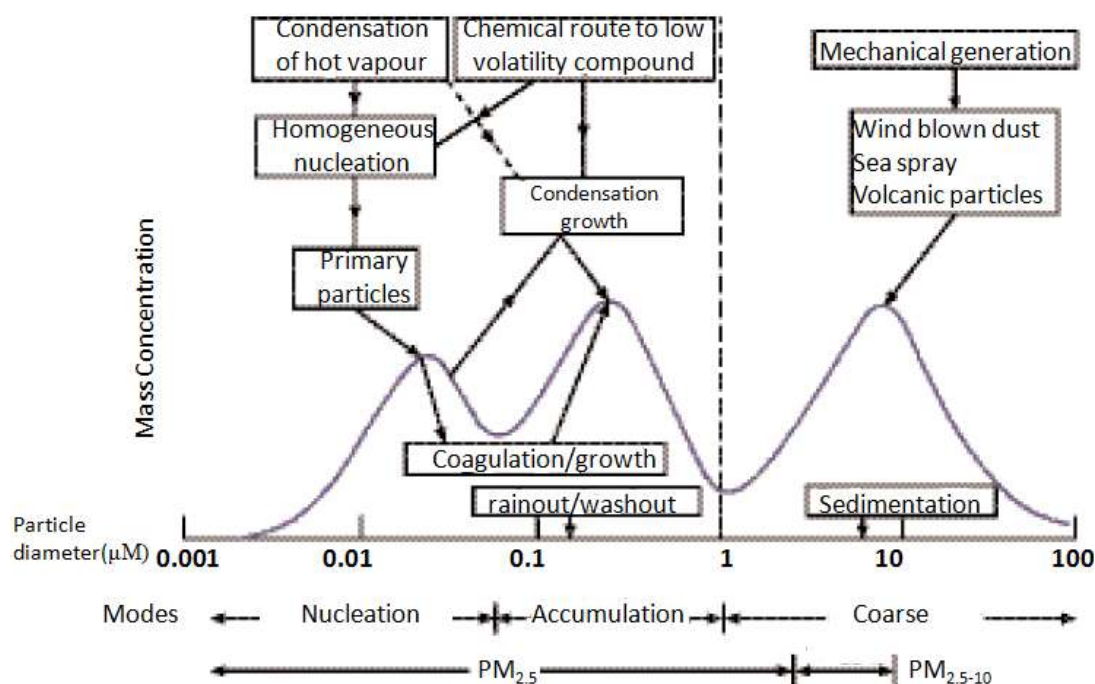


**Figure 3: Idealised Modal Classification, Adapted from AQEG [18]**

It should be noted that  $PM_{10}$  which is investigated in the thesis, includes all these PM fractions.

In accordance with the modal classification, PM composition varies, but overall it represents an amalgamation of organic and inorganic species, and typically it is comprised of organic carbon and elemental carbon [25], and also contains inorganic matter, sulphates, nitrates, water as well as radionuclides, polycyclic aromatic hydrocarbons, and numerous metals [26]. These last components have been implicated in PM's toxic effects [27]. Mass closure models can be utilized to assess the chemical composition of PM by measuring a subset of components and estimating the remaining species based on theoretical assumptions. In the UK, Harrison and colleagues (2004) used this approach and estimated that  $PM_{10}$  from roadside locations was comprised of elemental carbon (25.8%), organic compounds (23.2%), ammonium sulphate (10.2%), ammonium nitrate (5.7%), sodium nitrate (3.8%), sodium chloride (5%), calcium sulphate (3.4%), bound water (5.7), iron rich dust (17.2%). Interestingly, iron levels were significantly higher at roadside compared to equivalent background samples (18.3%) [28].

However, daily, seasonal and spatial variations are associated with compositional changes in PM. Daily variations are due to changes in meteorological conditions (e.g.

temperature, humidity, wind direction and speed), long range transport and local emissions. Seasonal variations reflect longer term changes in the aforementioned variables while spatial variations reflect proximity to sources [29]. Indicatively, in summer months there is increased photochemical reactivity which leads to degradation of PAHs and formation of oxygenated and nitrated derivatives [30], while in winter there is a more significant increase in PAHs associated with wood burning [31]. Also, the importance of location is shown at roadside sites as traffic related emissions are more prominent, for example higher heavy metal concentrations are consistently found in road dust, fuel, road wear products from tyres, and road construction materials [32]. Ultimately, these sources of variation highlight the complexity in investigating the toxicity of PM.

### 1.1.2 Permissible Levels and UK Compliance

The European Commission's Ambient Air Quality Directive (2008/50/EC) and the 4th Air Quality Daughter Directive (2004/107/EC) set the air quality standards for national and local authorities. The permissible NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> levels are summarized below, in **Table 1**.

| NO <sub>2</sub>                                   |                                                                                     | O <sub>3</sub>                                                                                                                     | PM <sub>10</sub>                                  |                                                                                 |
|---------------------------------------------------|-------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------|---------------------------------------------------------------------------------|
| <b>Annual limit value:<br/>40µg/m<sup>3</sup></b> | <b>Hourly limit value of<br/>200µg/m<sup>3</sup><br/>(18 permitted exceedances)</b> | <b>Maximum daily 8-hour mean:<br/>120µg/m<sup>3</sup><br/>(Not to be exceeded more than 25 times a year-averaged over 3 years)</b> | <b>Annual limit value:<br/>40µg/m<sup>3</sup></b> | <b>Daily limit value:<br/>50µg/m<sup>3</sup><br/>(35 permitted exceedances)</b> |

**Table 1: Permissible Concentrations Set by the EU**

It is interesting to note that O<sub>3</sub> does not have an annual limit, also, the other two air pollutants may have a yearly limit but the additional limits have a different time frame, hourly and daily for NO<sub>2</sub> and PM<sub>10</sub> respectively. It should be acknowledged that there are also air quality standards for further air pollutants including; PM<sub>2.5</sub>, sulphur dioxide and carbon monoxide.

The 2012 compliance assessment summary report by Defra clearly shows that  $\text{NO}_2$  is the principal pollutant that the UK is struggling to reduce. In 2012, 38 zones exceeded the annual  $\text{NO}_2$  limit, and 3 zones (London, Glasgow, North East Scotland) exceeded the hourly limit as well [33]. Meanwhile, there were no exceedances for  $\text{O}_3$ , although the long term objective for human health (i.e. no exceedance of the maximum daily 8-hour mean in a year) was not reached at all sites. In addition,  $\text{PM}_{10}$  concentrations appeared to marginally comply, however it should be noted that in the Greater London zone daily limit values of  $\text{PM}_{10}$  were exceeded on 46 days but this was reduced to 35 days (i.e. maximum number of allowed exceedances) following a subtraction of natural source contribution [33].

### 1.1.3 Concentration Patterns

There are certain variables the most important of which are season, location and time that can be predictive of the air pollutant's levels. Close to roadsides and on weekdays  $\text{PM}_{10}$  and  $\text{NO}_2$  concentrations tend to be high as there is an increased volume of traffic [18]. In contrast,  $\text{O}_3$  levels tend to be higher at background sites [34] and the  $\text{O}_3$  'weekend effect' term has been used often to describe the higher  $\text{O}_3$  levels at that time of the week. This increase can be attributed to higher VOC/ $\text{NO}_x$  ratio [35], however there is variability that is dependent on the  $\text{NO}_2/\text{NO}$  ratio [36].

In terms of season,  $\text{NO}_2$  and  $\text{O}_3$  have well documented opposing patterns that are directly related to their photochemistry. Specifically, in winter,  $\text{NO}_2$  levels present a zenith as days are shorter and there is lower solar energy and OH concentrations and heating related emissions are higher. In contrast, in summertime higher temperatures and sunlight lead to a peak of  $\text{O}_3$  levels [37]. Equally, these gaseous pollutants have opposite diurnal patterns;  $\text{O}_3$  slowly increases during sunrise, reaches a peak in the afternoon and levels persist to late afternoon [38]. In contrast,  $\text{NO}_2$  has two main concentration waves, a morning peak and an evening rush hour peak [38]. However, more diverse patterns may be observed during atypical meteorological conditions and pollution sources.  $\text{PM}_{10}$ 's seasonal and diurnal pattern is similar to  $\text{NO}_2$ 's levels, as it also presents higher concentrations during winter months due to lower ambient temperatures shifting the semi-volatile organic compounds from the gas phase into the particulate phase and due to higher emissions. In addition, during rush hour there is a peak due to increased primary emissions, though  $\text{PM}_{10}$  episodes usually occur in

summer months and tend to be the result of long range transport upsurge and secondary particle formation due to increased photochemistry and stagnation [39].

#### **1.1.4 How are pollutant concentrations changing in the UK?**

Systematic measurement of air pollutants begun in the 1970's, an analysis of the NO<sub>2</sub>, and PM<sub>10</sub> UK levels until 2012 reveals a sharp improvement in the country's average PM<sub>10</sub> concentrations (77% decrease in emissions) that has however stagnated since 2008. Similarly, NO<sub>2</sub> levels have presented a decrease overall but at a slower rate and are quite static over the past 6-8 years [40-42]. In contrast annual O<sub>3</sub> measurements globally but also in the UK have presented an increasing pattern. A comparison with older values (commencing in 1987) denotes that there is a significant increase in urban background concentration and no clear trend for the rural background locations [43]. Though, peak O<sub>3</sub> levels have been declining.

Predicting future trends in air pollutants is rather challenging, as, uncertainty is an inherent part of forecasting. This uncertainty stems from difficulties in accurately estimating potential climate and meteorological related changes, as well as emissions related to future technologies [18]. In addition, methodological changes in air pollutant measurements (especially PM) render comparisons with historical data and predictive models challenging [44]. The projected estimates have suggested that NO<sub>2</sub> concentrations will fall in the immediate future (2015) and a greater number of UK zones will comply with the air quality standards related to NO<sub>2</sub> [42]. However this prediction may be overly optimistic for roadside locations. While NO<sub>x</sub> levels have decreased at roadside locations the difference between NO<sub>2</sub> concentrations at roadside and at background sites has increased since 1998 [45]. This trend can be attributed to an increase in the NO/NO<sub>2</sub> ratio as a result of newer diesel vehicles that directly emit increased concentrations of NO<sub>2</sub> potentially due to after treatment PM control technologies and use of turbo-charging [46]. This observation is particularly important since the number of new diesel cars is increasing and the sales of new petrol cars are dropping [45]. The O<sub>3</sub> levels have generally shown a decrease in peak exposures but an overall increase in concentration, which is likely to further increase in the future. Urban O<sub>3</sub> levels are expected to rise in the next 20 years and to tend towards the rural O<sub>3</sub> concentrations [47]. The current and potential future increase can be attributed to increase of the NO/NO<sub>2</sub> ratio [48], higher inter-continental transport of O<sub>3</sub> in the northern hemisphere, non-methane VOC emissions from vegetation, and potential rise

of methane levels in Europe [49]. Finally, PM<sub>10</sub> levels over the last few years have been rather static and do not present a clear trend, thus it is the most complex pollutant among these three in terms of predictions. Although there are some older reports suggesting increase in concentrations [50] the limited number of sites monitoring PM composition across the UK render predictive calculations uncertain as changes in different sources can influence PM levels [18].

#### **1.1.5 Respiratory Inhalation/Absorbance/Deposition**

It is important to consider the anatomical site of impact of NO<sub>2</sub>, O<sub>3</sub> and PM as to an extent it determines their pathophysiological actions. The aforementioned pollutants are inhaled agents therefore the respiratory tract and more specifically the respiratory tract lining fluid (RTLFL) constitutes the first physical interface encountered. The gaseous pollutants examined are thought to diffuse within the RTLFL and the penetration is dependent on their reactivity towards the substrates of the RTLFL and especially the antioxidants. For example it has been estimated that most of the O<sub>3</sub> and NO<sub>2</sub> will not reach the respiratory epithelium as it will react with the RTLFL moieties [51-53]. Thus, gaseous absorbance within the RTLFL increases with higher gaseous oxidative potential, concentration, and diffusion coefficients. Their estimates of absorption can range depending on breathing parameters but are overall very high for both pollutants (>85%) [54-58]. The highest O<sub>3</sub> and NO<sub>2</sub> tissue dose occurs at the centriacinar pulmonary region but O<sub>3</sub> effects extend also more peripherally towards neighbouring alveolar walls and ducts potentially due to its higher toxicity and limited solubility [59]. Reactive gasses with low water solubility, such as O<sub>3</sub> can penetrate further in the respiratory tract and hence can come in contact with the smaller airways [60]

PM exhibits a more complex deposition pattern as it is a heterogeneous pollutant and there are also translocation and/or removal mechanisms to consider due to its structural dimension. The most important PM characteristic driving deposition rate and location is the size and aerodynamic properties of the particles (**Figure 4**) [61]. In general, particles with a diameter larger than 10µm can deposit in nasal airways by impaction and this process can be influenced by hair, bends of the airway passages mostly by impaction and air velocity [62]. Coarse particles correspond to the inhalable particles and deposit at the tracheobronchial region (trachea and bronchioles) and gravitational deposition (whereby PM in the airstream is settling due to gravitational forces) is among

the factors influencing the deposition rate [63]. Fine particles can deposit deeper in the respiratory tract, in the pulmonary region (small bronchi and alveolar sacs) mostly due to Brownian motion [64]. The total lung deposition efficiency decreases to about 20% for particles with aerodynamic diameter of 0.1-1 $\mu\text{m}$  as neither impaction/gravitational settling nor Brownian diffusion are particularly effective [65].

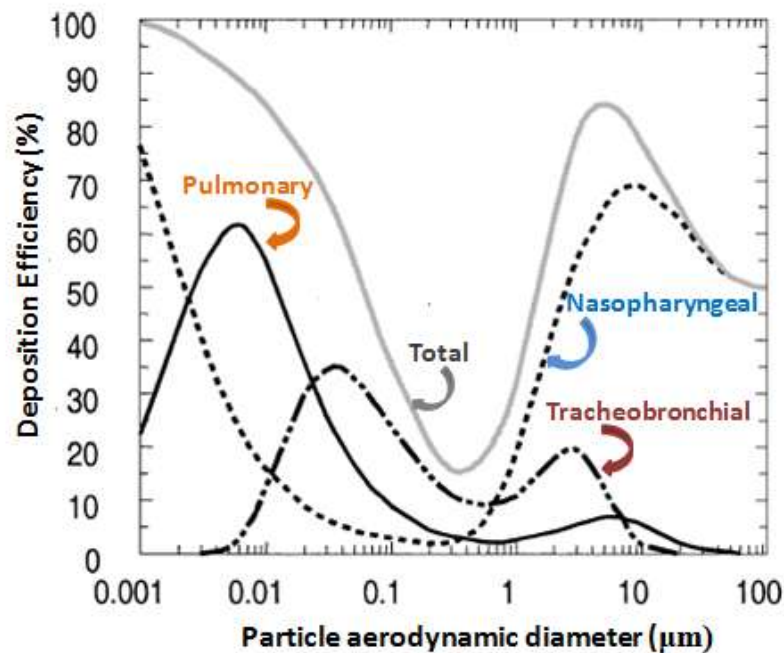


**Figure 4: Expected Particle Deposition Efficiencies in the Respiratory Tract**

The values are based on in an adult engaging low-level physical activity, larger particles remain in the head region and the smaller particles can reach the alveoli, adapted from Phalen, 2004 [60].

Further factors influencing the deposition of PM include changes in the anatomy of the airways and the geometric and temporal flow; examples include presence of respiratory disease, mode of breathing (oral vs. nasal) and breathing rate [66]. In general, increased breathing rate, oral mode, and respiratory illness result in higher deposition [66].

Once PM has been deposited in the airways, clearance mechanisms can limit the load, thus the efficiency of these mechanisms can determine the magnitude of the pathophysiological actions of PM. In the upper part of the respiratory tract, clearance can be facilitated by sneezing and coughing. The lung possesses two principal clearance

systems: the mucocilliary and the phagocytic systems. Particles deposited in the ciliated region of the tracheobronchial region are translocated by mucociliary action to the nasopharynx and from there to the gastrointestinal tract where they are cleared [67]. Important inter-individual differences exist in the process of clearance [68], the exact time will vary but it is thought to take approximately between a few to 24 hours [69]. Particles deposited in the terminal bronchioles, alveoli, alveolar ducts (i.e. below the ciliated epithelium) are cleared phagocytically by macrophages and type II pneumocytes and this is thought to be a slower process [67]. However, ultrafine particles may escape clearance and cross the alveolar-capillary barrier [70] or be breathed out again. Interestingly, the efficiency of the clearance mechanisms is thought to be depressed in the presence of air pollutants such as NO<sub>2</sub> [71] .

## **1.2 The Respiratory Tract Lining fluid**

Inhalation is the main route by which air pollutants enter the body. The human respiratory epithelium is covered by a complex, regionally heterogeneous aqueous layer; the respiratory tract lining fluid. In the proximal airways the RTLFL depth ranges between 1 to 10µm and is comprised of a two layer structure, an upper gel phase composed of glycosylated proteins (mucins) that also have antioxidant functions [72], and an aqueous sol phase which contains low molecular weight antioxidants and [73-75] and provides a low viscosity environment for the beating cilia [76]. In contrast, the distal region is 0.2-0.5µm thick. It consists of plasma derived exudate, secretions from underlying cells and a spectrum of antioxidants including; ascorbate, urate and reduced glutathione [72]. In addition, the RTLFL contains further antioxidant defences including: antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase [75, 77], and metal binding proteins such as lactoferrin, transferrin, albumin, ferritin and metallothioneins [77] therefore it contains a collection of antioxidant defences that can offer protection against pro-oxidants.

### **1.2.1 Antioxidants in the Respiratory Tract Lining Fluid**

Oxidative stress occurs as a result of an imbalance between production of reactive oxygen species and antioxidant defence mechanisms, a state that can damage sensitive biological targets [78]. The RTLFL has a strategically important position as it is the first physical and biochemical interface encountered by inspired agents such as O<sub>3</sub>, PM, and

NO<sub>2</sub> [79, 80]. It contains high concentrations of water soluble antioxidants ascorbic acid, reduced glutathione and uric acid [73, 74], and these provide a reducing environment which can act to neutralise direct and indirect (secondary oxidation products) effects of oxidants [81]. The impairment of these antioxidant defences is thought to be involved in respiratory injury [82-84].

#### **1.2.1.1 Ascorbic Acid**

Ascorbic acid (L-threo-hex-2-enomo-1,4-lactone), also known as Vitamin C was isolated and identified in 1928 by a Nobel laureate, Professor Albert Szent-Györgyi, a discovery which led to improved nutrition in the 20<sup>th</sup> Century [85]. Vitamin C deficiency can famously cause scurvy, but is also associated with defective connective tissue and wound healing [86]. It is a diacid and exists almost exclusively as the monoanion (ascorbate) at physiological pH range (pK<sub>1</sub>=4.2, pK<sub>2</sub>= 11.8) [87]. The conjugated structure of the five-membered lactone ring, offers stabilization of monodehydroascorbate, that is, the free radical one-electron oxidation product by delocalization of the unpaired electron [87]. It is a chain breaking antioxidant that can readily scavenge peroxynitrite, nitrogen dioxide radical, hypochlorous acid, superoxide anions and hydroperoxyl radical [88]. It can also reduce antioxidant radicals back to their reduced form such as Vitamin E, as well as reduced glutathione and urate upon O<sub>3</sub> or NO<sub>2</sub> exposure [89-91]. Oddly enough, the reaction of ascorbate with transition metals can lead to the formation of deleterious products such as hydrogen peroxide that can lead to increased oxidation as can be expected from Fenton chemistry [92]. However, it is a matter of great controversy as to whether this could occur *in vivo* [92].

Ascorbate is reversibly oxidised with the loss of one electron and forms the semidehydroascorbate radical, which is relatively unreactive due to its unpaired electron being in the delocalised  $\pi$ -system, further oxidation results in dehydroascorbate (DHA) which can be back reduced to ascorbate [93]. The ascorbyl radical will either disproportionate to generate ascorbate and DHA or it will be reduced to ascorbate by NADH-dependent semihydroascorbate reductase or it will be rapidly and irreversibly hydrolysed and thus depleted [93].

However, the mechanism of ascorbate transport in humans in general and the respiratory tract in particular are still under investigation. New ascorbate concentrations can only be acquired through the diet and are thought to be transported to the cerebrospinal fluid and blood stream via the Sodium Dependent Vitamin C Transporters

1 and 2 (SVCT1 and 2) [94]. SVCT1 is mainly expressed in the epithelial cells of the liver, kidney and intestine, whereas SVCT2 is mostly expressed in the lung, brain, spleen and liver [23]. The glucose transporters (GLUT 1, 3 and potentially GLUT4) have been shown to transport DHA via facilitated glucose transport [95], interestingly GLUT1 can even cross the blood brain barrier and provide an ascorbate source to the brain [96]. An elucidation of the exact transport mechanism of ascorbate in the lungs is of great interest as it could potentially provide a pharmacological target.

#### **1.2.1.2 Uric Acid**

Uric acid is a heterocyclic compound with the chemical formula  $C_5H_4N_4O_3$  and was discovered in 1776 by the famous German pharmacist Karl Scheele. In humans, it is formed by the oxidation of purine nucleotides xanthine and hypoxanthine by xanthine oxidase [93]. Uric acid is a weak acid ( $pK_{a1}$  5.4,  $pK_{a2}$  10.3) and therefore mostly exists in its ionised form, urate, at physiological pH, while very high concentrations in the serum are associated with crystal formation which leads to gout [97].

It is a natural antioxidant and at physiological concentrations it can act as a scavenger of oxidants such as singlet oxygen, hydroxyl radical, hydrogen peroxide and organic peroxide and also as a metal chelator stabilising ascorbate [98, 99]. In addition it has been shown to scavenge air pollutants such as  $O_3$  and  $NO_2$  [73, 100]. Importantly the unpaired electron in the urate radical is highly delocalized which results in the resonance-stabilised radical [101], but can react with ascorbate to regenerate urate [99]. Several protein transporters are candidates in urate transport but URAT1 and GLUT9 are the best characterized [102]. The exact mechanism of urate transport in the RTLF has not been delineated. Its RTLF concentration is high (100-400 $\mu$ M) and, despite inter-individual variability, the levels are thought to be highest in the upper airways [73, 74]. Interestingly lower levels have been shown in asthmatics [103].

#### **1.2.1.3 Glutathione**

Glutathione (GSH or l- $\gamma$ -glutamyl-l-cysteinyl-glycine) was discovered in 1888 by De Rey-Pailhade and is synthesized by two enzymes;  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS), which is the rate-limiting enzyme and glutathione synthetase [104]. Glutathione is a tripeptide composed of glutamic acid, glycine, cysteine and its active group is the thiol ( $-SH$ ) of the cysteine residue which serves as a proton donor [105]. It is present in all mammalian tissues and is involved in practically all important biological processes

including cell proliferation, apoptosis, enzyme detoxification and redox balance [105, 106]. It is a nucleophile and a reductant and therefore can react with electrophilic and oxidizing agents such as peroxynitrite, hydrogen peroxide, NO, lipid peroxide, superoxide and Fenton reaction products [107]. All the aforementioned reactions lead directly or indirectly to the formation of glutathione disulphide (GSSG) by GSSG reductase in an NADPH dependent pathway [108]. Therefore the GSH/GSSG ratio can be used as an indicator of oxidative state and has a value >10 under normal physiological conditions [109] and, interestingly, increased GSSG levels have been shown in asthmatics [82]. Glutathione is predominantly located in the liver (5-10mM) [110] but significant concentrations are also found throughout the RTLF (50-225 $\mu$ M) [73, 74].

### 1.2.2 Concentration of the Major Water Soluble Antioxidants

The concentration of the major water soluble antioxidants present in the RTLF varies along the RTLF, a large range of concentrations is provided by relevant studies [73, 74]. In the nasal airways urate is the prevalent antioxidant and it also appears to have a higher concentration range in the proximal and distal airways (**Figure 5**) However, due to the large deviations reflecting inter subject variations and the sensitivity of the methodology, previous studies modelling the effects of RTLF have utilised equimolar concentrations (200 $\mu$ M) of the three antioxidants [111-115] and this approach was also employed by the current study.

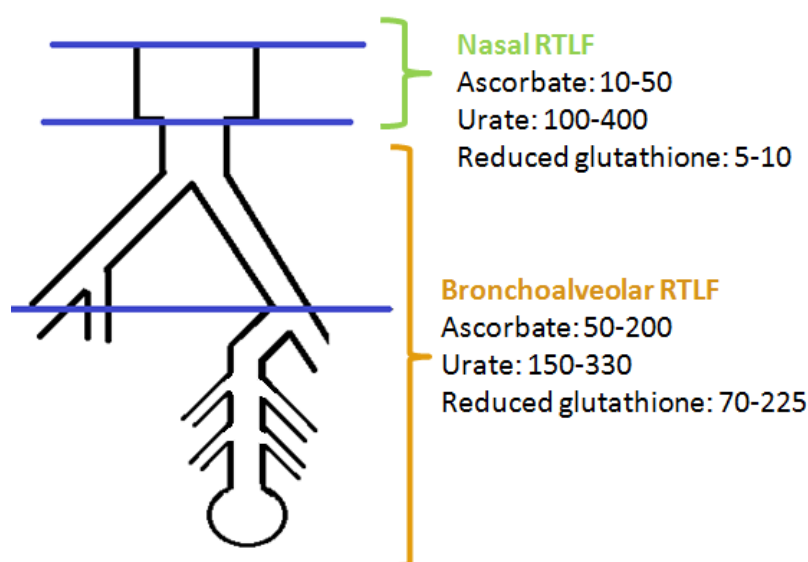


**Figure 5: Ascorbate, Urate and Reduced Glutathione Concentrations in the RTLF**  
Values ( $\mu$ M) are based on lavage samples of healthy adults and have been corrected for dilution [72, 73].

### 1.3 Respiratory Effects of Nitrogen Dioxide, Ozone and Particulate Matter

#### 1.3.1 Biochemical Basis of Toxicity

The aforementioned pollutants are oxidizing agents; NO<sub>2</sub> is a free radical, O<sub>3</sub> is a highly reactive allotrope of oxygen, and PM<sub>10</sub> also has free radical activity due to its components such as transition metals [116]. Due to their high oxidative potential they have the ability to induce free radical reactions such as lipid peroxidation and protein oxidation [117-121] and interestingly some *in vivo* evidence suggests that antioxidant supplementation can offer protection against their pathophysiological actions [122-124]. In addition, these pollutants have been shown to induce markers of oxidative damage in the airways of humans [125-127] and can modulate the antioxidant defences of the RTLF -as demonstrated in human chamber studies [128-132] discussed in section 1.4- which is the 'first line of defence' against inhaled oxidants [80]. Thus, it is believed that air pollutants can exert their pathophysiological actions, at least in part, via the induction of oxidative stress [79] (**Figure 6**). This state involves an imbalance between antioxidant defences of the RTLF and reactive oxygen species (ROS) which in turn allows the latter to react with sensitive biomolecules such as proteins, lipids, DNA and in this process there is secondary generation of reactive mediators and activation of signaling pathways that can escalate the pathophysiological response [79, 81]. Thus, a comparison of the interaction occurring between the principal antioxidants of the RTLF and the major ambient air pollutants is thought to contribute to an increased understanding of the pollutant's differential mechanisms of toxicity.

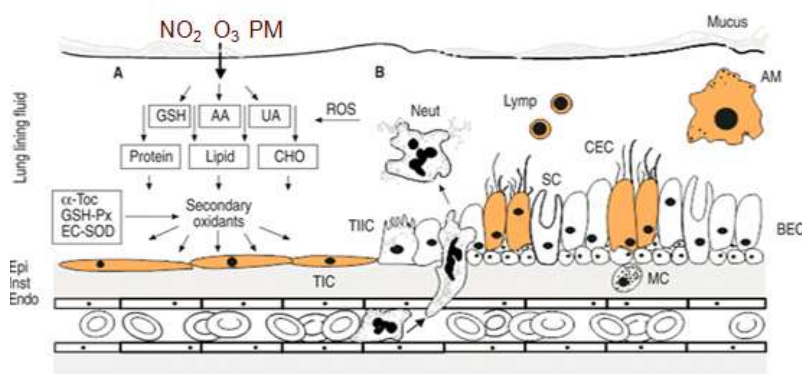


**Figure 6: Possible Mechanisms of Air Pollutant Induced Injury in the Lung**

Schematic representation of the interactions occurring between inhaled air pollutants and components of the RTLF. Ascorbate, urate, and reduced glutathione, react rapidly with the air pollutants and act as sacrificial substrates scavenging the oxidative species and therefore prevent the oxidation of sensitive macromolecules such as protein, lipids and carbohydrate. If these antioxidant defenses are overwhelmed the air pollutants can react with the aforementioned biomolecules and result in generation of secondary oxidants, other antioxidant defences such as  $\alpha$ -tocopherol can offer a second tier of radical scavenging. Airway inflammation results in the accumulation of neutrophils and macrophages which can further elevate the oxidative burden. Taken from: Kelly *et al.*, 2003 [78]

impact of air pollution. For example, the famous Harvard Six Cities Studies demonstrated that living in cities with higher air pollution resulted in approximately two years' reduction in life expectancy [134].

### **1.3.3 Inflammatory Response following Exposure to Air Pollutants**

Air pollutants can initiate the production of inflammatory mediators, a process which can increase the oxidative burden in the lungs, especially if the antioxidant defences that naturally endow the lungs are compromised. Therefore, the inflammatory response to O<sub>3</sub>, NO<sub>2</sub> and PM is relevant to the aims of this project and hence it will be briefly presented in this section.

Exposure of humans and animals to O<sub>3</sub> (0.1-2ppm) results in an influx of inflammatory cells, mainly neutrophils [135-137]. This process is thought to be partly stimulated by the neutrophil chemotactic factor, macrophage inflammatory protein 2 (MIP-2; [138]). The most likely sources of MIP-2 are thought to be the airway epithelium as well as the O<sub>3</sub> stimulated alveolar macrophages [139]. Interestingly, O<sub>3</sub> induced hyper-responsiveness was inhibited in dogs receiving hydroxyurea treatment (neutrophil depletion), thus the O<sub>3</sub> induced hyperresponsiveness may be depended on the mobilization of neutrophils into the airways [140]. The immune response to O<sub>3</sub> appears to be time dependent, indicatively; rats exposed to O<sub>3</sub> presented a MIP-2 peak at 6h followed by a neutrophilic infiltration peak at 24h post exposure [141]. In contrast, monocyte chemoattractant protein 1 (MCP-1) presented a zenith at 24h and this was followed by an increase in the number of macrophages in the BAL at 72h post exposure [141]. In addition, exposure to O<sub>3</sub> is known to cause elevated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) binding activity in type II epithelial cells and macrophages [142], a finding which is supported in O<sub>3</sub> studies presenting elevated levels of IL-1b [143], IL-17a [144], and IL-8 [145]. More recent studies have found differences in the O<sub>3</sub> induced inflammatory response of asthmatic and healthy individuals. Specifically, atopic asthmatics presented enhanced concentrations of IL-8, IL-6, IL-1β and increased TLR4 expression in sputum samples compared to the healthy control group [146]. However, other studies have not found differences in the bronchial mucosal inflammation of healthy participants and asthmatics once their baseline responses were taken into account [147]. Though, it should be acknowledged that asthmatics are a very diverse group of patients and interstudy comparisons are challenging.

There are less studies in the literature investigating the inflammatory actions of NO<sub>2</sub>, though, overall it appears to be causing a lower inflammatory response than O<sub>3</sub> and the magnitude of that contrast is study specific. Indicatively, in an *in vitro* study exposing human bronchial epithelial cells to ambient concentrations of O<sub>3</sub> and NO<sub>2</sub>, both air pollutants resulted in increased expression of RANTES (regulated on activation, normal T cell expressed and secreted), soluble intracellular adhesion molecule 1 (sICAM-1), IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF), though NO<sub>2</sub> presented an equivalent response at an approximately 4 times higher concentration [148]. In addition, healthy subjects exposed to O<sub>3</sub> concentrations as low as 100ppb (6h) presented increased levels of neutrophils, IL-6, and lactate dehydrogenase (LDH) protein, prostaglandin E2 (PGE2) and fibronectin in BAL samples [149], whereas, equivalent results are presented in chamber NO<sub>2</sub> studies utilising at least 15 times higher concentrations [150]. In another chamber study, NO<sub>2</sub> resulted in increased levels of neutrophils and IL-8 in the bronchial wash but there was no sign of inflammatory changes in the bronchial biopsies [151], thus suggesting that the major site of inflammation following exposure to NO<sub>2</sub> is the small airways and not the alveoli. In contrast, O<sub>3</sub> inflammatory effects can be extended more deeply in the respiratory tract [152] due to its higher toxicity. A common observation between the O<sub>3</sub> and NO<sub>2</sub> exposures is that asthmatics tend to present a greater inflammatory response. Specifically, exposure to NO<sub>2</sub> (1ppb, 3h) resulted in increased levels of thromboxane B2 and prostaglandin D2 and decreased levels of 6-keto-prostaglandin (the latter is associated with bronchodilation) in the BAL of asthmatics, while healthy subjects presented no change from baseline [153].

The inflammatory effects of PM are more complex than those of the gaseous pollutants, as its composition will vary and hence its toxicity. In general, healthy participants exposed to DEP present an increase of inflammatory cells and chemokines. Specifically, healthy participants exposed to DEP have exhibited an increase in the concentration of IL-6 and neutrophils in the sputum [154], as well as an increase of IL-13 in the bronchial epithelium [155] and an induction of neutrophils and alveolar macrophages in the BAL has been observed [156]. Further inflammatory effects in DE chamber studies include higher concentration of B lymphocytes in airway lavage, more neutrophils and platelets in peripheral blood and an upregulation of ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1), mast cells, CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in bronchial biopsies compared with the control group [157].

Interestingly, recent studies suggest that rats receiving tracheal injections of PM<sub>10</sub> collected in China and suspended in saline exhibited significant upregulation of inflammatory markers in the brain tissue [158], these results imply that the pro-inflammatory effects of PM are not confined within the lung, however, methodological limitations cast doubt on the validity of these findings.

Importantly, DE exposure can trigger an upregulation of NF- $\kappa$ B, activator protein-1 (AP-1) and upstream of mitogen activated protein kinases (MAPKs) [159]. Therefore, DE can activate redox sensitive transcription factors, which can in turn trigger the synthesis of pro-inflammatory cytokines, consistent with *in vitro* studies on particulates [160-162]. Interestingly though, addition of antioxidants in human bronchial cells can attenuate the expression of these molecular events, a finding which highlights the protective role of the antioxidant defences [163].

A common theme linking the inflammatory response of these different air pollutants is that inflammatory mediators can generate oxygen derived free radicals (superoxide anions, hydroxyl radicals) and metabolites (hydrogen peroxide, hypochlorous acid) [164]. Indicatively, neutrophils, mast cells, eosinophils, and macrophages have an exceptional capacity to produce oxidants. They can generate superoxide anions which are converted to hydrogen peroxide by superoxide dismutase, and Fenton chemistry can result in the production of hydroxyl radicals in the presence of metals [164]. This imbalance, can lead to oxidative stress and this in turn can lead to airway hyperresponsiveness [165]. In accordance with this premise, asthmatic inflammatory cells from the BAL and peripheral blood presented higher generation of superoxide anion radicals than the healthy individuals [166]. Therefore, ironically, the inflammatory response which constitutes a defence mechanism can lead to the activation of redox sensitive transcription factors such as AP-1 and NF- $\kappa$ B, gene expression of pro-inflammatory mediators, and chromatin remodelling [167]. Thus, there is a continuous reinforcement of redox imbalance which can lead to various chronic inflammatory diseases, and hence the RTLf antioxidants which are the focus of this thesis can possibly prevent this vicious cycle from occurring.

### **1.3.4 Major Study Designs**

The health effects of air pollutants can be investigated via short (acute) and long term (chronic) exposures and the most common study designs are the time series and cohort studies respectively. Time-series examine the associations between exposure to air pollutants and morbidity and/or mortality by utilising repeated observations of the dependent and the independent variables within a defined population and geographical area [168]. Typically, data are collected at regular time intervals (e.g. daily NO<sub>2</sub> levels and hospital admissions) and the aim is to delineate short term associations [169]. A major advantage of these studies is that they don't need to control for personal habits such as smoking and eating habits because they are not associated with air pollution levels and they do not vary from day to day [170]. However, such studies have to control for meteorological events and co-varying air pollutants [133]. Cohort studies are a type of longitudinal study, participants are selected based on their exposure status and are followed to monitor the development of a specific health effect (e.g. investigating mortality on a long-term basis in relation to an indicator of average exposure to NO<sub>2</sub>) and can be conducted retrospectively or prospectively [171]. A more rudimentary approach is offered by cross-sectional studies [168] as data are collected from different participant groups from areas with different levels of air pollution at one time period.

### **1.3.5 Acute Respiratory Effects**

Numerous time series studies have explored the association between daily increases in the levels of the aforementioned air pollutants and mortality in single pollutant models, while a very small number of studies have investigated respiratory mortality in multipollutant models. The effects tend to be higher in single than multipollutant models and on occasions the confounding is so pronounced that the estimates are insignificant when the latter approach is used. However, large variations in the estimated effects are observed across studies and the differences are even more pronounced for different geographic locations. Indicatively, Saez and colleagues (2002) found no independent association between photochemical air pollutants and respiratory mortality in data collected from 7 Spanish cities (EMECAM project) [172]. In contrast, in one of the few Asian multipollutant studies investigating the associations of NO<sub>2</sub>, O<sub>3</sub>, PM<sub>10</sub> and SO<sub>2</sub> with daily respiratory mortality, only the O<sub>3</sub> and SO<sub>2</sub> effects remained significant once the other pollutants were taken into account [173]. In a larger

European study, Samoli and colleagues (2006) utilised a database of 30 European cities (APHEA-2) and reported that an increase of  $10\mu\text{g m}^{-3}$   $\text{NO}_2$  (average lag 0 and 1) resulted in a 0.34% excess risk of respiratory mortality, this estimate was not significantly affected when adjusted for  $\text{PM}_{10}$  or  $\text{O}_3$  but was reduced when black smoke (0.28%) or  $\text{SO}_2$  (0.16%) were considered [174]. Much larger estimates were reported by Chiusolo and colleagues (2010) in the EpiAir study of 10 Italian cities. The authors used a more extended lag time (0-5days) and estimated that an increase of  $10\mu\text{g m}^{-3}$   $\text{NO}_2$  resulted in a 3.48% excess respiratory mortality risk. This estimate increased even further to 9.63% in the warm season (April-September),  $\text{O}_3$  and  $\text{PM}_{10}$  did not confound these results while other pollutants were not considered [175]. Further time series studies have investigated the association between daily increases in air pollutants and hospital admission for respiratory diseases. Pun and colleagues (2014) extended this question by investigating  $\text{PM}_{10}$  compositional characteristics that were associated with respiratory hospital admissions in Hong Kong. In this study, controlling for  $\text{NO}_2$ ,  $\text{O}_3$  and  $\text{SO}_2$  associated effects eliminated the significance of most associations, yet a  $0.02\mu\text{g}/\text{m}^3$  increase in sodium, aluminium and magnesium was correlated with an increase of 0.65, 0.56 and 0.63% respectively in respiratory hospital admissions [176]. Despite limitations in the  $\text{PM}_{10}$  collection and characterisation methodology such as collecting filters every six days and not always concomitantly from different stations, the study has a more interesting toxicological dimension. Indeed, the relationship between the gaseous pollutants and  $\text{PM}_{10}$  is a complex one, as adjusting the gaseous pollutants for  $\text{PM}_{10}$  and *vice versa* can lead often to study dependent effects. Indicatively, in a study examining the association between emergency respiratory hospital admissions and levels of  $\text{O}_3$ ,  $\text{NO}_2$ ,  $\text{PM}_{10}$  and  $\text{SO}_2$ , the two pollutant models presented significantly higher estimates than the single analyses [177], in other similar studies some of the estimates were attenuated in multipollutant models but significance remained [178, 179], whereas there are studies where the attenuation following co-pollutant adjustment was so extended that it resulted in loss of statistical significance, an example is the study conducted by Romieu and colleagues (2012) in Latin America where most  $\text{O}_3$  effects including hospital admissions were not significant following  $\text{PM}_{10}$  adjustment [180]. Thus, there is a great heterogeneity in study findings that may be based on methodological differences, different populations and airshed composition.

### 1.3.6 Chronic Respiratory Effects

The respiratory health effects of long term exposure to O<sub>3</sub>, NO<sub>2</sub> and PM<sub>10</sub> have also been investigated in cohort studies. In a recent meta-analysis and review of long term studies Faustine *et al.*, 2014 presented evidence of an independent effect of NO<sub>2</sub> on respiratory mortality. The pooled relative risk was 1.024 (95% CI 1.010–1.038) for NO<sub>2</sub> and 1.043 (95% CI 1.015–1.072) for PM<sub>2.5</sub> based on 9 studies, the authors noted that the effects persisted in multipollutant models [181]. However, the supplementary information reveals that only two studies had employed multipollutant models Hart *et al.*, (2011) controlled for PM<sub>10</sub> and SO<sub>2</sub> [182] and Cao *et al.*, (2011) adjusted for TSP [183] while calculated estimates had a high range; 5.60 (95% CI -12.00 - 26.60) and 2.10 (95% CI -1.00-5.30) [181]. Thus, although, this review offers support for the independent effect of NO<sub>2</sub> on health, further multi-pollutant analyses are necessary. The same holds true for PM<sub>10</sub> as the estimates and study designs vary [184]. There is also uncertainty surrounding the association between exposure to O<sub>3</sub> and mortality. Specifically, Jerrett *et al.*, (2009) reported positive associations between chronic ozone exposure and respiratory mortality in two pollutant models that included PM<sub>2.5</sub> [185]. However, their methodology was criticised as an annual average of 2 years data was used for PM<sub>2.5</sub> while daily maximum hourly levels from 3 year data were used for O<sub>3</sub> [186].

Stronger evidence supports the association between long term exposure to O<sub>3</sub>, NO<sub>2</sub> and PM<sub>10</sub> and the incidence of adverse respiratory symptoms and function. Indicatively, chronic O<sub>3</sub> exposure has been associated with onset of asthma in children [187], lung cancer in non-smoking adults [188] as well as reduced lung function in young adults [189, 190]. NO<sub>2</sub> and PM<sub>10</sub> have also been correlated with decreased lung growth in children [191, 192], as well as respiratory distress symptoms in women [193] and children [194]. Further studies exist supporting an association between the aforementioned air pollutants and respiratory morbidity in children, utilizing traffic-related metrics [195-201]. There are several potential reasons why there is stronger evidence of adverse air pollution related effects in children compared to adults based on lifestyle and physiological differences. For example, children tend to spend more time engaging in outdoor activities, are more active, have a higher breathing and metabolic rate, and the lung development and immune system are not fully developed [202]. However, it is unclear whether air pollution related adverse effects on children's

respiratory health can be overcompensated later on in adulthood. Interestingly, there are indications that children's respiratory health may be able to improve following long term exposure to improved air quality, thus supporting a causal role between air pollution and children's health [203] but also highlighting the significance of compensation and repair mechanisms.

### **1.3.7 Conclusion**

This section aimed to project the difficulties that epidemiological studies face in disentangling the impact of different air pollutants in the respiratory tract. Wherever possible studies considering the impact of copollutants in their analyses were considered, reviews of single pollutant studies can be found in reports such as those issued by the WHO. Single pollutant studies have often supported the respiratory morbidity of all air pollutants considered, however, the estimates are often decreased or even confounded in multipollutant studies. Other studies have used traffic related estimates (e.g. traffic load) as a marker of air pollution, while such studies have certainly provided evidence about the adverse health effects of air pollution and can benefit areas such as improved school site planning based on air pollution sources. Future studies can assist in the delineation of the air pollutant effects by employing more detailed and specific measurements. Also, a further point to consider about the current evidence is that the majority of the highly cited epidemiological studies of the last decade (e.g. Gauderman, Samoli) have based their calculation estimates on airshed measurements conducted in the 90's, thus it would be very interesting to see if the validity of these estimates is extended in more recent years. Overall, multi and single pollutant studies have associated NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> with adverse health effects but the large range of estimates especially in complex analyses creates doubts about the strength of the relationships and the causality of the effects.

## **1.4 Controlled Human Exposures**

In the literature there are a significant number of controlled human exposures investigating different health impacts of NO<sub>2</sub>, O<sub>3</sub> and PM (mainly cardiovascular). However, the focus of the information presented in the next paragraphs is related to the respiratory system which is in line with the study's objectives.

### 1.4.1 Pulmonary Function Effects

NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> appear to result in different pulmonary changes in controlled human exposures, however, it is important to bear in mind that a much larger number of exposures have focused on O<sub>3</sub> driven respiratory effects than the other two pollutants and thus there is an obvious bias. Nevertheless, the current scientific consensus clearly denotes O<sub>3</sub> being more reactive than NO<sub>2</sub> in the controlled human exposures.

In fact the literature suggests that healthy participants display no measurable adverse lung function effects when exposed to ambient concentrations of NO<sub>2</sub> [204]. Significant FEV decrements have only been observed at higher concentrations (1500ppb NO<sub>2</sub>) lasting 3h with intermittent exercise [205] as well as shorter exposures (1h) at higher concentrations ( $\geq 2000$ ppb) at resting conditions [206]. Orehek *et al* (1976) drew attention to the increased susceptibility of the asthmatics to NO<sub>2</sub> by reporting an increase in non-specific airway responsiveness (sRaw) at levels as low as 100ppb, though these findings have not been replicated and the statistical analyses has been heavily criticized as biased for subdividing the 20 participants into ‘NO<sub>2</sub> responders’ and ‘NO<sub>2</sub> non-responders’. Overall, the scientific evidence supports that asthmatics are much more sensitive to NO<sub>2</sub>’s respiratory effects. Specifically, this group of individuals presents pulmonary function deterioration (sRAW and FEV<sub>1</sub>) at ~250ppb after a 30 minute exposure during resting conditions [207-209], while studies employing exercise protocols (i.e. increased inhaled dose) have reported significant decrements after a 2h exposure to 200ppb NO<sub>2</sub> [210]. The heterogeneity of the chamber exposures conditions and the unequivocal diversity of asthmatic patients may explain contrasting findings presented by other studies [211, 212], while it should be noted that the aforementioned studies recruited mild asthmatics therefore the NO<sub>2</sub> effects could hypothetically be exacerbated in moderate and severe asthmatics.

It is unknown whether this contrast between asthmatics and healthy participants is reflected in their inflammatory biomarkers, or whether asthmatics have compensatory mechanisms like the ones observed in controlled diesel exhaust (DE) exposures. There is a distinctive lack of controlled exposures comparing these two groups under identical conditions, it has been suggested that it is unlikely for NO<sub>2</sub> concentration to elicit inflammatory responses in healthy individuals at concentrations below 1ppm [8], while at higher concentrations (2ppm, 4h) BW and BAL samples from healthy participants have shown significant increases of polymorphonuclear leukocytes (PMNS), interleukin (IL)-6, IL-8, tissue plasminogen activator,  $\alpha_1$ -antitrypsin, and significant

reduction of macrophage function compared to air control exposures in cross over study designs [213, 214]. Inflammatory responses of asthmatics following single exposures to NO<sub>2</sub> without allergen challenge are conflicting [215, 216] while a recent study suggests increased eosinophil counts in sputum following three consecutive exposures to 600ppb NO<sub>2</sub> for 30mins [217]. Thus, further studies are necessary in order to delineate the pulmonary inflammatory impact of NO<sub>2</sub>, investigation of healthy and asthmatic responses under the same controlled conditions could benefit this research field. In addition, unlike the O<sub>3</sub> effects, the current scientific evidence does not support clear dose dependent increments in lung function for NO<sub>2</sub>, this could reflect differences in pathophysiological mechanisms or such effects have not been elucidated due to less extensive amount of research on NO<sub>2</sub>.

Undoubtedly however, O<sub>3</sub> results in pulmonary dysfunction at lower concentrations than the ones described for NO<sub>2</sub>. Studies have consistently shown significant FEV<sub>1</sub> decrease in healthy individuals following 2h exposure to 500ppb O<sub>3</sub> at resting conditions [218, 219], while significant effects have also been reported at lower concentrations but the results have been conflicting. A significantly lower O<sub>3</sub> concentration (~70ppb) can elicit the same effects within the same time frame during exercise conditions [220-222]. Research studies examining the association between O<sub>3</sub> exposure and FEV strongly support that this relationship is consistent with a sigmoid-shaped mode, driven by the following parameters; (a) O<sub>3</sub> concentration, (b) duration of exposure and (c) minute ventilation [223]. These have a (physiologically negative) effect on FEV, until a plateau is reached [223]. Interestingly, the principal characteristics determining these parameters is exercise [224] and age, thus younger people are more susceptible to O<sub>3</sub>'s effects [223]. The exposure to O<sub>3</sub> is also associated with a rapid increase in neutrophils, prostaglandins and further inflammatory mediators with peaks reported between 3-18h post exposures, although it appears to be a complex response with different inflammatory mediators peaking at different time points and respiratory compartments [225]. Interestingly, in a meta-analysis of data from human chamber studies, a simple linear relationship was observed between O<sub>3</sub> dose (80-600ppb) and inflammatory markers in the BAL of healthy adults [224].

A further important point in relation to the controlled human exposures is a lack of extensive and consistent contrast in the inflammatory, oxidative and functional response between asthmatic and nonasthmatic participants exposed to O<sub>3</sub>, once baselines differences are taken into account [147, 226]. This observation could potentially reflect

a lower breathing rate and exercise performance by asthmatics which in turn results in a lower inhaled dose. Studies that have indeed found elevated airway obstruction in asthmatic participants [227] have showed great variation within each group, which in turn is representative of the differential susceptibility to O<sub>3</sub>. This well published phenomenon has not been fully explained, however, epidemiological studies have provided evidence of genetic differences governing susceptibility [228]. In accord with this line of research, increased activated innate immune function after O<sub>3</sub> exposure has been associated with muted immune cell trafficking pathways, thus genomics could potentially help elucidate the intersubject variability in O<sub>3</sub> associated inflammatory responses [229].

A further air pollutant that has been investigated in controlled human exposures is PM; such studies have been conducted in chambers connected to tailpipe diesel exhaust or more recently via concentrated ambient particles (CAPS) chambers. The latter methodology, involves drawing ambient air (NO<sub>x</sub>, O<sub>3</sub> scrubber is used) through a size selective inlet and towards concentrator stages in order to condense the particles at the specified size range (usually PM<sub>0.1-2.5</sub>) the output of this system is connected with the human exposure chamber [230]. The research field of controlled human exposures to PM was developed 20-30 years after the gaseous exposures. The first major studies were conducted in the 90's and a very large proportion of the published studies are conducted by the same research group (Umeå University, Sweden), therefore there is a considerably smaller number of studies and relatively limited diversity in experimental conditions. Also, there is an evident difficulty in distinguishing the PM-associated effects in the DE exposures, though the presence of NO<sub>x</sub> in the mixture renders these results relevant to the NO<sub>2</sub> exposures as well.

A major difference between these studies and the gaseous pollutant studies described above in terms of pulmonary function effects is that spirometric measurements (e.g. FEV<sub>1</sub>, FEF<sub>25-75</sub>) do not show any changes in healthy or asthmatic participants (PM<sub>10</sub> range of 100-300µg m<sup>-3</sup> [157, 231-233] while there is evidence that airway resistance is increased in both groups at equivalent concentrations [154, 231, 233]. Interestingly, there is a marked difference between healthy and asthmatic participant's inflammatory responses with the latter showing a lack of detectable inflammatory responses potentially due to compensatory mechanisms. Specifically, following exposure, BW samples of healthy participants have shown increased levels of neutrophils [234, 235] IL-6, IL-8, MPO [231, 236], as well as decreased macrophage levels in BAL and

sputum [154, 231, 232] and increased levels of adhesion molecules ICAM-1 and v-selectin [157]. In contrast, inflammatory changes in the asthmatics are limited to increased IL-10 levels in lung tissue (biopsies) [237], a change that does not occur in healthy participants [155, 231]. This increase in IL-10 is consistent with the lack of significant inflammatory response in asthmatics given that IL-10 has been shown to have an anti-inflammatory response by indirectly inhibiting the nuclear translocation of NF- $\kappa$ B [238].

Lastly, further studies have employed a field exposure design characterised however by the quantitative measurements used in the chamber studies. Specifically McCreanor *et al.*, 2007 exposed asthmatics to diesel traffic by walking along Oxford street for 2h or Hyde Park on a different occasion while performing real-time measurements (Average Oxford street values: 76.5ppb, NO<sub>2</sub>, 125  $\mu\text{g m}^{-3}$  PM<sub>10</sub>, 28.3  $\mu\text{g m}^{-3}$  PM<sub>2.5</sub> vs. Hyde park: 11.7 NO<sub>2</sub>, 72  $\mu\text{g m}^{-3}$  PM<sub>10</sub>, 11.9  $\mu\text{g m}^{-3}$  PM<sub>2.5</sub>,  $p < 0.05$ ) [239]. The Oxford street walk exposure resulted in significant reductions of lung function (FEV1 and FEVC), as well as elevation of airway acidification and sputum MPO, other inflammatory markers (neutrophils, IL-8) had an increasing pattern but differences were not significant [239]. These results indicate a higher respiratory impact of DE in asthmatics compared to the laboratory controlled chamber exposures, despite this study recording lower concentrations of air pollutants. Conclusive comparisons are not possible given the major differences in the study designs, however the findings imply that variables associated with real life DE exposures may enhance its adverse effects on the respiratory system.

**Table 2: Summary of Controlled Human Exposure Studies Quantifying Antioxidant Responses in the Respiratory Tract Following Exposure to O<sub>3</sub>, NO<sub>2</sub> and PM**

| Reference                                 | Pollutant       | Concentration                                                                      | # Participants<br>(state of health) | Exercise | Exposure Duration<br>(Endpoints) hours | Exposure Responses                                                                                                                                                            | Other Comment                                                                                                           |
|-------------------------------------------|-----------------|------------------------------------------------------------------------------------|-------------------------------------|----------|----------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|
| Kelly<br><i>et al.</i> , 1996<br>[127]    | NO <sub>2</sub> | 2ppm                                                                               | 44, healthy                         | Yes      | 4<br>(1.5, 6, 24)                      | 1.5h–BW: ↓ UA ↓ <b>AH</b> ↑ <b>GSH</b> (= GSH; in BAL)<br>6.0h; - BW: ↑ UA =AA ↑ <b>GSH</b><br>24h; -BW: =UA =AA = GSH (↑ <b>GSH</b> ; in BAL)<br>BAL: =MDA (all time points) | Each endpoint represented<br>a different BAL group, similar<br>hierarchy with BW                                        |
| Blomberg<br><i>et al.</i> , 1999<br>[210] | NO <sub>2</sub> | 2ppm                                                                               | 12, healthy                         | Yes      | 4 - 4 consecutive<br>(1.5, 6)          | 1.5and 6h: BW&BAL; =UA =AA =GSH =GSSG<br>=macrophages = lymphocytes = mast cells<br>↑ <b>neutrophils</b> (only in BW)                                                         | One time point (1.5h) was<br>examined for the air control and<br>was compared with both NO <sub>2</sub><br>time points. |
| Devlin<br><i>et al.</i> , 1999<br>[180]   | NO <sub>2</sub> | 2ppm                                                                               | 8, healthy                          | Yes      | 4<br>(16)                              | BAL: =UA =AA =GSH<br>↑ <b>GSH in BAL cells</b> (not fluid)<br>=FEV <sub>1</sub> = mast cells =lymphocytes                                                                     | Not enough BW<br>to quantify antioxidants                                                                               |
| Blomberg<br><i>et al.</i> , 1998<br>[130] | DE              | 300 µg/m <sup>3</sup> PM <sub>10</sub><br>(1.6ppm NO <sub>2</sub> ,<br>7.5ppm CO)  | 15, healthy                         | Yes      | 1<br>(NAL; 0, 1, 6)<br>(BW,BAL,BS;6)   | 1h NAL; =UA ↑ <b>AA</b> = GSH<br>6h NAL; = AA =UA = GSH<br>BW &BAL &blood; =AA = UA = GSH =MDA                                                                                | Repetitive sampling of NAL<br>resulted in significant washout<br>of GSH (air +DE) at 1h and AA<br>(air) at 1h and 6h.   |
| Mudway<br><i>et al.</i> , 2004<br>[203]   | DE              | 100 µg/m <sup>3</sup> PM <sub>10</sub><br>(0.6ppm NO <sub>2</sub> )                | 25, healthy                         | Yes      | 2<br>(NAL; 0, 1,6)<br>(BAL,BW; 6)      | 0,1h NAL: =UA =AA =GSH<br>6h NAL: = UA ↑ <b>AA</b> ↑ <b>GSH</b><br>6h BAL: = UA=AA=GSH<br>6h BW: =UA = AA ↑ <b>GSH</b> ↑ <b>neutrophil</b>                                    | Similar NAL washout effects<br>as study above.                                                                          |
| Behndig<br><i>et al.</i> , 2006<br>[131]  | DE              | 100 µg/m <sup>3</sup> PM <sub>10</sub><br>(0.4ppm NO <sub>2</sub> ,<br>10.4ppm CO) | 15, healthy                         | Yes      | 2<br>(18)                              | BAL: ↑ UA =AA ↑ <b>GSH</b> =IL-8 =MPO =IL-6<br>=neutrophils<br>BW: =UA =AA =GSH ↑ <b>IL-8</b> ↑ <b>MPO</b><br>=IL6(borderline p=0.06) ↑ <b>neutrophils</b>                    |                                                                                                                         |

NAL: Nasal Lavage, BW: Bronchial Wash, BAL: Bronchoalveolar Lavage, significant increase: ↑, significant decrease: ↓, =: no significant change. Statistically significant results are presented in bold to facilitate comparisons.

**Table 2: Summary of Controlled Human Exposure Studies Quantifying Antioxidant Responses in the Respiratory Tract Following Exposure to O<sub>3</sub>, NO<sub>2</sub> and PM (*contd.*)**

| Reference                                | Pollutant      | Concentration | Participants<br>#<br>(state of health)           | Exercise | Exposure Duration<br>(Endpoints) hours                                                     | Exposure Response                                                                                                                                                                                                                                      | Other Comment                                            |
|------------------------------------------|----------------|---------------|--------------------------------------------------|----------|--------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------|
| Mudway<br><i>et al.</i> , 1999<br>[128]  | O <sub>3</sub> | 0.2ppm        | 13, healthy                                      | Yes      | 2<br>NAL&blood; pre-expo.<br>1h into expo.<br>0, 1h post expo.<br>BS; 1.5h post expo.      | Pre-exposure: = UA = AA = GSH = neutrophils<br>1h into exposure: ↓ UA = AA = GSH = neutrophils<br>0h post exposure: ↓ UA = AA = GSH = neutrophils<br>1h post exposure: = UA = AA = GSH = neutrophils<br>↓ UA in NAL was correlated with ↑ UA in plasma | AA and UA decreases were observed due to washout.        |
| Mudway<br><i>et al.</i> , 1999<br>[129]  | O <sub>3</sub> | 0.2ppm        | 9, healthy                                       | Yes      | 2<br>BAL&blood; pre expo.<br>0, 6h post expo.                                              | BAL 6h post exposure: = UA ↓ AA = GSH<br>=MPO = neutrophils (but increasing pattern) =FEV<br>Association of AA with macrophage numbers (R=0.66, p=0.05) and total cell count (R=0.66, p=0.05)                                                          | Results of two participants were excluded, so .n=7.      |
| Mudway<br><i>et al.</i> , 2001<br>[102]  | O <sub>3</sub> | 0.2ppm        | 15, healthy controls<br>+<br>15, mild asthmatics | Yes      | 2<br>(6)                                                                                   | ↓ FEV ↑neutrophilia (but no difference between asthmatics and healthy) = UA<br>Baseline: AA & UA of asthmatics < healthy<br>6h post exposure, BW& BA: ↓ AA (healthy) = AA (asthmatic)                                                                  |                                                          |
| Behndig<br><i>et al.</i> , 2009<br>[205] | O <sub>3</sub> | 0.2ppm        | 15, healthy                                      | Yes      | 2<br>NAL&blood; pre-expo.<br>1h into expo.<br>0, 1h post expo.<br>BW&BAL&BS; 6h post expo. | NAL; =GSx ↓AA ↓UA –resolved after 6h<br>BW: ↑neutrophils=lymphocytes=macrophages(↑pattern)<br>BW: ↑UA ↑AA ↑GSx<br>BAL: ↑UA ↑AA ↑GSx ↑α-Toc ↑ EC-SOD<br>BS: ↑UA =AA =GSx                                                                                | NAL; only GSH had washout effects which persisted at 6h. |

NAL: Nasal Lavage, BW: Bronchial Wash, BAL: Bronchoalveolar Lavage, significant increase: ↑, significant decrease: ↓, =: no significant change. Statistically significant results are presented in bold to facilitate comparisons.

#### 1.4.2 Effect on Antioxidant Defenses

As mentioned in section 1.3.1 oxidative stress is thought to play a key role in the pathophysiological actions of the aforementioned pollutants, thus it is relevant to consider the pollutant's capacity to overwhelm antioxidant defences by reviewing their antioxidant impact within the controlled human exposure setting. A summary of relevant studies is presented in **Table 2**. These studies reveal complex antioxidant responses following exposure to air pollutants.

Indicatively, one of the few studies quantifying the response of low molecular weight antioxidants in the human RTLF to NO<sub>2</sub>, exposed healthy participants (n=44) to 2ppm of NO<sub>2</sub> for 4h in a double blind crossover design. Kelly and colleagues (1996) demonstrated that in the bronchial region there was a rapid decrease of uric acid and ascorbic acid at the 1.5h interval, at the following interval (6h) ascorbic acid concentration returned to baseline values while uric acid was elevated and the values returned to normal 24h after the exposure. Interestingly, the reduced glutathione levels showed a different pattern and were increased at both the 1.5 and 6h intervals [128]. Thus, this was one of the first studies to establish that NO<sub>2</sub> can modulate antioxidant responses in humans and that antioxidants present in the lung can react differently with NO<sub>2</sub> leading to diverse effects [128]. In a later study, the impact of consecutive exposures to NO<sub>2</sub> was investigated by exposing healthy participants to NO<sub>2</sub> on four successive days under the same conditions that were utilised in the aforementioned study. The results supported an attenuation of the antioxidant depletion capacity of the NO<sub>2</sub> as there were no significant changes in antioxidant concentration and only signs of increased neutrophil levels in the bronchial wash [240]. A similarly low reactivity of NO<sub>2</sub> was observed when healthy subjects were exposed to 2ppm of NO<sub>2</sub> for 4hours [214]. In this study there was no sign of respiratory obstruction (FEV), or changes in the antioxidant and inflammatory markers measured, there was however an increase in cellular glutathione of bronchoalveolar lavage at 16h [214]. Given that a single time point was used, prior changes cannot be excluded, however these studies support that NO<sub>2</sub> is less reactive than O<sub>3</sub>.

Indeed, exposure of healthy adults to O<sub>3</sub> at concentration levels that were 1/10 lower than the ones used for NO<sub>2</sub> resulted in a significant decrease of urate levels in nasal lavage collected 1h into the exposure, and immediately after the end of the study (2h) [129]. This response was interestingly correlated with an increase in urate plasma levels, potentially due to compensatory mechanisms [129]. The lack of ascorbate or

reduced glutathione oxidation may reflect the extreme washout of these moieties due to repetitive sampling and potentially an effect could have been masked [129]. A similar washout effect was not observed for urate possibly because it is known to be the dominant antioxidant in this region (by concentration) or because of different replenishment pathways. This heterogeneity of RTLF composition often results in different antioxidant responses depending on site of sampling. For example, this partly reflects why ascorbate was the only antioxidant to be significantly depleted in bronchoalveolar samples collected 6h post exposure [130]. Despite the reported changes in antioxidant levels following O<sub>3</sub> exposure, lung function decrements and baseline antioxidant concentrations do not appear to be predictive of the inflammatory and oxidative response [103, 241]. It is believed that rather than the absolute change in redox status, it is the exceedance of an adaptive response which leads to O<sub>3</sub>'s pathophysiological response, although the detailed mechanism requires elucidation.

A different antioxidant response pattern has been observed following human exposure to DE, although direct comparisons with the gaseous pollutants are not possible due to the compositional heterogeneity of the former. A small number of studies are available but these have utilised different time points and DE exhaust composition and thus conclusions are difficult to derive. It appears though that there is a flux of ascorbate and reduced glutathione following exposure to DE (100-300µg/m<sup>3</sup>, 1-2h) and these effects can persist until the last endpoint of 6h [131].

These studies show that DE exposure can modify the protective antioxidant pool in the respiratory system by causing the movement of antioxidants and by upregulating the transcription of redox-sensitive transcription. Given that NO<sub>2</sub> is one of the air pollutants present in DE, one could assume that not only can it have oxidative actions but, also its potency may increase when it is part of a mixture of air pollutants (i.e. lower concentration required to cause adverse health effects compared to single exposures). Nevertheless given that this is a mixture of air pollutants the results cannot be attributed to NO<sub>2</sub> *per se* lightly. Interestingly, Rudell *et al.* (1999) showed that healthy adults exposed to DE or DE filtered with a particle trap (reduced particles by 50%) did not differ in their BAL inflammatory response [156]. Thus, this study supported the toxicological importance of DE's gaseous component, unfortunately though antioxidant concentrations were not measured in this study. Replication of the same experimental protocols with and without the NO<sub>2</sub> component may shed light on the toxicity of NO<sub>2</sub>. Overall, given that there are single exposures reporting the oxidative actions of NO<sub>2</sub> it

seems appealing that NO<sub>2</sub> contributes to the oxidative effects observed in the aforementioned DE studies.

### 1.4.3 Conclusion

Controlled human exposures certainly offer a greater control of the dependent and independent variables than epidemiological studies. However, as the summary table indicates **Table 2** they have a small number of participants and limited time points. In addition almost all related respiratory studies have been conducted in healthy individuals during exercise conditions and such parameters are known to influence deposition [242]. Additionally, the participant selection criteria tend to be biased (usually university students associated with the study). Therefore, the generalisability of the findings is hindered.

Despite these limitations, chamber studies have elucidated the range of air pollutant concentrations that can lead to respiratory obstruction. In addition, chamber studies provide evidence that exposure to NO<sub>2</sub>, O<sub>3</sub> and PM modulates the antioxidant response in general, and the water soluble antioxidants ascorbate, urate and reduced glutathione in particular. A comparison of the gaseous exposures leads to the conclusion that O<sub>3</sub> is more reactive than NO<sub>2</sub> in the RTLTF as lower concentrations are required for a higher response. Though, given differences in experimental conditions (e.g. time points) it is not possible to specify the magnitude of that contrast. In addition, the difficulty in comparing the respiratory impact of the aforementioned gases with PM is highlighted in the presented studies. In controlled human exposures the compositional heterogeneity [243] of PM is further complicated by the presence of other pollutants in DE studies, while the more recently developed CAPs approach can be limited by the variability in size and composition [230]. Though, this last exposure model was not presented in the antioxidant section as there were no related investigations. Future studies, investigating the effects of the aforementioned pollutants by utilising identical protocols for the gaseous exposures and equivalent PM conditions could benefit the field.

## 1.5 Animal Studies

A prodigious number of studies have examined the impact of the gaseous pollutants of interest in animal models, studies utilised mainly rats and mice, but also other animals such as rabbits, sheep and dogs with the bulk of these studies occurring in the 60's and 70's (detailed reviews can be found by Mustafa [118] and Evans [244]) and clearly

demonstrate that most studies have employed concentrations outside the typical ambient range. A comparison of the two gaseous pollutant's effects reveals that O<sub>3</sub> can cause greater toxicological effects in the respiratory tract at lower doses [245]. The magnitude of their contrast is study dependent, though a 10-20 fold concentration difference is often cited [59]. In terms of site of action, NO<sub>2</sub> effects are more localized in the centriacinar region, while O<sub>3</sub> effects can extend more peripherally to include the entire alveolar duct and associated alveoli [245]. In contrast, the site of PM deposition is size dependent (as it was shown in **Figure 4**) and its toxic effects are heterogeneous and depend on its size and composition, with smaller (fine, ultra fine) city derived particles causing greater toxicological responses [243]. Despite compositional differences of the aforementioned pollutants, they are characterized by two common interrelated toxicological responses following exposure. Specifically, proinflammatory responses can be induced leading to increased permeability and oedema due to disruption of the respiratory epithelial barrier, processes which in turn are thought to be driven by the pollutant's oxidative properties [246-248].

#### **1.5.1 Protective Actions of Antioxidants in Animal Models**

Animal studies have shown that exposure to air pollutants can modulate the levels of antioxidant moieties and related compounds in the respiratory tract. Indicatively, several studies have reported increased levels of antioxidant defences following exposure to O<sub>3</sub> [249, 250], weekly exposures of rats (O<sub>3</sub>, 800ppb) elevated the concentration of reduced glutathione and glutathione peroxidase in bronchoalveolar lavage samples three days after the exposure, while the levels returned to baseline after another 7 days [251]. An increase of antioxidant defences was also observed in NO<sub>2</sub> and PM studies examining a potential association between exposure and markers of oxidative stress. For example, mice (13 weeks old) exposed to NO<sub>2</sub> (0.4, 1.2 and 4ppm) for four months presented 8, 35 and 72% higher lipid peroxidation than the control group and the oxidative effects were dependent on the concentration of NO<sub>2</sub> [252]. This increase in the oxidative marker was positively associated with elevated levels of the following protective enzymes; disulfide reductase, superoxide dismutase, 6-phosphogluconate dehydrogenase, glutathione reductase and peroxidase. However, the antioxidant enzymes presented a maximum at 4 weeks and then plateaued [252]. In a following study utilising the same experimental conditions, the long term pattern of antioxidant defences was investigated. Interestingly, the researchers observed that the

antioxidant enzymes associated with the NO<sub>2</sub> exposure drastically dropped after 9-18 months, reaching a full inhibition at the final measurement (27 months), this abrupt decrease was inversely associated with an abrupt augmentation of lipid peroxidation [253], though the exact pathway leading to the antioxidant enzyme's inhibition is not fully understood. In addition, at 27 months laboratory rats are towards the end of their life, thus it is especially challenging in animal studies to relate their age to human years. Similar short term changes of antioxidant defences have been observed for PM. In an innovative study, exposure of rats to CAPS (5h, 300µg/m<sup>3</sup>) resulted in significant increase of oxidative stress in the lung measured via *in situ* chemiluminescence [254]. Oxidant levels in the lung were linear with exposure time and were positively associated with the samples' metal content and levels of the antioxidant enzymes superoxide dismutase and catalase [254]. These studies provide evidence of the modulation of antioxidants *in vivo* by air pollutants and suggest a protective role but do not present direct evidence of protective actions.

This protective hypothesis was tested by Sun and colleagues (1988) and for this purpose the researchers supplemented mice with L-buthionine-S,R-sulfoximine, a compound which can inhibit by 50% glutathione production in tissues and exposed the animals to O<sub>3</sub> (0-1ppm, 3months) [255]. The results showed that the treatment group presented significantly higher proliferation and hypertrophy of non-ciliated epithelial cells in the terminal bronchioles and alveolar ducts as well as thickened walls and a higher influx of inflammatory cells [255]. While, the results provide evidence of the protective actions of reduced glutathione, unfortunately, the study did not quantify the levels of other antioxidant defences to determine if there was a compensatory upregulation. Further evidence of the shielding role of antioxidants was presented in a murine ovalbumin sensitisation model where the oxidative effects of adjuvant DEP intratracheal instillation were suppressed following intraperitoneal injection of thiols [256]. This supported the view, that antioxidant acquisition can inhibit air pollution driven oxidative stress. Indeed, a large number of studies have supported the protective role of an antioxidant rich diet against O<sub>3</sub>'s, NO<sub>2</sub>'s and PM's oxidative effects and likewise showed increased susceptibility associated with ascorbate and glutathione deficiency [257-261]. This body of evidence supporting antioxidant supplementation contradicts the plentiful negative findings in human exposures, though differences could be attributed to the added complexity of human exposures and potentially to interspecies differences.

### 1.5.2 Conclusion

The animal studies have provided detailed information of the morphological and biochemical changes occurring upon inhalation of air pollutants. However, the concentration range used overall is much higher than the human chamber and epidemiological studies; in addition the animals are in a resting physical condition which further complicates data extrapolation. Quantitative comparisons of the air pollutant's oxidative effects are rather challenging given differences in study methodologies. All in all, the data appear to support the causal role between air pollutant's health effects and oxidative stress. Future studies could quantitatively compare the major air pollutant's oxidative effects under the same controlled conditions in order to facilitate comparisons and delineate potential differences in oxidative pathways.

### 1.6 *In vitro* Studies

*In vitro* studies are fundamental in establishing the biochemical and molecular pathways leading to the biological outcomes observed in human and animal studies. The deleterious effects of NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> have been extensively investigated *in vitro*. The emphasis however of this section is again on the respiratory system given its association with the study's aims.

The pulmonary proinflammatory effects of O<sub>3</sub> and NO<sub>2</sub> *in vivo* are also evident *in vitro* [262-264]. In a comparative study, Bayram *et al.*, (2001) exposed bronchial epithelial cells of asthmatics and non asthmatics to O<sub>3</sub> (10-100ppb) and NO<sub>2</sub> (100-400ppb) for 6 hours [148]. The results showed that O<sub>3</sub> and NO<sub>2</sub> at concentrations higher than 50ppb and 200ppb respectively resulted in release of proinflammatory cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), soluble cell adhesion molecule 1 (sCAM-1) and interleukin 8 (IL-8). In addition, only asthmatic cells released RANTES a chemokine stimulating eosinophils, also GM-CSF and sICAM-1 levels were significantly higher [148]. Unfortunately, the authors did not utilize the same O<sub>3</sub> and NO<sub>2</sub> concentrations and did not compare statistically the effects of the two pollutants; it appears though that NO<sub>2</sub> caused the same responses as O<sub>3</sub> at a 10 times higher concentration. In accordance with these findings, DEP (50 to 100 micrograms/m<sup>3</sup>) resulted in equivalent pro-inflammatory effects and more elevated release of inflammatory mediators in asthmatic derived cells [265]. More recently, an increased inflammatory response of asthmatics was replicated in epithelial cells derived from

children, also, an important finding was that this susceptible group had significantly higher levels of a pro-remodelling cytokine (vascular endothelial growth factor) at baseline but also after exposure to ambient PM (SRM 1648a) [266]. Thus, *in vitro* studies support an elevation of pro-inflammatory pathways in response to air pollutants, these responses may be more elevated and complex in individuals with respiratory disease.

### **1.6.1 Oxidative Effects in Respiratory Tract Lining Fluid Models**

The interaction of the air pollutants with the RTLF is particularly important as the respiratory tract is the portal entry of air pollutants and this thin layer of fluid is its first line of defence. It has been demonstrated that NO<sub>2</sub> and O<sub>3</sub> uptake is entirely dependent upon the reaction of the gaseous pollutants with the antioxidant moieties a process that was firstly described by Postlethwait's group as 'reactive absorption' [52, 53]. Thus, the rate of absorption is positively associated with the antioxidant levels, however this does not imply a greater injury as oxidants can be quenched, indeed a lower rate of absorption may occur at lower concentrations but the reactive species can induce greater oxidative actions to the cell membrane [267].

*In vitro* RTLF models have shown that NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> induced antioxidant depletion. Specifically, Kelly and Tetley (1997) exposed RTLF samples taken as bronchoalveolar lavage (BAL) fluid from 12 volunteers to increasing concentrations of NO<sub>2</sub> (50-1000ppb) for up to 4 hours [268]. In addition, they investigated the effects of NO<sub>2</sub> in single and combined antioxidant solutions with relevant RTLF antioxidant concentrations under the same exposure conditions [268]. The researchers found that urate and ascorbate levels were depleted at similar rates across all three different models, while reduced glutathione consumption was significantly lower [268]. In support of the protective actions of antioxidant moieties, malondialdehyde (MDA) levels increased only after the 60min exposure, suggesting that lipid peroxidation was prevented until a significant decrease of the antioxidant levels had occurred [268].

In addition, Mudway *et al.*, (1998) exposed a similar RTLF model to O<sub>3</sub> and demonstrated time and concentration dependent depletion of urate, ascorbate and reduced glutathione, the latter antioxidant was again the least significant target while urate had a higher reactivity trend but did not differ significantly from ascorbate [269]. Further *in vitro* studies employing RTLF models denote that the reactivity hierarchy of the antioxidant moieties is air pollutant specific. Indicatively PM<sub>10</sub> has consistently

shown high reactivity towards ascorbate, less so towards reduced glutathione and no effect on urate levels [111, 112, 115, 270, 271]. These results suggest that the RTLF antioxidants are likely to act as sacrificial substrates *in vivo* in response to air pollutants.

### 1.6.2 Conclusion

The *in vitro* studies provide a level of simplification that facilitates the direct investigation of the air pollutants' interaction with the antioxidant moieties. Interestingly, despite more realistic air pollutant concentrations being utilised, the oxidative actions in the respiratory tract epithelium and fluid were still observed. However, it should be acknowledged that the *in vitro* studies are not able to consider the antioxidant replenishment that is likely to be taking place, cannot investigate the full spectrum of antioxidant defences present in the RTLF or the impact of chronic exposures. Nevertheless, a direct comparison of the pollutant's effects in these studies would be useful as it could provide information on their relative toxicological impact; unfortunately this is not possible as there are important differences in the exposure protocols. This research challenge has formed the basis of the present thesis.

## 1.7 Study Rationale

**Nitrogen Dioxide: simply a marker of traffic and air pollution or a significant respiratory health threat on its own right?**

Air pollution is obviously a fundamental problem; it can affect all facets of life from the environment and politics to health and life expectancy [272]. Therefore, the role of regulating bodies such as the European Commission that set the permissible levels for the different air pollutants is of imperative importance. However, setting effective regulations that will protect the public implies an excellent understanding of the toxicity and pathophysiological actions of the different air pollutants. Such an understanding has not been reached for NO<sub>2</sub> and this is the basis of the current PhD project.

Specifically, there are three major air pollutants that warrant consideration from a public health viewpoint; O<sub>3</sub>, PM and NO<sub>2</sub>. Studies examining the respiratory effects of O<sub>3</sub> have shown that it can affect lung function and cause respiratory symptoms [73]. In addition studies have demonstrated that PM –a diverse suspension of fine liquid and/or liquid particles - can have significant negative respiratory effects [273] although its effects depend on its composition which is characterized by seasonal and temporal variation [274]. The toxicological information on NO<sub>2</sub> is far more limited while the

existing studies do not offer tangible information on its toxicity and mechanisms of action. As presented in the literature review above, there are questions over the chosen concentrations for both the animal and *in vitro* studies while the epidemiological studies offer tentative conclusions.

Given that the levels of NO<sub>2</sub> and the other air pollutants are highly correlated a key issue is how can we possibly ascertain that the effects observed in the epidemiological studies are due to the actions of NO<sub>2</sub> *per se*? One can possibly argue that NO<sub>2</sub> is acting as a surrogate. At the same time, one cannot exclude the possibility that unexplored additive effects are taking place. Thus, the three major air pollutants need to be tested within the same experimental system in order to collect reliable data on the relative toxicity of NO<sub>2</sub> and this is precisely the aim of the current PhD project. During this project the aforementioned air pollutants were tested within the same experimental set up, while also concomitant exposures were considered.

The respiratory tract lining fluid (RTLFL) was modelled given the fact that it is the first physical interface that the air pollutants encounter when they are inhaled. It constitutes a 'first line of defence' [80] which protects the underlying tissue from inflammatory reactions [275]. It consists of varied groups of substances and it covers the respiratory tract epithelial cells (RTCEs) from the nasal mucosa to the alveoli [80]. Specifically, it has been shown that it contains proteins, mostly albumin [80, 81], antioxidant enzymes and metal binding proteins [276] as well as antioxidants, including, ascorbic acid, reduced glutathione, and uric acid [74]. The presence of these antioxidant defences is particularly important (**Figure 6**) given that air pollutants are thought to cause their pathophysiological actions through oxidative stress [79].

Indeed previous studies have demonstrated the oxidative actions of the aforementioned pollutants in RTLFL models [268, 269, 277], however, no study thus far has utilized identical exposure conditions for the gaseous pollutants and equivalent for PM<sub>10</sub>. Comparisons tend to be restricted in the laboratory conditions without considerations of the pollutants' real life concentrations. In addition, a major challenge has been the investigation of PM effects within the same experimental model and a comparison of its effects with the gaseous pollutants due to a) PM<sub>10</sub>'s solid state which is different from the gaseous pollutant's state, b) PM<sub>10</sub>'s compositional heterogeneity.

These scientific challenges were addressed by quantifying the antioxidant depletion capacity of PM<sub>10</sub> samples concomitantly collected by two contrasting locations and by developing an antioxidant depletion rate expression which allowed the consideration of

the pollutants' real life concentrations and ultimately direct comparison of the antioxidant depletion capacity of the different pollutants in the same RTLF model.

In summary, the aims of this thesis were the following:

- ✚ Quantification of the antioxidant depletion capacity of  $\text{NO}_2$ ,  $\text{O}_3$  and  $\text{PM}_{10}$  in the same RTLF model containing realistic concentrations of the water soluble antioxidants, plausible ambient concentrations of the air pollutants and physiological conditions (temperature, pH).
- ✚ Delineation of the reactivity hierarchy of the three major water soluble antioxidant moieties towards the air pollutants examined.
- ✚ Direct comparison of  $\text{O}_3$ 's and  $\text{NO}_2$ 's antioxidant consumption on a unitary basis by employing identical exposure conditions.
- ✚ Development of an experimental protocol allowing direct comparison of  $\text{O}_3$ 's,  $\text{NO}_2$ 's and  $\text{PM}_{10}$ 's oxidative effects based on the pollutants' real life concentrations.

## **2 Materials and Methods**

### **2.1 Materials and Antioxidant Quantification Methods Utilized for the Exposure of a Human RTLF Model to Different Air Pollutants**

#### **2.1.1 Chemicals**

All chemicals used were obtained from Sigma Chemical Company Ltd (Poole, UK) and were of high pressure liquid chromatography grade. For chemical antioxidant depletion analysis, 18.2 MΩ-cm Millipore distilled deionised water was used, ultra-filtered using an Elga-stat filtration system and further treated with Chelex-100 resin to eliminate any background metal contamination.

#### **2.1.2 Chelex Water Preparation**

All exposures were carried out using Chelex treated water in order to minimise the presence of metal contaminating ions. It was prepared by the addition of 3g of Chelex-100 (Sigma C7901) to deionised Elga-star water (100ml). The solution was left to stir for 24 hours in a plastic beaker. Afterwards, the Chelex resin was removed by vacuum filtration using a 0.45µm cellulose nitrate membrane. Finally, the pH was corrected to pH 7 using 1M Chelex treated hydrochloric acid (HCL) and 1M Chelex treated sodium hydroxide (NaOH). The Chelex-treated water was stored at 4°C for no longer than one month.

#### **2.1.3 Synthetic Respiratory Tract Lining Fluid (SynRTLF)**

Physiologically relevant concentrations of the three major water soluble antioxidants present in the RTLF; ascorbic acid, uric acid and reduced glutathione were prepared based on their reported concentrations. It should be noted that while urate may potentially have a higher concentration than the other two antioxidants, uncertainty surrounds the antioxidant's absolute concentrations given that these values are characterized by very large standard deviations, and there is also a large range of values associated with different sites of the respiratory tract. For this reason, more recent synRTLF studies, including this one, have utilized an equal concentration (200µM) of antioxidants in their model [111, 112, 271, 278-281], as this approach is more representative of the entire RTLF.

#### **2.1.3.1 Materials for Stock Preparation**

1. Ascorbic acid
2. Reduced glutathione
3. Uric acid
4. 50% Metaphosphoric acid
5. 1M NaOH
6. 1M HCL

#### **2.1.3.2 Stock synRTLf preparation**

SynRTLf stock was prepared containing 2mM ascorbic acid, reduced glutathione and uric acid. This stock was diluted 1 in 10 (450µl Chelex treated water and 50µl stock) on the day of the exposure in order to give a physiologically representative concentration of 200µM [73, 74]. For the synRTLf stock preparation, high concentration stocks (50mmol/L) of ascorbic acid and reduced glutathione were prepared by the addition of 440mg and 767mg respectively in 50ml of Chelex treated water (pH 7). Aliquots were dispensed into individual 15ml falcon tubes and stored at -80 °C. The concentration of the stocks was verified against standards using the High Pressure Liquid Chromatography (HPLC) analysis for ascorbic acid and the glutathione assay for GSH (the HPLC and glutathione methods are described further down). Uric acid (33.33mg) was added to 90ml of ultrapure Chelex resin treated water and was heated until the boiling point was reached. Once uric acid was dissolved it was removed from the hot plate and was left to cool at room temperature. Soon after, the quantified stocks of ascorbic acid and reduced glutathione were added in order to give a final concentration of 2mM. The pH was adjusted to pH7 using 1M Chelex treated HCL and 1M Chelex treated NaOH. Finally, 5ml aliquots were dispensed into individual 15ml falcon tubes and stored at -80 °C.

#### **2.1.4 Quantification of Ascorbic Acid and Uric Acid Concentration**

Determination of ascorbic acid and uric acid concentrations were performed using reverse phase HPLC with electrochemical detection [282].

##### **2.1.4.1 HPLC Analysis; Materials**

1. Dipotassium phosphate
2. Octane sulphonic acid

3. Ortho-phosphoric acid
4. Metaphosphoric acid
5. Ascorbic acid
6. Uric acid
7. HPLC grade water

#### **2.1.4.2 HPLC Analysis; Pollutant exposed synRTLF sample Preparation**

On the day of the experiment, HPLC vials were filled with 400µl ice cold HPLC grade water and 50µl ice cold 50% metaphosphoric acid (MPA) just before the addition of 50µl of the exposed synRTLF solution or controls, as this is known to stabilise ascorbic acid [283]. Vials were frozen at -80°C and placed on the HPLC for analysis within a week.

#### **2.1.4.3 Preparation of Standard Curve**

1mM uric acid was prepared in 5% MPA (0.0168g in 100mls 5%MPA) and was heated until boiling point was reached. This solution was then allowed to cool until it had equilibrated to room temperature. At that point 8.8mg of ascorbic acid was added to the 50mL uric acid 5% MPA solution. Further 50mls of the uric acid stock solution were added, resulting in a total volume of 100ml (final stock concentration of 500µM ascorbic acid and 1mM uric acid). Standard curves were constructed by dilution of the stock concentration with 5% MPA solution. The precise dilutions can be found on **Table 3**. 700µls of each standard were aliquoted into HPLC vials and stored at -80°C, typical standard curves are shown in **Figure 7**.

|                              | Std. 1 | Std. 2       | Std. 3 | Std. 4 | Std. 5 | Std. 6 |
|------------------------------|--------|--------------|--------|--------|--------|--------|
| 5% MPA (mls)                 | 10     | 5            | 9.938  | 9.875  | 9.75   | 9.5    |
| 500µM AA/ 1mM UA stock (mls) | 0      | 5mls of Std3 | 0.0625 | 0.125  | 0.25   | 0.5    |
| Final [AA] (µM)              | 0      | 1.57         | 3.13   | 6.25   | 12.5   | 25     |
| Final [UA] (µM)              | 0      | 3.13         | 6.25   | 12.5   | 25     | 50     |

**Table 3: Final Ascorbate and Urate Concentrations for the Six Standards**

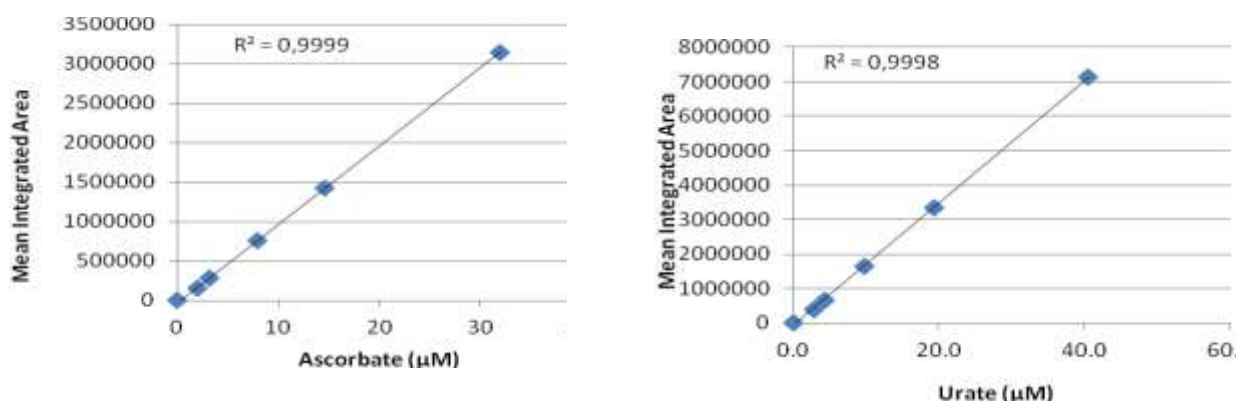


**Figure 7: Typical Standard Curves for Ascorbate and Urate**

#### **2.1.4.4 Running Conditions of HPLC**

A thermostatically cooled Gilson model 231 auto-sampler was used to inject 20μL of each sample onto a 25cm, 10 x 300nm, 5μm C18 column, eluted with a 0.2 mol/L K<sub>2</sub>HPO<sub>4</sub>-H<sub>3</sub>PO<sub>4</sub> buffer containing 0.25 mM octanesulphonic acid (pH 2.1) at a flow rate of 1.5ml/min. Electrochemical detection was performed using an EG&G electrochemical detector (Jones Chromatography Ltd, Hengoed, Wales) and was set at a 1100mv electrode voltage, time constant of 5 seconds, and had cathodic output and sensitivity of 1μA. The Unipoint system software was used in order to integrate the chromatograms, sample concentration was proportional to the standard curve.

#### **2.1.4.5 Intra- and Inter- Assay Variation**

Given that a very large number of HPLC runs were conducted in relation to this project and the runs also varied in length depending on the number of samples quantified it was important ensure that there was stability within each run and across different ones. For this reason, standards were re-injected at the middle and end of every run. These controls revealed that within each run, standard reinjection did not differ significantly from the initial chromatograms and similarly across different runs these values differed by less than 5%, thus ensuring internal and external reliability.

### 2.1.5 Quantification of Reduced Glutathione and Oxidised Glutathione Levels

Reduced glutathione (GSH) and glutathione disulphide (GSSG) levels of the synRTLTF samples exposed to PM, O<sub>3</sub>, and NO<sub>2</sub> were quantified according to the method described by Baker (1990) [284]. This assay was conducted in two stages: the first stage measured total glutathione (GSx), while the second was limited to quantifying only the GSSG concentration. By measuring these two species, the remaining GSH concentration could then be calculated: by using the following formula: **GSH = GSx - 2GSSG**.

Determination of GSx and GSSG were based on the 5,5-dithiobis-2-nitrobenzoic acid (DTNB). Briefly, GSH reacts non-enzymatically with DTNB to generate GSSG and the highly coloured ion 5-thio-2-nitrobenzoic acid (TNB) which can be followed spectrometrically [285]. In addition, the GSSG formed is back-reduced to GSH by glutathione reductase coupled to NADPH oxidation, and this reaction also reduces any GSSG originally present to GSH. The concentrations of reactant concentrations are chosen so that the rate of colour formation is linear with time (slope line:  $\Delta\text{absorbance}/\Delta\text{time (min)}$ ;  $\Delta A/\Delta t \text{ (min)}$ ) [285]. This provides a measurement of both GSH and GSSG, effectively providing the quantification of GSx. To determine GSSG concentration, 2-vinyl pyridine was added to samples and standards to conjugate any GSH present and prevent its reaction with DTNB. Any TNB present in this second stage will have come only from reduction of GSSG. Thus, the GSH present in the exposed synRTLTF solutions was calculated by the subtraction of GSSG (x2) from the total GSx concentration.

#### 2.1.5.1 Quantification of Glutathione Levels; Materials

1. NADPH
2. DTNB
3. 2-vinyl pyridine
4. Glutathione disulphide
5. Glutathione reductase
6. Sodium phosphate buffer

### 2.1.5.2 Preparation of Standard Curves for Glutathione Analysis

A series of GSSG standards were prepared by diluting a 3.3  $\mu\text{M}$  stock solution; ranging between 0-165pmol/L, equivalent to 0-330pmol/L GSx. Typical standard curves and the exact GSx and GSSG concentrations can be found at **Figure 8** and **Table 4** respectively

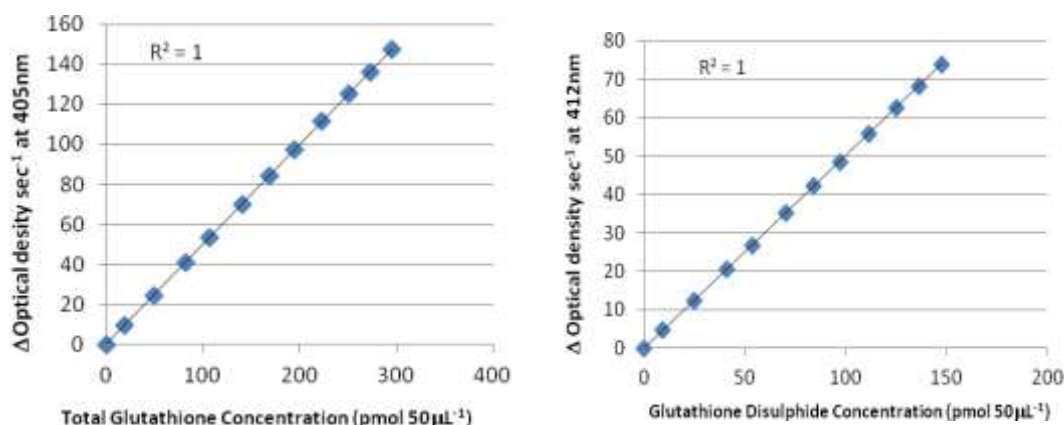


**Figure 8: Typical Standard Curves for Reduced Glutathione and Glutathione Disulphide**

| Standard                               | 1    | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12   |
|----------------------------------------|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| GSSG stock ( $\mu\text{L}$ )           | 0    | 91  | 182 | 273 | 364 | 455 | 546 | 636 | 727 | 818 | 909 | 1000 |
| $\text{PO}_4$ buffer ( $\mu\text{L}$ ) | 1000 | 909 | 818 | 727 | 636 | 546 | 455 | 364 | 273 | 182 | 91  | 0    |
| GSx pmols/50 $\mu\text{L}$             | 0    | 30  | 60  | 90  | 120 | 150 | 180 | 210 | 240 | 270 | 300 | 330  |

**Table 4: Exact Concentrations of the Glutathione Standards**

### 2.1.5.3 Quantification of Glutathione Assay Procedure

16.7 $\mu\text{L}$  aliquots (particle free supernatant in the case of  $\text{PM}_{10}$ ) were added to 2ml microcentrifuge tubes containing 983 $\mu\text{L}$  of 100mM sodium phosphate buffer (pH 7.5, 1mM ethylenediaminetetraacetic acid). The eppendorfs were vortexed and 50 $\mu\text{L}$  were transferred into a well on a 96 well-plate. Immediately afterwards a reaction mixture containing DTNB (0.15mM), 1U glutathione reductase (Baker's Yeast) and daily

prepared NADPH (0.2mM), was added and the well-plate was placed on the microplate reader (405nm, SpectraMAX 190; Molecular Devices) for two minutes at 30°C.

The concentration of GSSG was determined by conjugation of undiluted 2-vinyl pyridine (5  $\mu$ L) and 130  $\mu$ L of samples or controls in 2 mL micro-centrifuge tubes. This solution was briefly vortexed for 5 seconds and was incubated for 1 hour at room temperature in a fume cupboard. Following incubation, aliquots of 50 $\mu$ L of the derivatised standards and samples were transferred into a new 96 well plate and the concentration of GSSG was measured as an increase in absorbance at 412nm over two minutes at 30°C. All samples were analysed in duplicate.

The GSSG was then subtracted from the total GSH to give the reduced GSH using the equation described earlier.

## **2.2 Modelling the Interactions of Nitrogen Dioxide, Ozone, and Particulate Matter with the Synthetic Respiratory Tract Lining Fluid**

The study rationale and general hypothesis to be tested in the thesis has been outlined previously (Chapter 1). Briefly, quantification of the antioxidant depletion capacity of NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> in an RTLFL model was chosen as this is the first physical interface encountered by any inspired agent and for its content of a range of protective biochemical species [51, 73, 79, 81, 286, 287].

In the following sections the two principal challenges were addressed a) developing a protocol that quantified the antioxidant depletion capacity of the aforementioned pollutants in an RTLFL model while employing the same experimental conditions to the best possible extent b) expressing these findings in a way that allowed quantitative comparisons between the gaseous exposures and PM.

### **2.2.1 Exposure of the Respiratory Tract Lining Fluid Model to the Gaseous Pollutants**

Exposures were performed using a 5.6L purpose built chamber flushed with known O<sub>3</sub> and concentrations (0, 50, 150, 400, 1000ppb), at a flow rate of 3Lmin<sup>-1</sup>. Three independent exposures were performed for each concentration. PTFE tubing was used as it is a flexible, thermoplastic and highly resistant to oxidizing agents, and similarly all fittings were stainless steel. Inside the chamber a 24 well plate (1.9cm<sup>2</sup> round wells, Nunc<sup>TM</sup>, Thermoscientific) made of oxidizing resistant material was placed and 2mL of

synRTLF (200 $\mu$ M) were transferred in three wells (for triplicate collection at each time point). In addition, the chamber was maintained at 37°C throughout the exposure using a heating pad and the mixing was carried out by placing the exposure chamber on an orbital shaker (rotary shaker, Rotatest *R100*, Luckham) at a frequency of 2.3Hz. At set time intervals (0, 30, 60, 90, 120mins) the chamber was very speedily opened and samples were collected in triplicate in order to quantify the change in antioxidant depletion, after practice this process took 30s, and were immediately placed in a -80°C freezer. A schematic representation of the gaseous pollutant exposures is provided in **Figure 9**.

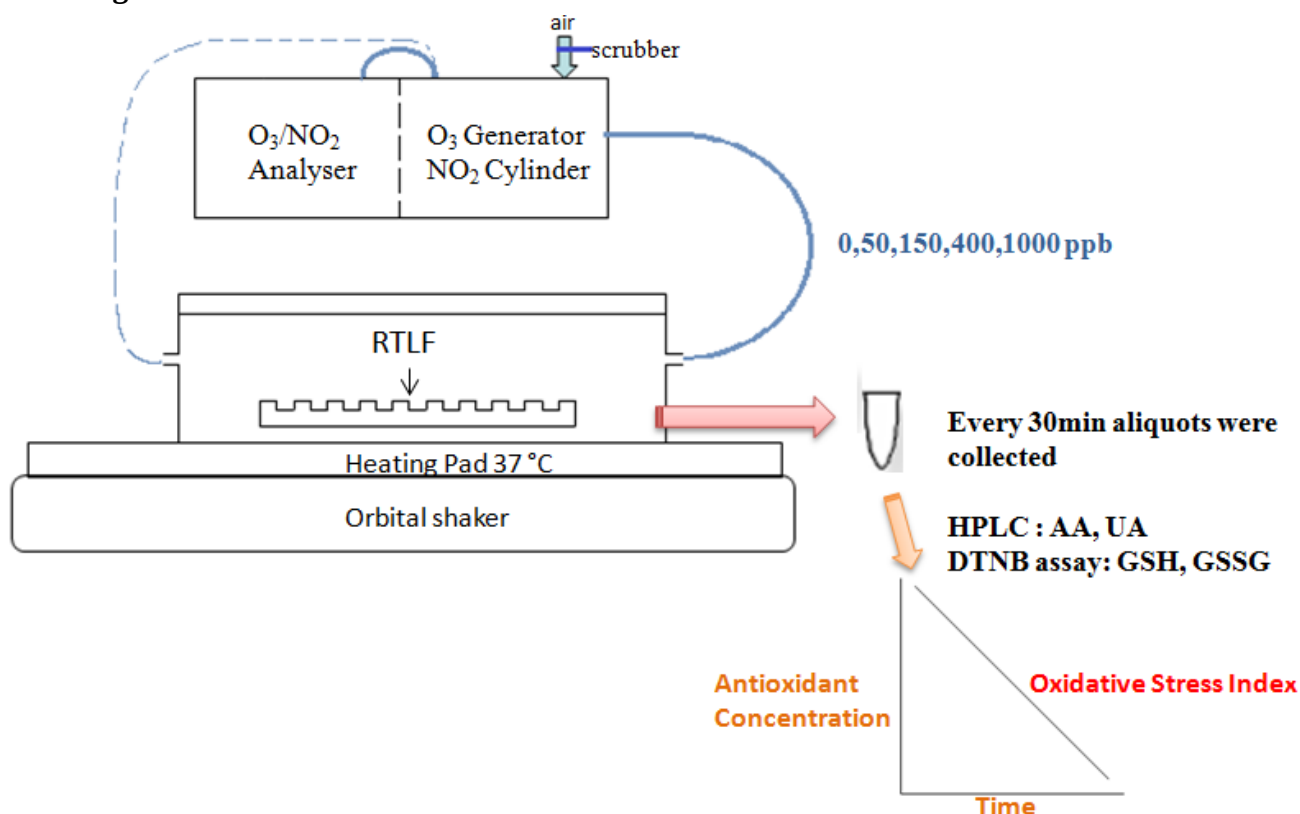


**Figure 9: Schematic Representation of Apparatus and Methodology used for the Exposure of the RTLF Model to the Gaseous Pollutants.**

Briefly, O<sub>3</sub> was generated by UV radiation of air source, and similarly an NO<sub>2</sub> cylinder of certified concentration was used for the NO<sub>2</sub> exposures, the cylinder's output was mixed with air in order to achieve the desired concentration (50,150,400,1000ppb). The gaseous levels of the generating sources were monitored via a connecting tube loop presented in the diagram. Further tubing connected the gaseous source to the exposure chamber containing the RTLF model. The dotted tubing line demonstrates that the chamber exhaust was monitored in pilot studies and further control experiments as well as further random points. An automatic regulation of the gaseous concentrations based on the exhaust measurements was not chosen following consultation with the manufacturer's engineer, as it could potentially lead to an overestimation of the gaseous concentrations. The temperature and mixing rate were controlled. At set time intervals, synRTLF samples were removed and transferred to an -80C freezer. Analysis of the samples allowed the quantification of antioxidant depletion over time.

### ***2.2.1.1 Choice of Concentrations***

The same concentrations were used for the O<sub>3</sub> and NO<sub>2</sub> experiments in order to facilitate comparisons of exposures (0, 50, 150, 400 and 1000ppb) and these were chosen on the basis of a) presenting realistic to extreme but attainable concentrations, b) there was an interest in replicating the concentrations used by previous relevant studies [268, 269] c) in pilot studies, the accuracy of the O<sub>3</sub> analyser and generator dropped below the 50ppb point as the UV lamp was flickering and the equipment could unexpectedly shut down. Lastly, it should be noted that the chosen concentrations constitute the lowest ones in this research area.

### ***2.2.1.2 Ozone Generation***

Ozone was generated by means of a mercury vapor lamp producing ultraviolet light at the 185nm wavelength (Dasibi Ltd, Dynamic Gas Calibrator, model 5008). The predetermined O<sub>3</sub> air flow (3L/min) was controlled by an internal needle valve and was monitored by an electronic flow sensor, the reading error estimate for the air flow was  $\pm 2\%$ . The amount of ozone produced was proportional to the intensity of the 185nm light, which is in turn dependent on lamp current. The intensity of the generators' lamp was controlled by the internal U.V. photometer. The latter operated by detecting the absorption of U.V. radiation at the 245nm wavelength by O<sub>3</sub> molecules as they passed through the absorption cell. The photometer-feedback feature measured the O<sub>3</sub> concentration output of the generator and sent a signal back to the O<sub>3</sub> generating lamp until the desired concentration was achieved. The reading error estimate was  $\pm 5\%$ . The photometer- feedback system was employed rather than the detector feedback system, where the chamber output is connected to the equipment, as reaction of O<sub>3</sub> with the antioxidants can potentially result in a lowering of the O<sub>3</sub> levels and thus a false feedback system that would result potentially in an unwanted increase in the ozone concentration and, thus, an overestimation of the effect. While, the O<sub>3</sub> output was not monitored continuously, the gaseous concentration was recorded prior to the start of the experimental series (in pilots replicating the experimental conditions) as well as during random points of the experiments and after the completion of all exposures in order to a) verify the system stability after lamp usage b) investigate the time required for the chamber to regain the concentration that it had prior to the fast opening for sample collection. The results showed that the system was behaving in a steady, predictable

way and for the highest concentrations the concentration in the chamber was regained within 3 minutes (**Figure 10**).

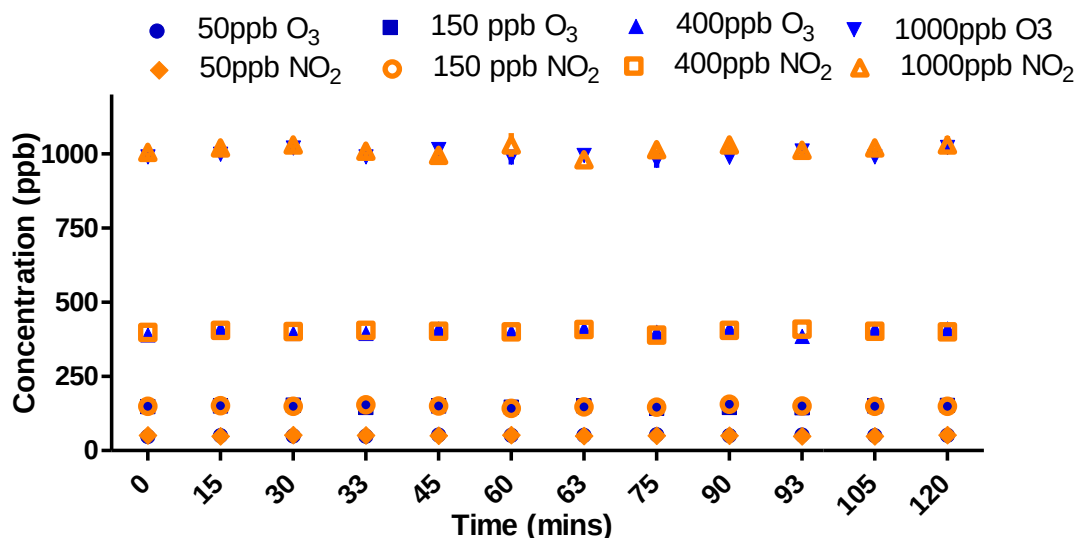


**Figure 10: Verification of Gaseous Chamber Stability**

Data represent mean  $\pm$ SD, n=5. A major target of the project was to maintain gaseous exposure stability, quantification of the gaseous concentrations showed that the levels were within 5% of the set concentrations at all tested time points, and within 3 minutes of opening the chamber concentration had been regained. Constant measurement of the gaseous levels inside the chamber was not possible for reasons given in the methods section; however other important data (e.g. proper functioning of mercury lamp, correct measurement of NO<sub>2</sub> from the certified cylinder by the NO<sub>x</sub> analyser) supported this. The gaseous concentration inlet was measured constantly; this tubing was briefly disconnected and attached to the exhaust in order to measure the output of the chamber. The results above present the quantification of all major time points at five exposures (2 pilots with synRTLTF in chamber before the start of the experiments in order to establish the well functioning of the equipment, 1 pilot without synRTLTF in well plate, 2 experiments). Further measurements were conducted at random points in the experimental series (and pilots) and were in agreement with the stability and reliability of the equipment.

Furthermore, it should be noted, that an accurate mechanical operation of the equipment was certified by a specialist prior to the start of the experimental series. Also, the relationship between the lamp intensity and O<sub>3</sub> generation was examined and indeed there was a linear, consistent function presented in **Figure 11**.

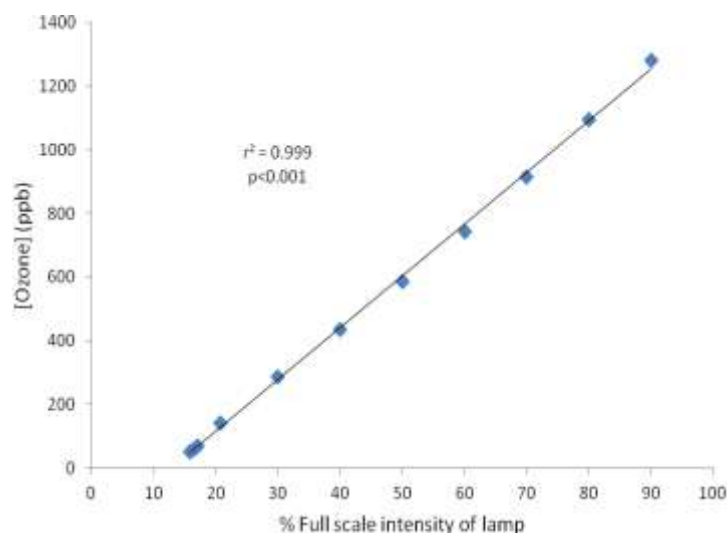
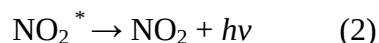


**Figure 11: Ozone Production vs. Mercury Lamp Intensity**

A highly linear and consistent relationship ( $n = 6$ ) was observed between the intensity of the mercury lamp (percentage of full scale) and the  $O_3$  generation

### 2.2.1.3 Nitrogen Dioxide Generation

$NO_2$  was derived from a cylinder (1500ppb in synthetic air, Air Liquid UK Ltd) fitted with a two stage gas regulator, allowing consistent pressure flow. PTFE tubing was connected via a t- junction with an equivalent tube carrying scrubbed air (kynar body filled with granular adsorption material capturing airborne chemicals and gaseous pollutants). The output of the air and  $NO_2$  sources was adjusted so that a total  $3L\ min^{-1}$  flow was achieved (the same as in the  $O_3$  exposures) and the correct concentration was established through the use of a chemiluminescent nitrogen oxides ( $NO_x$ ) analyzer (Dasibi, model 2108). Concentrations of  $NO_2$  were quantified indirectly through the chemiluminescent reaction of nitric oxide ( $NO$ ) with  $O_3$ . Specially, the instrument measures the light intensity of the chemiluminescent gas phase reaction of nitric oxide as follows:



As shown in the first equation (1) the reaction of  $NO$  with  $O_3$  results in electronically excited  $NO_2$  molecules. These excited  $NO_2$  molecules release their excess energy by emitting a photon and dropping to a lower energy level as shown in the second equation (2). This light intensity produced is directly proportional to the  $NO$  concentration present. Specifically, the analyser samples the gas stream and measures the  $NO$

concentration by digitising the signal from the analyser's photomultiplier tube. A valve then routes the sample stream through a converter containing heated (315°C) molybdenum (Mo) to reduce any NO<sub>2</sub> present to NO ( $3 \text{ NO}_2 + \text{Mo} \rightarrow 3 \text{ NO} + \text{MoO}_3$ ). The built in computer subtracts the NO<sub>x</sub> and NO values, thus yielding the NO<sub>2</sub> concentration. The equipment's proper functioning was investigated prior to every exposure by flushing the analyser with NO derived from a cylinder of certified NO concentration. This concentration verification preceded the exposures and lasted 30 minutes in order to ensure stability, an NO<sub>2</sub> concentration which was within  $\pm 5\%$  of the desired concentration was deemed acceptable. Similarly to the O<sub>3</sub> generator, the NO<sub>x</sub> analyser had undergone service prior to the commencement of the experimental series. Also, similarly to the O<sub>3</sub> exposures, the NO<sub>2</sub> output from the chamber was not monitored continuously, checks had been conducted at the start, end, as well as further random time points via the NO<sub>x</sub> analyser detector system in order to a) verify the system stability, b) investigate the time required for the chamber to regain the concentration that it had prior to the fast opening for sample collection. The results showed that the system was behaving in a stable, expected manner and NO<sub>2</sub> concentrations was regained within 3 minutes of the chamber opening.

#### ***2.2.1.4 Set Up Used for Combined Gaseous Exposures***

The RTLF model was concomitantly exposed to O<sub>3</sub> and NO<sub>2</sub>, a simplified diagram of the exposure set up is provided below. The principal air flow meters are also marked. The NO<sub>2</sub> source (1500 ppb) was mixed with air in order to achieve a constant concentration (1000ppb or 100ppb) at a set air flow (1.5 lmin<sup>-1</sup>), similarly the O<sub>3</sub> generator was programmed to produce O<sub>3</sub> at a set concentration and air flow (1.5 lmin<sup>-1</sup>). The two separate gaseous stream lines were connected via a T junction that was attached on the exposure chamber containing RTLF in this case, or PM disc cuts in the PM related experiments. The chamber was placed on a rocking platform and a heating pad (37°C). Besides having airflow checks at different points, the total airflow of the O<sub>3</sub> and NO<sub>2</sub> mixture was always checked in order to verify that it added up to 3l min<sup>-1</sup> (**Figure 12**).

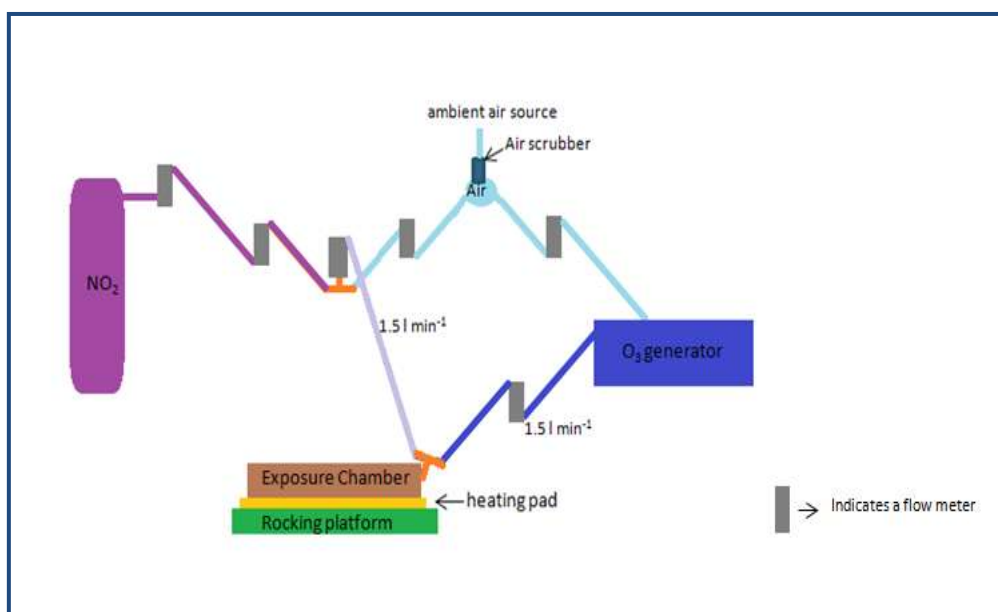


**Figure 12: Diagrammatic Representation of the Experimental Set Up Used for the Concomitant Gaseous Exposures**

This set up was chosen in favour of the O<sub>3</sub> and NO<sub>2</sub> tubes being connected prior to entering the chamber as the mixing would have been potentially insufficient. Alternatively, the tubing could have been changed to increase significantly the diameter, but in this case the experimental conditions would be different from the single pollutant exposures.

#### **2.2.1.5 Particulate Matter Disc Cuts Exposed to Nitrogen Dioxide or Ozone**

The hypothesis that O<sub>3</sub> or NO<sub>2</sub> may alter the antioxidant depletion capacity of the PM disc cuts was tested by exposing PM disc cuts originating from the same filter to a) air b) 1000ppb O<sub>3</sub> c) 1000ppb NO<sub>2</sub>. The protocol can be briefly summarised as follows: 5mm disc cuts were performed on each PM filter, triplicates were used for each of the three conditions (NO<sub>2</sub> or O<sub>3</sub> or air) and time points (0, 1, 2 hours). Each individual disc cut was removed with very clean metal-free forceps/ microspatulas and placed into individual pre-labelled round wells of the 24 well-plate. For the 0 time point the disc cuts (n=3) were placed inside the 3 wells but were removed prior to the start of the exposure in pre-labelled 'O' ring Simport microcentrifuge tubes, carefully using metal-free forceps. This step was only performed to control for any effect that the transfer of the PM disc cuts from the well plate to the microcentrifuge tubes could have had (Figure 13).

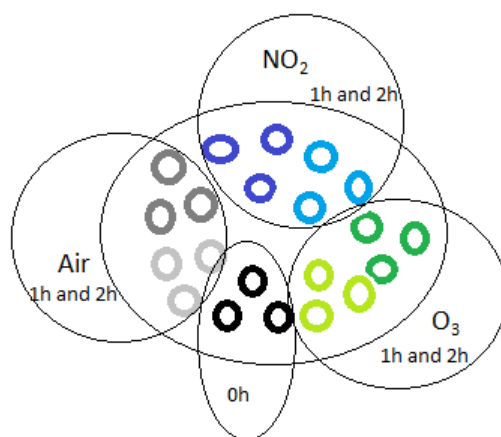


**Figure 13: Diagrammatic Representation of Disc Cuts Performed on PM Filters Exposed to Gasses prior to Incubation in synRTLF.**

Twenty one, 5mm disc cuts were performed on each of the 40 PM filters. Six disc cuts (1 triplicate for the 1h time point and a second triplicate for the 2h time point) were prepared for the three conditions; NO<sub>2</sub>, O<sub>3</sub>, air. Also, one triplicate was used for the 0h time point due to limited material.

The disc cuts of 40 PM filters from background and roadside location were exposed to either air or O<sub>3</sub> or NO<sub>2</sub> (3l min<sup>-1</sup>, 1000ppb) at a random order. Specifically a random orders generator (<http://www.random.org/>) was used to choose the order of exposure in order to avoid order effects. For all exposures the air flow was 3lmin<sup>-1</sup>, and details about the operation of the equipment were given on the relevant manufacturer manuals, and lab protocol 37. NO<sub>2</sub> and O<sub>3</sub> concentrations within  $\pm 5\%$  of the desired concentration were deemed acceptable; exposures were not carried out outside this range. Following the gaseous exposure to NO<sub>2</sub>, O<sub>3</sub> and air, the PM filter disc cuts were then incubated in synRTLF by following the same conditions that were utilised in the standard PM exposures.

#### ***2.2.1.6 Particulate Matter Disc Cuts Exposed Concomitantly to Nitrogen Dioxide and Ozone***

The hypothesis that concurrent O<sub>3</sub> and NO<sub>2</sub> exposures could alter PM antioxidant depletion capacity was tested by exposing PM disc cuts to a) standard exposure (i.e. no air stream) b) air c) 1000ppb O<sub>3</sub> and 1000ppb NO<sub>2</sub>. In addition, a 1in10 dilution was investigated, thus further conditions included d) 1000ppb O<sub>3</sub> and 100ppb NO<sub>2</sub> e) 100ppb O<sub>3</sub> and 1000ppb NO<sub>2</sub>. For the concomitant exposures the tubing system used

was identical to the one used for the RTLTF exposures (2.2.1.4) and the disc cut exposure conditions followed the protocol presented above (2.2.1.5).

#### ***2.2.1.7 Controlling for Leakages***

All structures were cautiously assembled to ensure the highest possible construction quality. In order to ensure that there were no leakages all connections were covered by PTFE tape thread sealant (Swagelok). This special, non reactive tape strengthened the argument that all connections were not leaking. To further establish that there were no losses all connections (t-junctions, flow meters, chamber connections etc) were further covered by liquid leak detector (Snoop). This product is a gas leak detector in liquid form that has sufficient viscosity to successfully detect gas leakages. In the presence of leaks, it forms bubbles, and indeed none were formed once the exposure system set up was finalized.

#### ***2.2.1.8 Controlling for evaporation***

Pilot studies revealed significant evaporation of the synRTLTF in the 4 hour exposures, and also some variation in evaporation due to the 24 well plate not being placed at exactly the same spot within the chamber. These, confounders were overcome by decreasing the total exposure time in 2 hours (as the depletion was linear with time, this would not make a difference in the final calculation), and marking the exact spot that the plate was positioned. In addition, at each individual experiment and at each time point changes in weight were recorded by running in parallel preweighed RTLTF samples and transferring them in preweighed volcanic tubes. The change in weight was used as a suitable correction index for evaporative loss. A constant 1% loss of RTLTF weight was observed for each time point, importantly the evaporation was consistent irrespective of the gaseous pollutant concentration. It should be noted that this minimal, constant evaporation was only noted in the gaseous exposures but not in the PM exposures possibly because there was no air stream in the latter.

#### ***2.2.1.9 Running Conditions***

For both gaseous exposures the relevant equipment and heating pad were left turned on 40 minutes prior to the start of the experiment so that stability was achieved. All exposures were performed inside an externally extracted fume cabinet to ensure

protection from the toxic exhausts. The fume hood was placed away from the windows in order to avoid daylight and indeed it was verified that NO<sub>x</sub> species were not generated as a by-product of the irradiation of the zero air source under any of the O<sub>3</sub> concentrations employed using the NO<sub>x</sub> analyser to measure the output.

### 2.3 Modelling the Interaction of Particulate Matter with the Respiratory Tract Lining Fluid

The current project exposed PM<sub>10</sub> directly PM<sub>10</sub> to synRTL<sub>F</sub> while a mixed interfacial exposure system was used for the gases. This aimed to model the *in vivo* exposure differences, that is, PM<sub>10</sub> is believed to get trapped in the RTL<sub>F</sub> [288] while gaseous diffusivity is thought to be dependent on the gaseous solubility and antioxidant reactivity [51-53, 289]. Understandably, this model constitutes an oversimplification of the PM<sub>10</sub> deposition within the RTL<sub>F</sub> as several factors such as ventilation rate, breathing pattern and respiratory health [290-292] are believed to affect the deposition, nevertheless, the current model is an appropriate representation given the *in vitro* constraints (**Figure 14**).

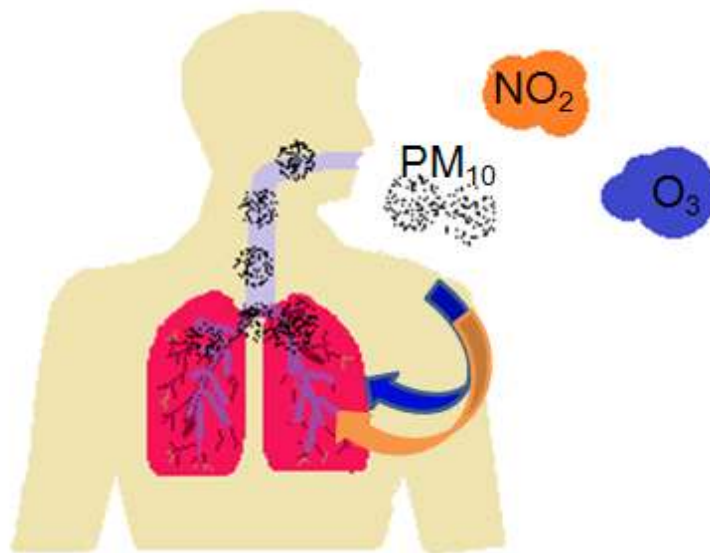


**Figure 14: Diagrammatic Representation of Expected Differences in Air Pollutant Uptake**

PM<sub>10</sub> is thought to get deposited within the RTL<sub>F</sub>, while the NO<sub>2</sub> and O<sub>3</sub> diffusivity is believed to be primarily dependent on the gaseous pollutants' reactivities towards the antioxidants of the RTL<sub>F</sub>.

### 2.3.1 Sites of Particulate Matter Collection

Determining the oxidative potential of representative PM samples is rather challenging, as its composition has a temporal and spatial variability [243]. For this reason two contrasting London locations were chosen; a roadside site (Marylebone Road), and background site (North Kensington).

Marylebone Road is major route in and out of Central London, running north-east to south-west and carrying over 80,000 vehicles per day (51°31'21.1"N 0°09'16.6"W) and the tall buildings on both sides of the road form a broad street canyon [293]. The monitoring site is located 1m from the kerb on the southern side of the road, that is, opposite the Madame Tussauds museum. In contrast, the North Kensington site is located within the Sion Manning School in St Charles Square (51°31'15.8"N 0°12'48.6"W) which is a mainly residential area [294].

### 2.3.2 Particulate Matter Sampling Campaign

Ambient PM<sub>10</sub> (particulate matter in which 50% of particles have an aerodynamic diameter of less than 10µm) was collected on partisol filters (Emfab, Teflon coated glass fibre) over a period of 24hours (Partisol-Plus 2025 Sequential Air Sampler). The Partisol 2025 is a gravimetric technique that draws air through a type-approved aerodynamic sampling inlet and collects the samples into a pre-weighed filter. This is then re-weighed under standard conditions in order to determine the mass collected. Filters were maintained in 5cm diameter sterile petri dishes at room temperature in dark conditions until exposure to synRTLF. Daily filters were collected concurrently at the background and roadside locations between January and December 2010. A total of 44 filters were retrieved from each location, spanning all seasons, thus giving a combined total of 88 PM<sub>10</sub> samples.

### 2.3.3 Protocol Development

An exposure protocol was developed in order to allow direct quantitative comparisons between the oxidative potential of PM<sub>10</sub> and the gases. The potential effect of extraction processes in altering the physical and chemical properties of particles [295-298], along with an increased sensitivity of these filters to sonication (physical destruction) prompted the development of an *in vitro* protocol that directly exposed PM to the RTLF model.

### 2.3.3.1 Materials

1. HPLC grade methanol
2. 8mm, 2mm and 5mm bore punches
3. non-metal forceps and micro spatula
4. Pre-labelled 'O' ring Simport microcentrifuge tubes

### 2.3.3.2 Performance of Disc Cuts on Particulate Matter filters

The bore punch, forceps and microspatula were pre-cleaned thoroughly in HPLC grade methanol. The methanol was allowed to dry off. Without removing the partisol filter from the petri dish, disc cuts were made in triplicate, following a triangular pattern as shown in **Figure 15**. Each individual disc cut was removed with thoroughly cleaned, metal-free forceps and microspatulas and placed into an individual pre-labelled Simport microtube, to be analysed independently. The filter discs were placed in the same fashion in all tubes, that is, lying vertical. Blank controls were always run to control for accidental carry over of PM from one experiment to another and confounding (see section on controls).

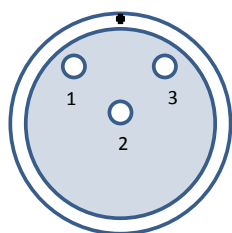


**Figure 15: Layout of Filter Disc Cuts**

The filter has a diameter of 47mm and a deposition diameter equal to 39mm represented by the light blue colour. Three disc cuts were utilized for each time

### 2.3.3.3 General Exposure Conditions

The disc cuts were performed the day before the exposure to RTLf and were kept at room temperature and in a dark place. On the day of the exposure 450µl Chelex-treated water was added, and samples were briefly vortexed. It was checked that all disc cuts were fully immersed in the solution.

The PM-filter solutions were then pre-incubated at 37°C (Stuart Scientific Incubator S160) for 10 min prior to a short vortex mixing, the addition of 50µl of the 2mM RTLf stock solution (containing 2mM AA, GSH and UA; see RTLf section) to each incubation tube and a further vortex mix. This process resulted in a final starting concentration of 200µM antioxidants.

The resulting reaction mixtures were incubated for up to 2 hours at 37°C with gentle mixing throughout (Vortex Genie-2, shaking mode). Controls were also incubated (see relevant section). For each time point samples were centrifuged at 13,000rpm for 1 hour at 4°C and the supernatant analysed for remaining antioxidant (HPLC and Glutathione analysis sections).

#### ***2.3.3.4 Investigating the Size of Disc Cuts and Particulate Matter Distribution on Filters***

Previous studies have often utilised extraction and resuspension protocols in order to extract PM from filters and later expose it to RTLTF models. These processes however could potentially affect the chemical composition of PM. In addition, an aqueous extraction process, was not possible with the current partisol filters (Emfab™) as pilots demonstrated that the filters were destroyed in this process, this potential disintegration presented a major confounder as the antioxidants could a) get absorbed onto the surface, b) the degrading material could potentially lead to the release of glass fiber chemical constituents such as alkali metals; magnesium, calcium and potassium, thus either case could lead to an overestimation of PM's effects (disc cut controls were always included in my exposures see section 2.3.3.6). For this reason I investigated whether performing disc cuts on the filters and incubating them in synRTLTF was a viable option. At first, pilots were run to investigate which size of disc cuts would be most appropriate. Three different diameter disc cuts were investigated 2mm, 5mm and 8mm and these were tested in triplicate from each partisol filter (3 disc cuts \* in triplicate = 9 disc cuts on each filter). Each disc cut of the triplicate covered the area near the edges of the filter as well as the central area. This pattern was carefully followed for all triplicates across the different filters in order to investigate if PM was uniformly distributed across the filter surface. In the same pilot the most appropriate disc cut for the specific locations was determined by performing 2, 5 and 8mm disc cuts (each in triplicate from different parts of the filter in order to test the deposition uniformity as well) and incubating them in RTLTF for 4 hours. Complete antioxidant depletion would demonstrate that this disc cut diameter may be unsuitable as rate calculations would not be possible and similarly a limited reactivity would signify that the particular disc cut size would not be ideal for the same reason.

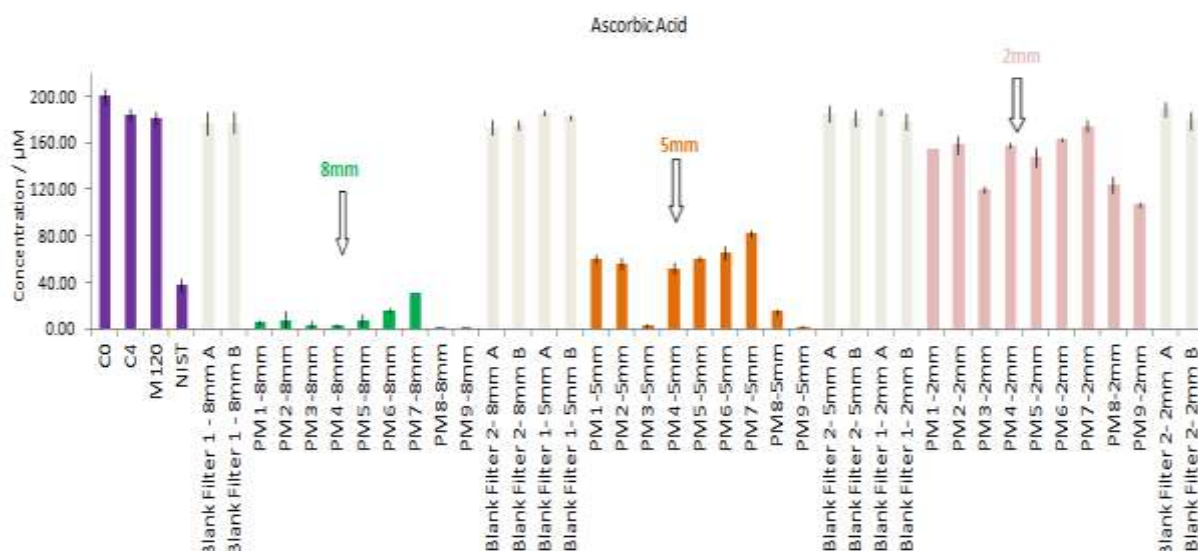


**Figure 16: Verification of Uniform PM Deposition on Filter and Selection of Appropriate Disc Cut Size.**

Three different disc cut diameters were tested; 8, 5 and 2mm on the same PM filters. In this figure the results for ascorbic acid are presented. The 8mm filters caused a near complete depletion for all filters and the 2mm filters were quite unreactive. Therefore, the 5mm disc cuts were tested further in the 2h protocol and were confirmed to be a good choice in later experiments. In addition the small standard deviations demonstrated that potential differences in PM deposition were not significant. The white colored bars represent blank filters that were used for carry over effect controls in this and all other PM related experiments. Indeed there was no cross contamination of PM from one experiment to another since these blank controls did not differ from the background oxidation controls.

Indeed the analysis of the results demonstrated that the standard deviation for each of the triplicates was within 5% and thus supported a uniform distribution of PM (. Also, since the 8mm PM filter cuts caused almost complete antioxidant depletion on certain filters and the 2mm filters a very minimal depletion over a period of 4 hours, both these disc cut diameters were assumed inappropriate in calculating the antioxidant depletion of PM at different time points and the 5mm disc the blank filters of different diameter did not differ, thus providing evidence that the filter material was not a confounder. Further samples were run successfully and this protocol was finalised, a schematic representation of the protocol is shown in **Figure 17**.

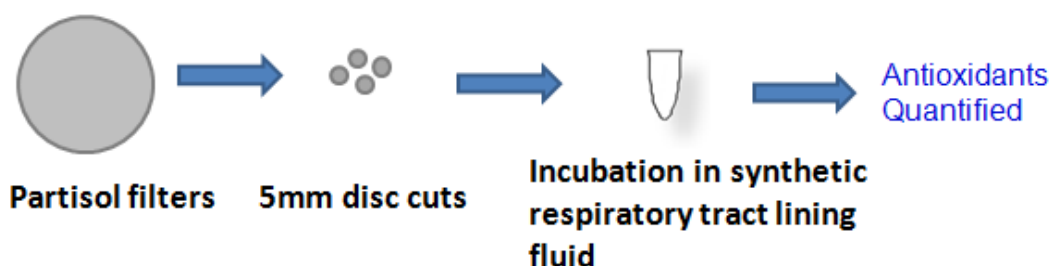


**Figure 17: Simplified diagram of the exposure protocol**

5mm disc cuts (n=3) were performed on the partisol filters for each time point, the discs were incubated in synRTLf. At different time points the antioxidant concentration was quantified and thus a rate of depletion could be calculated.

### **2.3.3.5 Background Oxidation Control**

The antioxidant depletion capacity of PM, O<sub>3</sub> and NO<sub>2</sub> in an RTLf model was investigated in the current experimental series. However, oxidation is a process that constantly and continually occurs in aerobic environments. ‘Oxidative damage remains an inescapable outcome of aerobic existence’ [299], this is because atomic oxygen is a free radical and molecular oxygen is a bi-radical [299]. Therefore it was very important to establish the oxidation that was caused by factors unrelated to the exposure of synRTLf to the aforementioned pollutants, this type of oxidation can be described as background oxidation. For PM, this was calculated by incubating particle free controls concurrently with the PM exposures. These ‘blank controls’ were prepared using the same materials and exposure methods as the PM exposures with the exception that a PM filter disc cut was not added. It is noteworthy, that these ‘blank controls’ did not differ from the ‘disc cut controls’, thus indicating that there was a consistent background oxidation and that the filter material was not being a confounder (Figure 18). Similarly, for the gaseous exposures, the RTLf model was exposed to scrubbed air (i.e. 0ppb exposure). Both the gaseous and PM exposures showed the same consistently low background oxidation for ascorbate (5%) urate (3.5%) and reduced glutathione (1.8%) within the 2h exposure. The background oxidation was subtracted from the antioxidant depletion effects caused by the air pollutants examined in order to present the pollutant-specific effects.

#### **2.3.3.6 Oxidative Activity of Disc Cuts**

The background antioxidant depletion was taken into account as described above. However, one could argue that the disc cuts alone could result in antioxidant depletion either by absorption of the antioxidants onto the filter or by the disc cut structure affecting the reaction kinetics, and especially during vortexing. For this reason particle free controls containing blank disc cuts were also run concurrently with the PM exposures. These were prepared in the same way as the PM disc cuts with the exception that no PM was deposited on the partisol filters. The blank disc cuts caused an antioxidant depletion that was consistent across different exposures and did not differ from the background oxidation controls (**Figure 18**).

#### **2.3.3.7 Control of Carry Over Effects**

The disc cuts were prepared as described on the relevant section through the use of a bore disc cutter. The latter was carefully cleaned with methanol and dried before using different filters. However, it was important to test for carry over effects. It is defined as a process by which ‘materials are carried into a reaction mixture to which they do not belong’ [300]. In the current experimental series the materials would be PM residues from previously prepared filters. This potential confounding was addressed by cutting blank discs (in triplicate) at the start and end of the PM filter cutting process. The results of these blank disc cuts did not differ from the background control or the blank free disc cuts assessing the oxidative activity of the disc cuts *per se* therefore, it was concluded that carry over effects were well controlled (**Figure 18**).

#### **2.3.3.8 Positive Control**

A positive control was used for all PM<sub>10</sub> exposures in order to verify that the experimental set up was functioning and in order to establish consistency between different exposures. Standard Reference Material 1648a of the National Institute of Standards and Technology (NIST) was used as a positive control. NIST SRM1648a is characterized as atmospheric particulate matter collected in an urban area and represents in the current experimental series ambient particulate matter with an established oxidative potential for our model. It contains several metals, predominantly; iron, aluminium, and potassium, as well as high concentrations of silicon dioxide and sulphate [301].

NIST SRM1648a was weighed and suspended in chelexed water containing 5% to a final concentration of 500ug/ml, the addition of methanol aimed to facilitate suspension. In order to verify that this does not affect the redox potential of the controls, 'blank NIST' controls were also run, these contained the 5% chelex water used for the NIST dilutions and consistently it was shown that this very small methanol concentration had no effect.

The resulting NIST suspension was vortexed vigorously for 5 minutes and then sonicated for 30 seconds at an amplitude of 15 microns. Aliquots of 1ml were stored in Simport 'O' ring microtubes at -80 °C. Before exposure these stocks were diluted to 55.56 µg/ml through a two step dilution. This concentration was chosen because on the day of the exposure 50µl of the 2mM RTLF stock is added to 450µl of each 55.56µg/mL PM suspension, resulting in concentrations of 200µM and 50µg/mL respectively. For the dilution, firstly 1ml of the 500ug/ml aliquots were transferred in a falcon tube containing 2,333mls chelexed water (5% methanol) and were sonicated for 30 seconds at 15microns. The resulting 150µg/ml concentration was diluted to 55.56µg/ml by the addition of 555µl of the aforementioned solution in eppendorfs containing 944µl chelex treated water. NIST controls were run concurrently to the PM exposures and the antioxidant quantification methods. They resulted in consistent antioxidant depletion that was statistically significant when compared to the background oxidation controls (**Figure 18**).

#### **2.3.3.9 Negative Control**

A model carbon black particulate sample (M120) was used as the negative control. It has been characterised to be 50 nm in diameter with an associated surface area of 32m<sup>2</sup>g<sup>-1</sup> [277]. Electron-probe X-ray microanalysis studies have demonstrated that M120 is composed of carbon black, chloride and sulphur with no evidence of surface associated metals [302]. Therefore, potential confounding effects of the particle surface and size on antioxidant quantification due to absorbance were investigated through the use of the negative control concurrently to the PM testing. The preparation was the same as the positive control (see section on NIST), that is, a 500µg/ml stock was diluted to 55.56µg/ml solution for the exposures. Results demonstrated that the antioxidant depletion caused by M120 in the 2 hour exposure did not differ from the oxidation caused by the background controls (**Figure 18**).

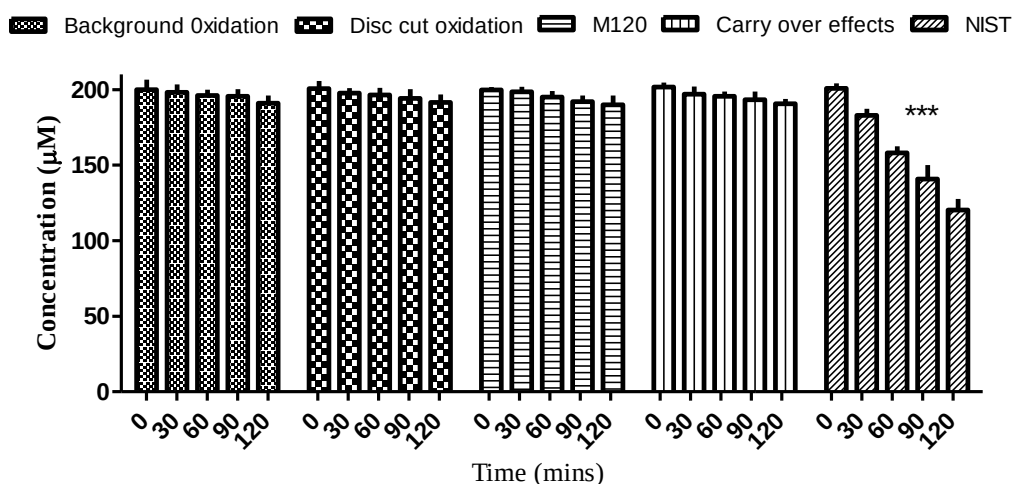


**Figure 18: Summary of Control Results in the Particulate Matter Exposures**

The diagram presents the overall ascorbate depletion mean  $\pm$  SD by the different controls, NIST caused the highest antioxidant depletion ( $p < 0.001$ ), while the remaining controls did not differ from the background oxidation control. Therefore, it can be concluded that the disc cut incubation and the related methods (carry over test) did not confound the results.

#### 2.3.3.10 Sample pH

pH is an important experimental factor affecting the rate of oxidation as shown previously [303, 304]. A pH of 7 was chosen for the synRTLF as it represents a physiological value. Based on the antioxidant's dissociation constants one could predict that in the synRTLF at more acidic concentrations reduced glutathione would exhibit higher antioxidant depletion while in a more basic environment ascorbic acid would present higher rates. The solutions used for the exposure (Chelex treated water, synRTLF) had a pH of 7. At the end of the exposures pH test strips with a colour scaling (pH test strips; 0-14 pH, Sigma-Aldrich) were immersed in each of the 'O' ring simport microcentrifuge tubes containing synRTLF exposed to 5mm PM disc cuts to examine any changes or equivalently in the 24 well plate containing RTLF exposed to gases. The colour of the test strips was compared with the chart on the package. The pH strips were consistently orange, which signified a pH of 7. While it is true that colorimetric pH test strips do not detect very slight changes in pH, it is highly unlikely that changes of that magnitude would influence significantly the predicted rates. The lack of any significant changes in the pH can be attributed to the presence of the antioxidants, the latter being reducing agents.

#### **2.3.3.11 Replications**

Each time point was quantified by exposing three disc cuts to synRTLf. Given that there were 5 time points, a total of 15 disc cuts were utilised from each filter (i.e.  $5 \times 3 = 15$ ). Similarly, triplicates were used for the gaseous exposures, aliquots were collected from the exposure chamber from three different wells, but also, each of the five tested concentrations was investigated in three separate exposures ([3 aliquots  $\times$  5 time points]  $\times$  3 separate exposures per concentration). The standard deviation of these replications is a measure of random error, experimenter error and systematic error.

#### **2.3.3.12 Normalisation**

For PM<sub>10</sub>, the raw values depicting the antioxidant depletion and glutathione disulphide formation were copied onto the same normalization template (Microsoft Excel, 2007). On that spreadsheet the average and standard deviation of all the controls (section on controls) was recorded in order to ensure that there was consistency across different exposures. The starting concentration of all gaseous exposures/ particle free controls was divided by 200  $\mu$ M which was the intended starting concentration to adjust for variation in starting concentration. The resulting factor was used to divide the remaining time points of all the exposures on the normalization template. It should be noted however, that there were never great deviations on starting concentrations of synRTLf, given that when prepared, a synRTLf that deviated by more than 5% from 200  $\mu$ M would have been discarded. After this normalization step, the background oxidation as indicated by the background controls was subtracted for each time point and finally the antioxidant concentrations were divided by the respective weight of the 5mm disc. Similarly, for the gases, data were transformed to give a starting concentration of 200  $\mu$ M in case there were any very small divergences and the background was subtracted.

#### **2.3.3.13 Experimenter Blinding**

Experimental bias can prevent the unprejudiced examination of research questions [305]. This can arise through the researcher's expectations for the study, without it being necessarily a conscious process [306]. This bias can be eliminated through experimenter blinding methodologies, where the researcher is deliberately unaware of

certain information related to the experimental conditions that can lead to conscious or subconscious bias [306]. In the current experiments, the PM filters were coded by the lab manager and the codes were only ‘broken’ once the exposures and quantification processes were completed by the experimenter. Thus I was not aware if I was analysing roadside or background samples or which dates. A similar blinding process was not possible for the gaseous exposure as I had to set up the equipment and monitor the concentration levels, however all quantifications were reviewed by at least one further lab member.

#### **2.3.3.14 Calculating the Weight of the 5mm Partisol Disc**

The total weight of the PM on the filter was provided after the quantification of the results (see section 2.3.3.13 on experimenter blinding). The total deposition diameter on the partisol filter was 39mm. The area of a circle can be calculated as  $\pi r^2$  therefore the total deposition area was  $19.5^2 * 3.14 = 1193.985\text{mm}^2$ . Similarly the 5mm disc cut has an area that equals  $19.625\text{mm}^2$ . Therefore one can calculate the weight of the 5mm disc cut, given that PM is deposited uniformly on the filter. For example, a partisol filter weighing  $816\mu\text{g}$  would signify that there are  $816\mu\text{g} / 1193.985\text{mm}^2 = 0.6834\mu\text{g}$  of PM per  $\text{mm}^2$  of the partisol filter and thus  $0.6834 * 19.625 = 13.41 \mu\text{g}$  of PM on a 5mm disc cut.

#### **2.3.4 Developing a Rate Calculation Method for the Particulate Matter exposures**

Once the ideal size of the PM disc cut (5mm) was established and a uniform distribution of PM across the filter was shown (section above) and since the controls were behaving as expected (section on controls) the next question that was investigated was whether antioxidant depletion could be quantified at different time points in order to generate a rate of antioxidant consumption for PM. For these pilot studies the same exposure conditions were employed. Antioxidant depletion was quantified (as described in the HPLC and glutathione sections) by collecting samples at 0, 30, 60, and 120 minutes of exposure. Antioxidant depletion was dependent on exposure time. Given that the results were quantifiable this method was further improved by adding a time point at 90 minutes, for consistency between the gaseous and PM exposures. The rate of antioxidant depletion was calculated by performing a linear regression analysis of the change in antioxidant concentration ( $\mu\text{M}$  per  $\mu\text{g}$  of PM)  $* 10^{-6} = \text{mol/l per } \mu\text{g of PM}$

over time (0, 30, 60, 90, 120 minutes = 0, 1800, 3600, 5400, 7200, seconds). This resulted in an antioxidant depletion rate expressed as mol/l/s per  $\mu\text{g}$  of PM. SPSS (14.0 statistical software) was used to run the linear regressions ( $y_i = \beta_0 + \beta_1 x_i + \epsilon_i$ ).

### 2.3.5 Antioxidant Depletion Rates of Particulate Matter; Sample Calculation

| Steps →          | 1                                                                          | 2                                                                                         | 3                                                                                                                        | 4                                                                                             | 5                                                                                                |
|------------------|----------------------------------------------------------------------------|-------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| Time point (min) | Average Normalised Concentration ( $\text{AH}_2$ ; $\mu\text{M}$ , $n=3$ ) | Average Normalised Background Oxidation Control ( $\text{AH}_2$ ; $\mu\text{M}$ , $n=3$ ) | Subtract step - 1 from Step 2 i.e. calculate PM driven antioxidant consumption ( $\text{AH}_2$ ; $\mu\text{M}$ , $n=3$ ) | Division of Remaining Antioxidant Depletion by Discut Weight (3.5 $\mu\text{g}$ in this case) | Rate calculation<br>Convert $\mu\text{M}$ to mol/l and mins to seconds and run linear regression |
| 0                | 194 $\pm$ 4.94                                                             | 200 $\pm$ 4.18                                                                            | 5.84 $\pm$ 4.94                                                                                                          | 1.64 $\pm$ 1.39                                                                               | 1.34( $\pm$ 0.22) $\times 10^{-9}$<br>mol/l/s per $\mu\text{g}$ of PM                            |
| 30               | 186 $\pm$ 1.67                                                             | 199 $\pm$ 2.30                                                                            | 13.13 $\pm$ 1.67                                                                                                         | 3.75 $\pm$ 0.47                                                                               |                                                                                                  |
| 60               | 174 $\pm$ 1.76                                                             | 196 $\pm$ 4.72                                                                            | 23.24 $\pm$ 1.76                                                                                                         | 6.64 $\pm$ 0.49                                                                               |                                                                                                  |
| 90               | 162 $\pm$ 2.30                                                             | 194 $\pm$ 3.15                                                                            | 31.73 $\pm$ 2.30                                                                                                         | 9.07 $\pm$ 0.65                                                                               |                                                                                                  |
| 120              | 152 $\pm$ 3.29                                                             | 191 $\pm$ 4.11                                                                            | 39.82 $\pm$ 3.29                                                                                                         | 11.38 $\pm$ 0.93                                                                              |                                                                                                  |

**Table 5: Sample Particulate Matter Rate Calculation**

For clarity purposes a sample calculation of the rate of antioxidant depletion of one of the filters is provided.

## 2.4 Comparison of Nitrogen Dioxide's, Ozone's and Particulate Matter's Oxidative Potential

The daily average  $\text{PM}_{10}$ ,  $\text{O}_3$  and  $\text{NO}_2$  concentrations at the North Kensington and Marylebone road sites were downloaded from the LAQN website (<http://www.londonair.org.uk/LondonAir/Default.aspx>) for the days that PM antioxidant depletion rates had been calculated and were then copied into an excel file. The concentrations were downloaded as  $\mu\text{g}/\text{m}^3$ , for all air pollutants. However the gaseous antioxidant depletion rates were expressed per ppb (mol/l/s/ppb). For this reason, the concentrations for nitrogen dioxide and ozone were converted on the Excel file to the ppb equivalent by dividing them by 1.88 and 1.96 respectively (WHO conversion

factors). Afterwards, the antioxidant depletion rates could be multiplied by the respective concentration and the duration of the exposure, in this case one day (i.e. 86400 seconds). Therefore, the antioxidant depletion of all three antioxidants was expressed as mol/l, thus allowing direct comparisons (**Figure 19**).

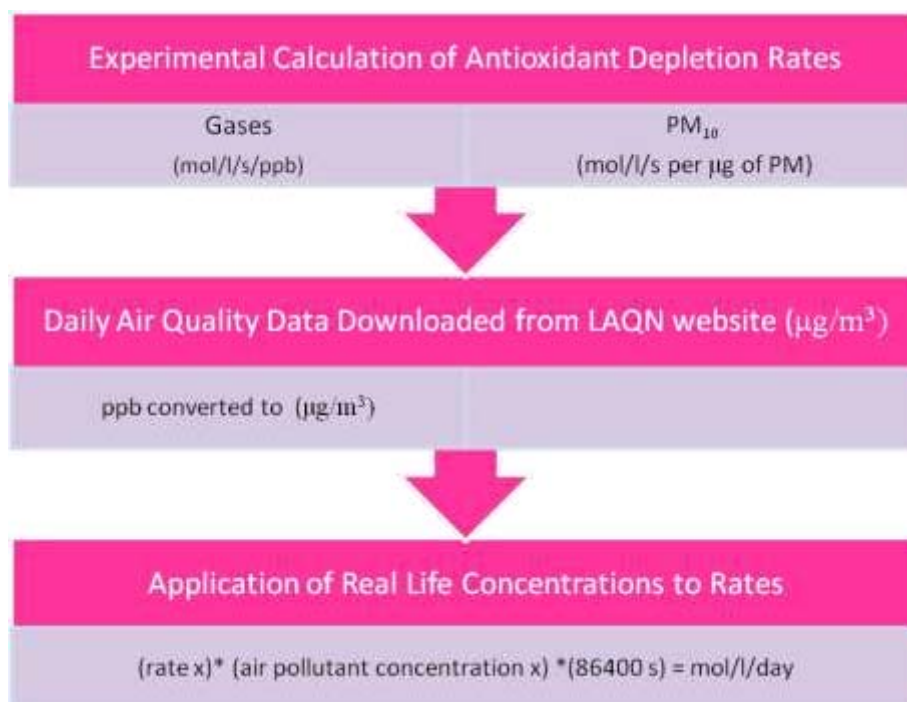


**Figure 19: Diagrammatic Description of the Rate Application Methods**

## 2.5 Statistical Analyses

All data are represented as mean  $\pm$  standard deviation values unless otherwise noted. All analyses were conducted using the SPSS statistics software package. Briefly, prior to the analyses the data were tested using the Shapiro-Wilk normality test in order to determine if the samples were drawn from a normally (parametric) or non-normally (non-parametric) distributed population. In general, only the values related to the PM exposures were non parametric. Differences in antioxidant consumption of NO<sub>2</sub> or O<sub>3</sub> concentrations versus their respective control exposure (0ppb) were investigated using a one-way ANOVA with post hoc Dunnet's test as the latter is

recommended when one of the groups is a control. Differences across the antioxidant depletion capacity of the two gaseous pollutants was examined using a one-way ANOVA with post hoc Tukey's test as it is believed to be more appropriate in a large set of means (i.e.  $>5$ ). The non parametric equivalent of the one-way ANOVA, that is, a Kruskal-Wallis –one way analysis of variance with Dunn's post test was used for the PM related multiple group comparisons (e.g. background antioxidant depletion vs. roadside antioxidant depletion). Also, Pearson-product-moment correlation coefficient was used in order to investigate correlations among parametric data, and for non parametric data its equivalent, that is, a Spearman Rank order correlation was used.

### **3 A Direct Comparison of Nitrogen Dioxide's and Ozone's Antioxidant Depletion Capacity in a Human Respiratory Tract Lining Fluid Model**

#### **3.1 Introduction**

Air pollution is made up of a complex and heterogeneous mixture, therefore disentangling the independent health effects of each pollutant can be very challenging [307]. *In vitro* studies can potentially reduce the uncertainty of such extrapolations and can assist in comparing their pathogenic mechanisms of injury [308]. O<sub>3</sub> and NO<sub>2</sub> are the most plentiful photochemical oxidants in ambient air and amongst the most potent gaseous pollutants in terms of respiratory impact [309]. For this reason these two air pollutants were chosen to investigate the oxidative changes that they instigate in a model of the extracellular antioxidant screen within the respiratory tract.

NO<sub>2</sub> is an oxidising, corrosive, water soluble gas with a reddish brown colour and a characteristic pungent odour [310]. It is one of the large and complex nitrogen oxides group of compounds (NO<sub>x</sub>), and from this group it is the principal pollutant of concern for human health [311]. In ambient situations, NO<sub>2</sub> is predominantly formed through the oxidation of NO in photochemical reactions; a more detailed description of the formation pathways is given at 1.1.1. NO<sub>2</sub> is a very interesting air pollutant for several reasons. Firstly, it can cause negative effects on human health, including on the respiratory system [312, 313]. Secondly, it absorbs solar radiation throughout the wavelength region of 300 - 650nm and thus contributes to climate change [314] and impaired atmospheric visibility [315]. Thirdly, due to these solar energy absorption properties NO<sub>2</sub> is the most important source of atomic oxygen in the troposphere and consequently dictates the O<sub>3</sub> levels in the atmosphere [314].

Therefore, O<sub>3</sub> is a secondary pollutant and while it is not a radical molecule it is the third most potent oxidant ( $E^{\circ} = +2.076$ ) with only fluorine and persulphate being more powerful oxidants [316]. O<sub>3</sub> is a contentious gas because while its actions are protective in the stratosphere due to the absorption of hazardous B and C ultraviolet radiations in the troposphere [317], it is associated with negative respiratory indices [73]. Its formation is driven by energy transferred to precursor pollutants during solar light radiation; a detailed description of these reactions is given in section 1.1.1

These two gaseous pollutants of interest exhibit opposing seasonal patterns, O<sub>3</sub> concentrations are higher in summertime, especially during increased ultraviolet

radiation, high temperatures, and stagnating high pressure conditions [318]. In contrast, highest ambient NO<sub>2</sub> concentrations tend to be found in wintertime during stagnant, high congestion conditions [319], it is noteworthy that 12% of the EU urban population lives in areas where the WHO air quality guidelines and annual EU limit values are exceeded [320]. The UK, like many other EU countries is in breach of the legally binding EU limits for NO<sub>2</sub>, indicatively, in 2010 and 2011, 40 out of the 43 UK zones did not fully comply [321], while London has been shown to have the highest NO<sub>2</sub> levels in Europe [320].

The respiratory tract is the portal entry of air pollutants, thus it constitutes a major target for NO<sub>2</sub> and O<sub>3</sub>. Epidemiological studies have associated exposure to NO<sub>2</sub> or O<sub>3</sub> with respiratory symptoms or parameters of pulmonary dysfunction following short and long term exposure [184, 187, 190, 191, 197, 198, 322-343]. However, these positive associations, vary largely in magnitude, and have not always been replicated in other studies [344-353], while a very small fraction of the epidemiological studies have used multiple pollutant models in their analyses.

Indeed, O<sub>3</sub> and NO<sub>2</sub> result in similar functional, biochemical, cytodynamic, and morphological respiratory changes [118, 354, 355] and these effects can be partly attributed to their oxidative actions. Specifically, O<sub>3</sub> toxicity has been ascribed to several reactions, including direct oxidation of biomolecules such as membrane phospholipids and unsaturated phospholipids or radical dependent formation of non-radical species such as ozonides and aldehydes [356]. Also, O<sub>3</sub> exposure has been reported to lead to neutrophilic activation [357], as well as hydrogen peroxide and oxygen radical generation in aqueous medium [358]. Similarly, NO<sub>2</sub> is thought to be an important contributor of nitrosative stress [91], exposure to this air pollutant has been associated with antioxidant depletion and lipid peroxidation *in vivo* and *in vitro* [253, 268, 359-361], as well as impaired superoxide radical release [362], and an imbalance in glutathione metabolism [363] [364]. However, the concentrations used in animal and *in vitro* experiments investigating the effects of O<sub>3</sub> and NO<sub>2</sub> have mostly been well above 2ppm and thus drawing confident conclusions about more realistic scenarios is challenging [365]. In addition, the magnitude and direction of the effects is species and age related [366-368], also the data extrapolation to humans is difficult [369] and there is a limited number of more recent toxicological studies.

The observed oxidative actions of NO<sub>2</sub> and O<sub>3</sub> render the respiratory tract lining fluid (RTLFL) of particular importance, as it is a 'first line of defence' richly invested with the

low molecular weight aliphatic antioxidants: ascorbate, urate, and glutathione [74, 79-81]. It has been postulated that an imbalance between the air pollutant derived oxidative burden and the antioxidant defences can ultimately lead to impaired respiratory function [79]. Previous studies have shown that the antioxidant moieties can act as sacrificial substrates for O<sub>3</sub> [122, 129, 260, 269, 370-379] and NO<sub>2</sub> [128, 257, 258, 268, 380, 381], therefore they can prevent the formation of oxidation products and hence conserve sensitive targets from oxidation [79]. However, the magnitude of the observed effects is different, as O<sub>3</sub> presents more deleterious actions than NO<sub>2</sub>, that is, a higher exposure dose is required by the latter in order to present equal effects [382]. While O<sub>3</sub> presents a higher redox potential than NO<sub>2</sub> it has a lower solubility in tissue fluids and thus a lower absorption in the respiratory tract [118, 383]. Therefore, a direct comparison of NO<sub>2</sub>'s and O<sub>3</sub>'s interaction with the RTLTF antioxidants requires an identical experimental set up. A very limited number of laboratory studies have compared the aforementioned pollutant's effects under equivalent conditions, and from these it can be concluded that the magnitude of the differences are study specific. Indicatively, *in vitro* studies have reported O<sub>3</sub> to be one [384], four [148], ten to fifteen [382, 385], or even three thousand times [386] more toxic than NO<sub>2</sub>. In addition, important differences in experimental protocols within and between experimental studies render direct comparisons of the aforementioned pollutants rather challenging. For these reasons, the main aim of this project was to quantitatively compare the antioxidant depletion capacity of the two major gaseous air pollutants and evaluate their antioxidant reactivity hierarchies in a biochemical respiratory fluid model under the same controlled conditions.

The aim of the experiments presented in this chapter was to investigate the interaction of O<sub>3</sub> and NO<sub>2</sub> with the major water soluble antioxidants by utilising an *in vitro* respiratory tract lining fluid model. The air pollutant concentration and time of exposure constituted the independent variables, while the dependent variables were the antioxidant depletion and glutathione disulphide formation. I sought to define the relative rates of consumption of each of these antioxidant moieties in order to arrive at a reactivity hierarchy that would postulate what happens *in vivo* and also carried out direct comparisons between the O<sub>3</sub>, NO<sub>2</sub>. Several distinct experimental approaches have been undertaken previously in order to study the reaction of O<sub>3</sub> and NO<sub>2</sub> with the synRTLTF antioxidants [128, 268, 269, 286, 371, 387-390]. The outcomes of these previous findings and their correlation with our results will be examined in more detail

in the discussion section. A major point differentiating this study from the existing literature is that the experimental conditions were identical for the two gases in order to achieve direct comparisons. The principal hypothesis was that O<sub>3</sub> would result in higher antioxidant depletion than NO<sub>2</sub> in the synRTLFL and ascorbate and urate would be more important targets than reduced glutathione. Samples were exposed to either O<sub>3</sub> or NO<sub>2</sub> using identical conditions.

### **3.2 Experimental Design**

A detailed description of the experimental conditions and an illustration of the set up can be found at 2.2.1. Briefly, physiologically relevant concentrations of the three major water soluble antioxidants associated with the RTLFL were prepared as described in 2.1.3.2. Exposure of synthetic RTLFL was carried out in a 5.6 litre purpose-built Perspex chamber, flushed with NO<sub>2</sub> or O<sub>3</sub> at 50, 150, 400 or 1000ppb, using the general conditions described in section 2.2.1. At 30, 60, 90 and 120 minute intervals throughout the exposure, the chamber was opened very briefly, samples removed and stored at -80°C for up to a week prior to analyses described in 2.1.4 and 2.1.5.

### **3.3 Results: Exposure of a Human Respiratory Tract Lining Fluid to Nitrogen Dioxide**

synRTLFL was exposed to four environmentally relevant NO<sub>2</sub> concentrations and scrubbed air, the latter was used as background oxidation/vehicle control since scrubbed air was also used in the dilution of the NO<sub>2</sub> source exposures as a mean of achieving different final concentrations (see Methods Chapter). Data represent the average of three independent exposures. The antioxidant consumption of ascorbate, urate and reduced glutathione by NO<sub>2</sub> with time is illustrated in **Figure 20**. The initial concentration was equal for all antioxidants (200µM).

There was a clear indication that antioxidant consumption was dependent on NO<sub>2</sub> concentration and time. The antioxidant consumption could be described as a linear regression ( $r^2$ : 0.93-0.99), and the determining factor was NO<sub>2</sub> concentration. The consistency of the linear drop in antioxidant consumption with time supports the contention that the reaction was zero order with respect to the antioxidant concentration over the 30-120 minute time period. The exposure did not result in full depletion as the exposures lasted only 120 minutes in order to limit evaporation as a confounding factor

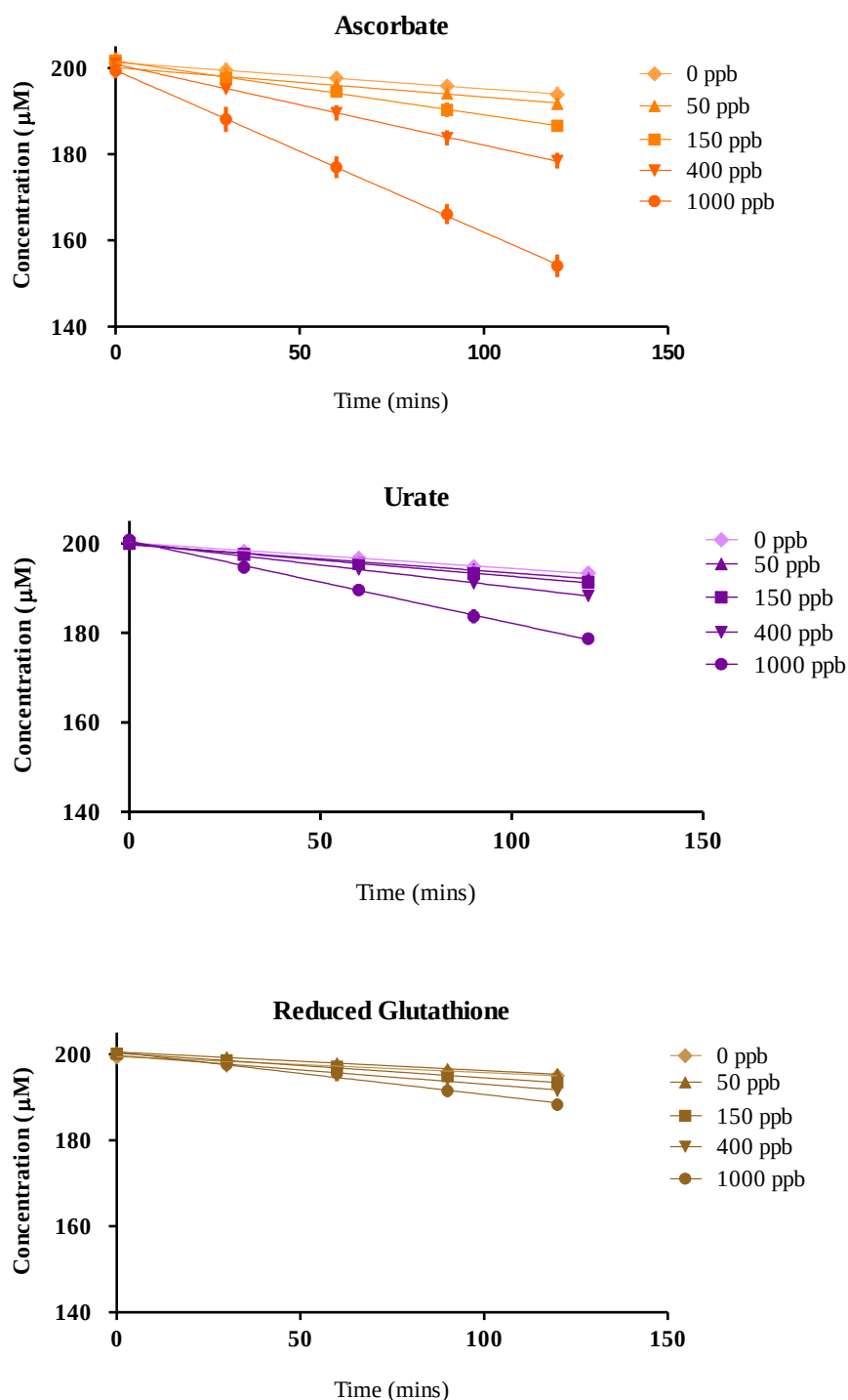


**Figure 20: Antioxidant Consumption of Ascorbic Acid, Uric Acid and Glutathione in the synRTLTF by NO<sub>2</sub>.**

SynRTLTF was exposed to a range of NO<sub>2</sub> concentrations and zero air control. Each point represents the mean and standard deviation of three independent replications (note y axis starts at 140  $\mu$ M for sake of clarity). Ascorbate and urate depletion rates were significantly higher than the control group (0ppb) at NO<sub>2</sub> concentrations that were equal or higher than 150ppb ( $p < 0.001$ ). Reduced glutathione was a less important target as significant effects were observed at NO<sub>2</sub> concentrations that were equal or higher than 400ppb ( $p < 0.01$ ; Table 6).

(2.2.1.8). The dependent variable was antioxidant concentration (converted from  $\mu\text{mol l}^{-1}$  to  $\text{mol l}^{-1}$  i.e. multiplied by  $10^{-6}$ ) and the independent variable was time (converted from minutes to seconds). All antioxidants followed the same pattern of antioxidant depletion, that is, the first  $\text{NO}_2$  exposure time point (30 mins) and lowest  $\text{NO}_2$  concentration (50ppb) presented the lowest level of antioxidant depletion and in contrast the highest antioxidant consumption was observed at the last time interval (120 mins) of the highest concentration (1000ppb). However, the antioxidant reactivity towards  $\text{NO}_2$  was not equal for all antioxidants. An initial visual scanning of **Figure 20** leads to the observation that ascorbate depletion appeared the highest and it was followed by urate and then reduced glutathione. Indicatively, ascorbate, urate and reduced glutathione consumption at the last time point of the 1000ppb exposure were 26, 8, and 4 % greater than the equivalent concentrations observed under control concentrations.

These point comparisons did not allow full appreciation of the antioxidant depletion capacity of  $\text{NO}_2$  with time and thus rate calculations were undertaken. In order to further compare and define the antioxidant consumption against  $\text{NO}_2$  concentration, the gradient of the linear regressions presented in **Figure 20** were calculated. For each concentration the rate was equivalent to the slope of the linear regression ( $y = a + bx$ ;  $b = ((n\sum Xy - (\sum x)(\sum y)) / (n\sum x^2 - (\sum x)^2)$  where the dependent Therefore, the resulting depletion rates for each concentration were denoted as  $\text{mol l}^{-1}\text{s}^{-1}$  and the results are presented in **Table 6**. Also, the background oxidation and the three respective antioxidants and the results were plotted in **Figure 21**.

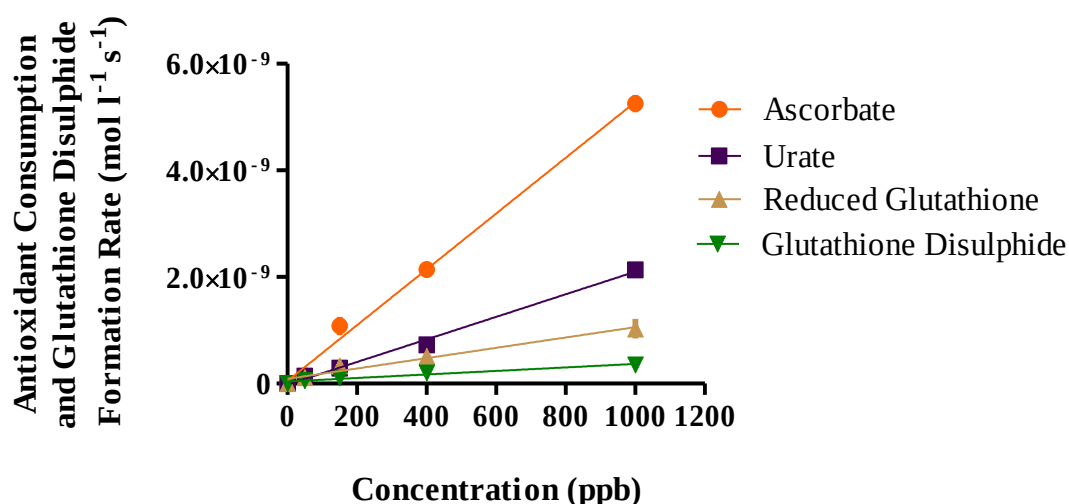


**Figure 21 Relationship between the Rate of Antioxidant Consumption and Glutathione Disulphide Formation with  $\text{NO}_2$  Concentration.**

The rates presented in Table 6 have been plotted against the respective concentration  $n=3$ . An overall antioxidant consumption rate can be calculated from this relationship and it is presented in Figure 22.

**Table 6: Antioxidant Consumption and Glutathione Disulphide Formation of synRTLF exposed to NO<sub>2</sub>**

| NO <sub>2</sub><br>(ppb) | Ascorbate                                                     |                |         | Urate                                                         |                |         | Reduced Glutathione                                           |                |         | Oxidised Glutathione                                        |                |         |
|--------------------------|---------------------------------------------------------------|----------------|---------|---------------------------------------------------------------|----------------|---------|---------------------------------------------------------------|----------------|---------|-------------------------------------------------------------|----------------|---------|
|                          | Consumption<br>Rate<br>(mol L <sup>-1</sup> s <sup>-1</sup> ) | r <sup>2</sup> | p       | Consumption<br>Rate<br>(mol L <sup>-1</sup> s <sup>-1</sup> ) | r <sup>2</sup> | p       | Consumption<br>Rate<br>(mol L <sup>-1</sup> s <sup>-1</sup> ) | r <sup>2</sup> | p       | Formation<br>Rate<br>(mol L <sup>-1</sup> s <sup>-1</sup> ) | r <sup>2</sup> | p       |
| <b>0</b>                 | 0.98 ± 0.03 x 10 <sup>-9</sup>                                | 0.99           | < 0.001 | 0.91 ± 0.11 x 10 <sup>-9</sup>                                | 0.99           | < 0.001 | 0.60 ± 0.10 x 10 <sup>-9#</sup>                               | 0.99           | < 0.001 | 0.15 ± 0.06 x 10 <sup>-10</sup>                             | 0.92           | 0.006   |
| <b>50</b>                | 1.14 ± 0.12 x 10 <sup>-9</sup>                                | 0.99           | < 0.001 | 1.06 ± 0.05 x 10 <sup>-9</sup>                                | 0.99           | < 0.001 | 0.71 ± 0.07 x 10 <sup>-9#</sup>                               | 0.99           | < 0.001 | 0.21 ± 0.09 x 10 <sup>-10</sup>                             | 0.99           | < 0.001 |
| <b>150</b>               | 2.08 ± 0.17 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 1.21 ± 0.09 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 0.95 ± 0.16 x 10 <sup>-9*#</sup>                              | 0.99           | < 0.001 | 0.26 ± 0.13 x 10 <sup>-10</sup>                             | 0.94           | 0.007   |
| <b>400</b>               | 3.14 ± 0.08 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 1.65 ± 0.02 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 1.12 ± 0.11 x 10 <sup>-9*#</sup>                              | 0.98           | < 0.001 | 0.35 ± 0.10 x 10 <sup>-10*</sup>                            | 0.92           | 0.01    |
| <b>1000</b>              | 6.25 ± 0.16 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 3.06 ± 0.03 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 1.60 ± 0.18 x 10 <sup>-9*#</sup>                              | 0.93           | 0.007   | 0.50 ± 0.11 x 10 <sup>-10*</sup>                            | 0.98           | 0.002   |

Rates are expressed as mean ± SD (n=3), rates were derived from the gradients of the antioxidant consumption vs. time regression for each individual NO<sub>2</sub> concentration. \* denotes a significant increase in the antioxidant consumption and glutathione disulphide formation rate compared with the 0ppb NO<sub>2</sub> control. # indicates that the reduced glutathione depletion was significantly lower than the equivalent value for urate and ascorbate.

The oxidation controls were subtracted from each calculated rate of the three respective antioxidants in order to clarify the relationship between the calculated consumption rates and the NO<sub>2</sub> concentration (**Figure 21**). The results were highly linear over this concentration range and overall, ascorbate appeared to be the primary target, followed by urate and then reduced glutathione. Quantitative comparisons of the antioxidant reactivities at all concentrations were achieved through the calculation of a ‘pseudo’ overall rate, that is indicative of the reactivity of each antioxidant towards NO<sub>2</sub>. This was achieved by calculating the gradient of the antioxidant consumption rates presented in **Figure 21**. The calculation can be written as;  $\Delta$  antioxidant consumption rate ( $\text{mol l}^{-1} \text{s}^{-1}$ ) /  $\Delta$  NO<sub>2</sub> concentration (ppb)  $\rightarrow \text{mol l}^{-1} \text{s}^{-1} \text{ppb}^{-1}$ . Overall antioxidant depletion and glutathione disulphide formation rates and statistics are presented in **Figure 22**.

All rate comparisons were significantly different (one way ANOVA;  $p < 0.0001$ ). Ascorbate antioxidant depletion was significantly higher than urate and reduced glutathione consumption, while urate exhibited significantly higher antioxidant consumption than the equivalent value for reduced glutathione. This suggested an overall hierarchy  $\text{AH}_2 \gg \text{UA} > \text{GSH}$ .

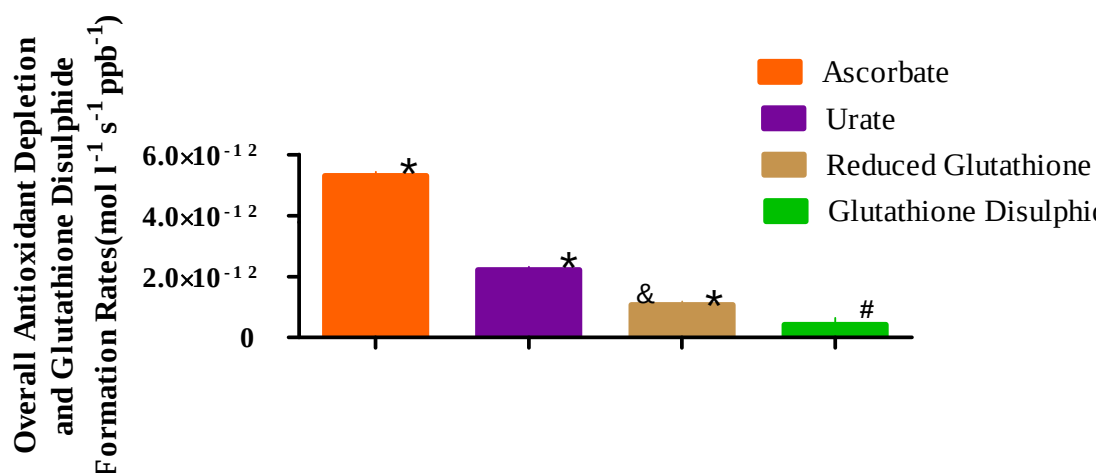


**Figure 22: Overall Antioxidant Depletion and Glutathione Disulphide Formation Rates of synRTLf exposed to NO<sub>2</sub>**

Values represent mean  $\pm$  SD ( $n = 3$ ). The overall consumption rates were calculated from the gradient of the consumption rates vs. the concentrations. \* indicates a significant difference between  $\text{AH}_2$  vs. UA and GSH. & indicates a significant difference between UA and GSH. # indicates a significant difference between  $\text{AH}_2$ , UA, GSH vs. GSSG.

Formation of oxidation products was followed only with respect to the reduced glutathione reactivity towards NO<sub>2</sub>. Similarly to the consumption of its reduced form, the formation of glutathione disulphide occurred in a time and NO<sub>2</sub> concentration

dependent manner (**Table 6**) and could be depicted using a linear representation (**Figure 21**). The overall calculated rate was  $0.42 \pm 0.22 \times 10^{-12} \text{ mol l}^{-1} \text{ s}^{-1} \text{ppb}^{-1}$ . This rate was approximately one half of the overall corresponding rate for the reduced form  $1.07 \pm 0.11 \times 10^{-12} \text{ mol l}^{-1} \text{ s}^{-1} \text{ppb}^{-1}$ . This finding supports the contention that the classical 2:1 reaction stoichiometry was occurring during the NO<sub>2</sub> exposure of the *in vitro* synRTLF model. Therefore, the overall NO<sub>2</sub> antioxidant depletion and glutathione disulphide formation rate can now be depicted as:  $\text{AH}_2 \gg \text{UA} > (\text{GSH} = \text{GSSG}/2)$ .

### 3.4 Results: Exposure of a Human Respiratory Tract Lining Fluid to Ozone

Exposure of synRTLF to O<sub>3</sub> resulted in time and concentration dependent antioxidant depletion (**Figure 23**). The highest depletion was observed at the maximum time point, 120 minutes and at the maximum concentration, 1000ppb. The relationship between antioxidant depletion over time was highly linear with time ( $R^2$  0.90-0.99). The consumption of ascorbate and urate were closely matched while reduced glutathione was affected considerably less. Specifically, ascorbate and urate were depleted by 28% and 27% respectively at the maximum point (1000ppb, 2h), while reduced glutathione was depleted by 16% for the same time point and concentration. Complete or near complete depletion was not observed, as the exposures lasted just 2hours, for this reason the lowest chosen concentration on the y axis of **Figure 23** was 140μM, this allowed for a more clear representation of the depletion. The stringent exposure conditions (see gaseous exposure set up) resulted in only small deviations among different exposures. For each time point, samples were collected in triplicate in order to ensure low intra-experimental variation, and each of the five concentrations was tested in three independent experiments in order to establish inter-experimental variation.

A principal objective was to calculate the rate of antioxidant depletion as this value provides a more elaborate perspective on the oxidative potential of O<sub>3</sub>. For each concentration this rate is equivalent to the slope of the linear regression in **Figure 23** ( $y = a + bx$ ;  $b = ((n\sum Xy - (\sum x)(\sum y)) / (n\sum x^2 - (\sum x)^2))$  where the dependent variable was antioxidant concentration (converted to mol l<sup>-1</sup> i.e. multiplied by 10<sup>-6</sup>) and the independent variable was time (converted to seconds i.e. multiplied by 60). Therefore, the resulting depletion rate for each concentration was denoted as mol l<sup>-1</sup>s<sup>-1</sup> and the results are presented in **Table 7**. The same method was applied to oxidised glutathione in order to investigate the rate of formation of the oxidation product and its relationship with the depletion of the reduced form of glutathione.

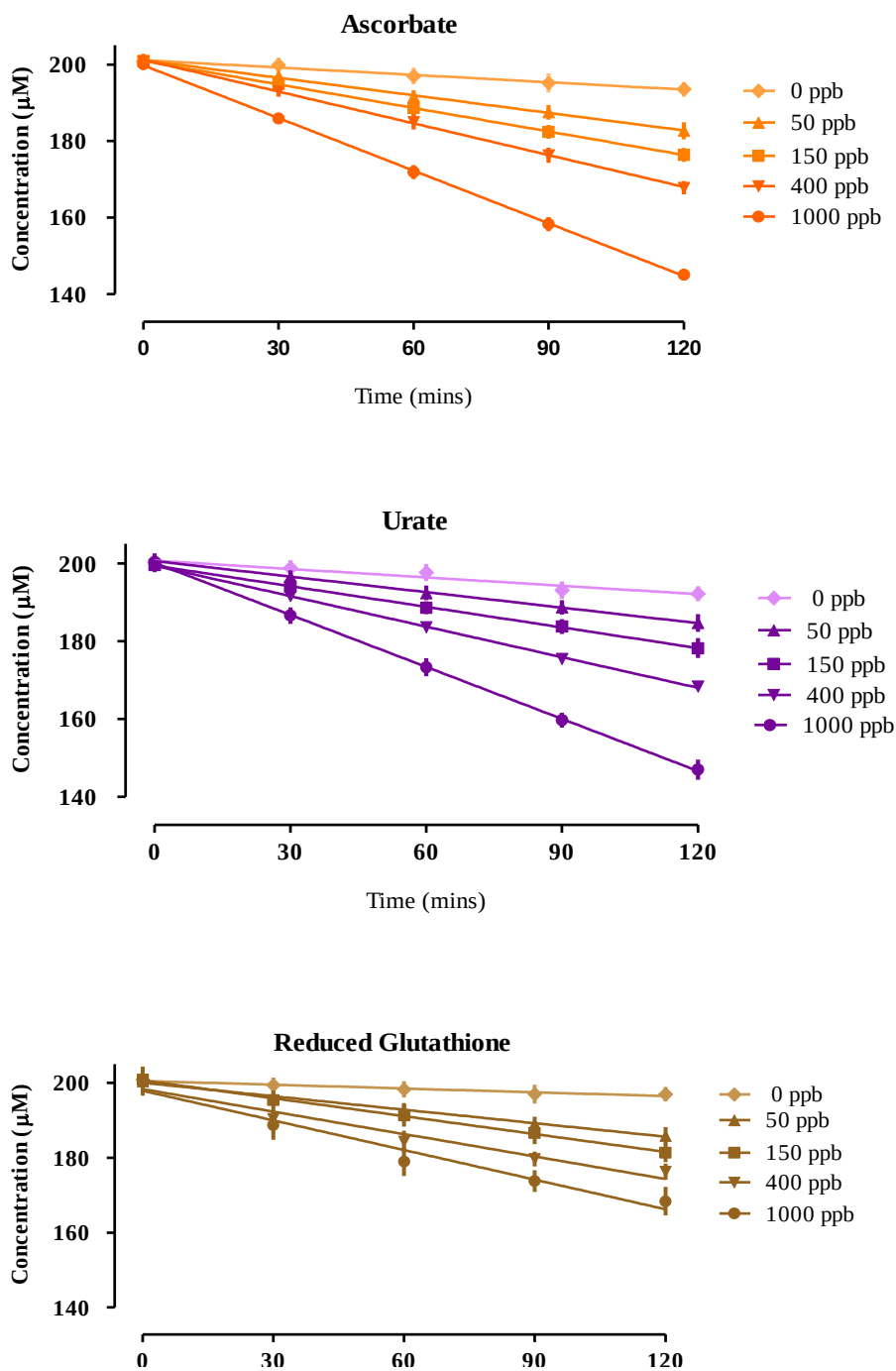


**Figure 23: Antioxidant Consumption of Ascorbic Acid, Uric Acid and Glutathione in the synRTLf.**

SynRTLf was exposed to a range of O<sub>3</sub> concentrations and zero air control. Each point represents the mean and standard deviation of three independent replications. For clarity purposes the lowest concentration on the y axis is 140μM. Antioxidant depletion rate was significantly higher than the control group (0ppb) at all tested O<sub>3</sub> concentrations ( $p < 0.001$ ; Table 7).

The rates presented in **Table 7** reflected the pattern and assumptions gained from the untransformed data, that is, the relationship between antioxidant consumption and time was highly linear and positive in character and the same held true for glutathione disulfide formation. For the three antioxidants, the lowest correlation was observed in the absence of O<sub>3</sub> (0 ppb) although it was still significant and positive in character ( $r^2$  0.90). Interestingly, the rate of consumption at all tested O<sub>3</sub> concentrations was significantly higher than the 0 control. The depletion at 0ppb reflects the action of oxidative species present in the air, that is, primarily oxygen and secondarily due to compounds such as; NO<sub>2</sub>, O<sub>3</sub>, SO<sub>2</sub> etc. For ascorbate and urate the oxidation in the presence of air is very similar at 1.05 and 1.20 mol l<sup>-1</sup>s<sup>-1</sup> respectively. However, reduced glutathione presented a significantly lower consumption rate for the same conditions ( $p=0.0001$ ). The kinetics of the O<sub>3</sub> reaction increased with increasing concentration, thus, for all antioxidants the lowest rate was observed at 0ppb and the highest at 1000ppb. The relationship between concentration and rate was examined in more detail in **Figure 24**.

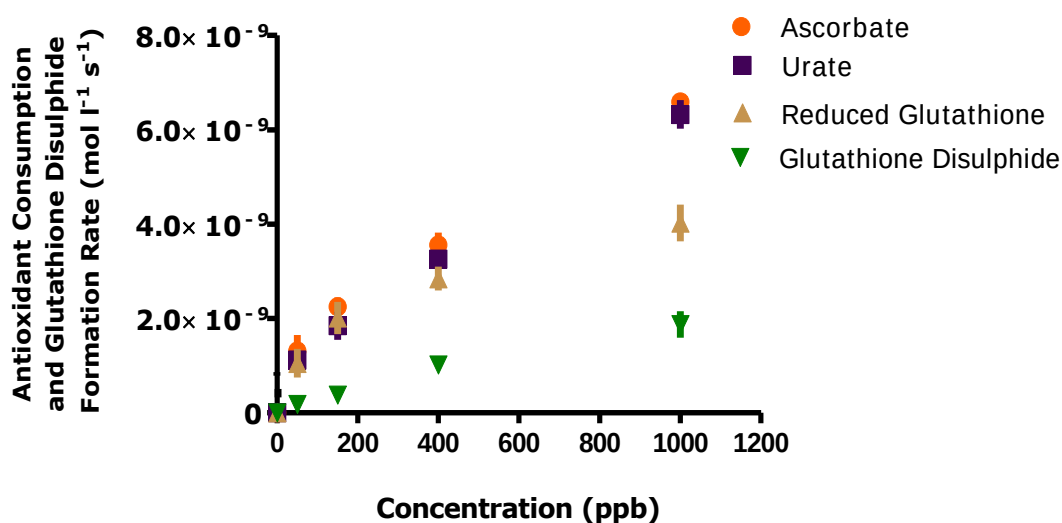


**Figure 24: Relationship between the Rate of Antioxidant Consumption and Glutathione Disulphide Formation with Ozone Concentration.**

The rates presented in Table 7 have been plotted against the respective concentration  $n=3$ . An overall antioxidant consumption rate per ppb of O<sub>3</sub> can be calculated from this relationship and is presented in Figure 25.

Specifically, in order to delineate the relationship between the calculated consumption rates and the O<sub>3</sub> concentration, the individual consumption rates (presented in **Table 7**) were plotted against their respective ozone concentration. The antioxidant consumption

**Table 7: Antioxidant Consumption and Glutathione Disulphide Formation following exposure of synRTLF to O<sub>3</sub>**

| O <sub>3</sub><br>ppb | Ascorbate                                                     |                |         | Urate                                                         |                |         | Reduced Glutathione                                           |                |         | Oxidised Glutathione                                        |                |         |
|-----------------------|---------------------------------------------------------------|----------------|---------|---------------------------------------------------------------|----------------|---------|---------------------------------------------------------------|----------------|---------|-------------------------------------------------------------|----------------|---------|
|                       | Consumption<br>Rate<br>(mol L <sup>-1</sup> s <sup>-1</sup> ) | r <sup>2</sup> | P       | Consumption<br>Rate<br>(mol L <sup>-1</sup> s <sup>-1</sup> ) | r <sup>2</sup> | P       | Consumption<br>Rate<br>(mol L <sup>-1</sup> s <sup>-1</sup> ) | r <sup>2</sup> | P       | Formation<br>Rate<br>(mol L <sup>-1</sup> s <sup>-1</sup> ) | r <sup>2</sup> | P       |
| 0                     | 1.05 ± 0.08 x 10 <sup>-9</sup>                                | 0.90           | 0.01    | 1.10 ± 0.10 x 10 <sup>-9</sup>                                | 0.91           | 0.01    | 0.52 ± 0.17 x 10 <sup>-9**</sup>                              | 0.90           | 0.01    | 0.15 ± 0.14 x 10 <sup>-9</sup>                              | 0.87           | 0.01    |
| 50                    | 2.37 ± 0.38 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 2.21 ± 0.21 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 1.57 ± 0.30 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 0.33 ± 0.14 x 10 <sup>-9</sup>                              | 0.92           | 0.008   |
| 150                   | 3.30 ± 0.20 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 2.94 ± 0.30 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 2.53 ± 0.34 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 0.53 ± 0.08 x 10 <sup>-9*</sup>                             | 0.92           | 0.008   |
| 400                   | 4.61 ± 0.26 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 4.36 ± 0.01 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 3.37 ± 0.25 x 10 <sup>-9*</sup>                               | 0.98           | < 0.001 | 1.16 ± 0.08 x 10 <sup>-9*</sup>                             | 0.91           | 0.001   |
| 1000                  | 7.64 ± 0.05 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 7.42 ± 0.30 x 10 <sup>-9*</sup>                               | 0.99           | < 0.001 | 4.55 ± 0.38 x 10 <sup>-9*</sup>                               | 0.96           | < 0.001 | 2.02 ± 0.28 x 10 <sup>-9*</sup>                             | 0.99           | < 0.001 |

Rates are expressed as mean ± SD (n=3), rates were derived from the gradients of the antioxidant consumption vs. time regression for each individual O<sub>3</sub> concentration. \* denotes a significant increase in the antioxidant consumption and glutathione disulphide formation rate compared with the 0ppb O<sub>3</sub> control. \*\* indicates that the reduced glutathione depletion was significantly lower than the equivalent value for urate and ascorbate.

at 0ppb (background oxidation) was subtracted from the consumption at each concentration, so that the results depicted only the effects of experimentally controlled O<sub>3</sub> exposures (**Figure 24**). The fact that reduced glutathione had a significantly lower depletion rate than the other two antioxidants (**Table 7**) resulted in a lowering of the antioxidant depletion contrast once the background was removed, that is, the three antioxidants presented very similar depletion rates at the lower O<sub>3</sub> concentrations, in fact the lower the O<sub>3</sub> concentration the smaller was the difference between reduced glutathione and the other antioxidants, potentially due to slower kinetics decelerating the recycling of reduced glutathione. Ascorbate and urate rates did not differ significantly at any concentration, while ascorbate was significantly higher than reduced glutathione at 400ppb ( $p = 0.016$ ) and both ascorbate and urate were more reactive than reduced glutathione at 1000ppb ( $p < 0.0001$ ).

The relationship between concentration and rate of depletion was linear and positive in character for ascorbate ( $r^2$  0.88) and urate ( $r^2$  0.92), while reduced glutathione presented a plateau effect at the highest concentrations, and could be presented better with a second order polynomial. This was the only antioxidant to fail the F test of the linear regression as the  $p$ -value was smaller than the significance level  $\alpha = 0.05$ , thus signifying a lack of linear fit ( $p = 0.01$ ) and the change at any concentration point could be calculated through the derivative of the equation ( $y = 1.08 \times 10^{-12} * x - 6.82 \times 10^{-15} * x^2$ ). This response resembled second order reaction kinetics ( $r^2$  0.93), and thus indicates a more complex stoichiometry potentially due to interactions with other antioxidant moieties in the solution or oxidative degradation products. These findings are compared with previous studies in the discussion section of this chapter.

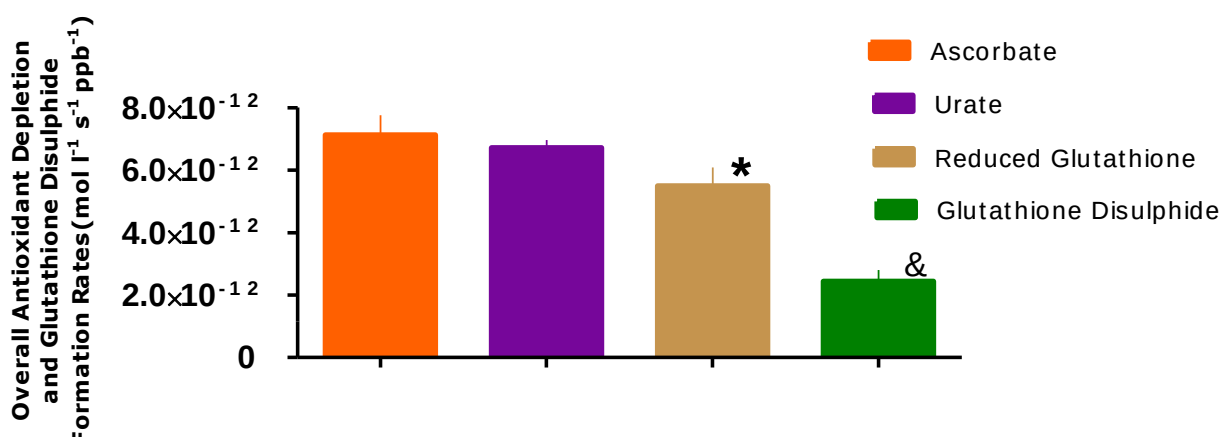


**Figure 25: Overall Antioxidant Depletion and Glutathione Disulphide Formation Rates.** Values represent mean  $\pm$  SD ( $n = 3$ ). The overall consumption rates were calculated from the gradient of the consumption rates vs. the concentrations. \* indicates a significant difference between AH<sub>2</sub> and UA vs. GSH. & indicates a significant difference between AH<sub>2</sub>, UA, GSH vs. GSSG.

The gradients of the regression lines that passed through the consumption rate data sets presented in **Figure 24** were calculated in order to identify the ‘overall antioxidant depletion rate’ expressed as  $\text{mol l}^{-1} \text{ s}^{-1} \text{ ppb}^{-1}$ . This value serves as an indicator of the overall reactivity of each substrate and allows for direct comparisons on a unitary basis. These overall consumption rates are presented in **Figure 25** and reveal a hierarchy in antioxidant depletion. The depletion rates of ascorbate and urate were very similar, but both were significantly higher than the depletion rate of reduced glutathione ( $p = 0.02$ ). In addition, antioxidant consumption was in all cases significantly higher than glutathione disulphide formation ( $p < 0.0001$ ). The latter presented a reduced/oxidised ratio that was within the expected 2:1 stoichiometry ( $5.49 \pm 0.60 \times 10^{-12} / 2.44 \pm 0.36 \times 10^{-12}$ ). Overall, the reactivity hierarchy can be denoted as  $\text{AH}_2 \geq \text{UA} > \text{GSH}$ .

### **3.5 Results: Direct Comparison of the Ozone’s and Nitrogen Dioxide’s Antioxidant Depletion Capacity in a Human Synthetic Respiratory Tract Lining fluid Model**

synRTLF was exposed to  $\text{NO}_2$  or  $\text{O}_3$  using identical experimental conditions and quantification protocols. Both air pollutants presented time and concentration dependent effects. Specifically, the consumption of each antioxidant under the conditions employed could be described by linear regressions through the untransformed data sets (Chapter 3; **Figure 20** and **Figure 23**) thus suggesting that the rate of consumption was unaltered as the substrate concentration decreased.  $\text{NO}_2$  and  $\text{O}_3$  presented consistent antioxidant depletion with time and for this reason the reactions can be described as zero order with respect to the antioxidant concentration. This finding was consistent across all concentration levels. As a result of the limited exposure time in this study (a longer period was not chosen due to evaporation; see methods section) and the realistic gaseous concentrations, complete consumption within this time course was not observed for either pollutant. However, the linearity of the response would appear to support the contention that this would indeed be the case. Indicatively, extrapolation of the regression lines through the ascorbate concentration data set to a zero concentration under equivalent conditions for  $\text{NO}_2$  or  $\text{O}_3$  (1000 ppb) would predict complete consumption at 399 mins and 480 mins respectively. The magnitude of that difference is greater when urate or reduced glutathione are considered as is discussed further on. These estimates are based rates from the brief controlled

exposure windows used in these experiments, representing a minimum time period for complete consumption, as concentrations can never be negative. In practice, the zero order kinetics would eventually progress to a different order upon limitations to substrate replenishment.

In order to quantitatively compare the oxidizing actions of NO<sub>2</sub> and O<sub>3</sub> in this model their antioxidant consumption and glutathione disulphide formation rates were calculated from the gradients of the linear regressions through the untransformed data sets and are summarized in **Table 8**. With respect to the consumption rate data, there was a similar autoxidation rate for the O<sub>3</sub> and NO<sub>2</sub> control exposures. Specifically, the 0ppb controls did not differ significantly between the two gaseous groups, thus implying reliability, reproducibility and consistency. In addition, significant increases in antioxidant depletion were observed at 50ppb for O<sub>3</sub> and 150ppb for NO<sub>2</sub> when compared to their respective control exposures (0ppb). The observed rate of NO<sub>2</sub> antioxidant consumption was significantly less than the equivalent O<sub>3</sub> values at all gaseous concentration levels (50-1000ppb) and for all antioxidants examined. Similarly, O<sub>3</sub>'s glutathione disulphide formation rates were significantly higher than the matching NO<sub>2</sub> values at concentrations equal and higher than 150 ppb. The rate of glutathione disulphide formation was not significant under an atmosphere of 150ppb O<sub>3</sub> and below; for NO<sub>2</sub> it was not significant at 400ppb and below.

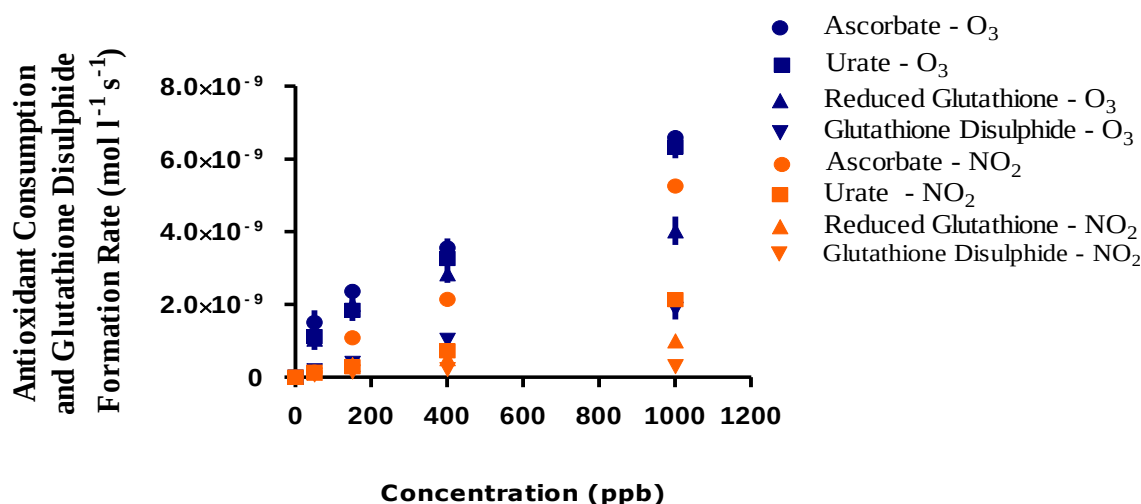


**Figure 26: Relationship between the Rate of Antioxidant Consumption and Glutathione Disulphide Formation in synRTLF exposed to Ozone or Nitrogen Dioxide Concentration.**

The rates presented in **Table 8** have been plotted against the respective pollutant concentration n=3. An overall antioxidant consumption rate can be calculated from this relationship and these data are presented in **Figure 27**.

**Table 8: Antioxidant Consumption and Glutathione Disulphide Formation rates ( $\text{mol l}^{-1} \text{ s}^{-1}$ ) of synRTLTF Samples Exposed to  $\text{O}_3$  or  $\text{NO}_2$  at Different Concentrations**

| ppb | Ascorbate                           |                                   |  | Urate                               |                                   |  | Reduced Glutathione                 |                                   |  | Glutathione Disulphide              |                                   |  |
|-----|-------------------------------------|-----------------------------------|--|-------------------------------------|-----------------------------------|--|-------------------------------------|-----------------------------------|--|-------------------------------------|-----------------------------------|--|
|     | $\text{O}_3$                        | $\text{NO}_2$                     |  | $\text{O}_3$                        | $\text{NO}_2$                     |  | $\text{O}_3$                        | $\text{NO}_2$                     |  | $\text{O}_3$                        | $\text{NO}_2$                     |  |
| 0   | $1.05 \pm 0.08 \times 10^{-9}$      | $0.98 \pm 0.03 \times 10^{-9}$    |  | $1.10 \pm 0.10 \times 10^{-9}$      | $0.91 \pm 0.11 \times 10^{-9}$    |  | $0.52 \pm 0.17 \times 10^{-9}$      | $0.60 \pm 0.10 \times 10^{-9}$    |  | $0.15 \pm 0.14 \times 10^{-9}$      | $0.15 \pm 0.06 \times 10^{-9}$    |  |
| 50  | $2.37 \pm 0.38 \times 10^{-9} * \&$ | $1.14 \pm 0.12 \times 10^{-9}$    |  | $2.21 \pm 0.21 \times 10^{-9} * \&$ | $1.06 \pm 0.05 \times 10^{-9}$    |  | $1.57 \pm 0.30 \times 10^{-9} * \&$ | $0.71 \pm 0.07 \times 10^{-9}$    |  | $0.33 \pm 0.14 \times 10^{-9}$      | $0.21 \pm 0.09 \times 10^{-9}$    |  |
| 150 | $3.30 \pm 0.20 \times 10^{-9} * \&$ | $2.08 \pm 0.17 \times 10^{-9} \#$ |  | $2.94 \pm 0.30 \times 10^{-9} * \&$ | $1.21 \pm 0.09 \times 10^{-9} \#$ |  | $2.53 \pm 0.34 \times 10^{-9} * \&$ | $0.95 \pm 0.16 \times 10^{-9} \#$ |  | $0.53 \pm 0.08 \times 10^{-9} * \&$ | $0.26 \pm 0.13 \times 10^{-9}$    |  |
| 400 | $4.61 \pm 0.26 \times 10^{-9} * \&$ | $3.14 \pm 0.08 \times 10^{-9} \#$ |  | $4.36 \pm 0.01 \times 10^{-9} * \&$ | $1.65 \pm 0.02 \times 10^{-9} \#$ |  | $3.37 \pm 0.25 \times 10^{-9} * \&$ | $1.12 \pm 0.11 \times 10^{-9} \#$ |  | $1.16 \pm 0.08 \times 10^{-9} * \&$ | $0.35 \pm 0.10 \times 10^{-9} \#$ |  |

Rates are expressed as mean  $\pm$  SD (n=3), rates were derived from the gradients of the antioxidant consumption vs time regression for each individual  $\text{O}_3$  or  $\text{NO}_2$  concentration, values are expressed as  $\text{mol l}^{-1} \text{ s}^{-1}$ . \* denotes a significantly higher antioxidant consumption or glutathione disulphide formation rate compared with the equivalent  $\text{NO}_2$  value. & indicates a significant increase in the antioxidant consumption or glutathione disulphide formation rate compared with the 0 ppb  $\text{O}_3$  control. # represents a significant increase in the antioxidant consumption or glutathione disulphide formation rate compared with the 0 ppb  $\text{NO}_2$  control. Further details of each regression analysis: coefficient of determination ( $r^2$ ) and  $p$  value can be found in Chapters 3 and 4 respectively, in summary, all values were highly linear and statistically significant. In all cases significance was assigned when  $p < 0.05$ .

The relationship between the antioxidant consumption rates and the NO<sub>2</sub> or O<sub>3</sub> concentrations were further clarified by plotting the individual antioxidant consumption rates against their respective gaseous concentration once the background oxidation was subtracted (**Figure 26**). The relationship between concentration and rate of depletion was linear and positive in character for all antioxidants exposed to NO<sub>2</sub> ( $r^2$  0.91-0.99), while in the case of O<sub>3</sub> there was a linear effect for ascorbate ( $r^2$  0.88) and urate ( $r^2$  0.92) but not reduced glutathione. These findings indicate that, other than for reduced glutathione, the reactions were first order with respect to both the NO<sub>2</sub> and O<sub>3</sub> concentration.

A further question that was examined was whether O<sub>3</sub> and NO<sub>2</sub> presented an equal affinity towards all the antioxidant moieties. A brief examination of Figure 20 and Figure 23 indicates that this was not the case. Therefore, in order to compare the antioxidant depletion rates of O<sub>3</sub> and NO<sub>2</sub> at all concentrations, the gradients of the regression lines presented in Figure 26 were used to generate the ‘overall calculated consumption rate’ expressed as mol l<sup>-1</sup> s<sup>-1</sup> ppb<sup>-1</sup>.

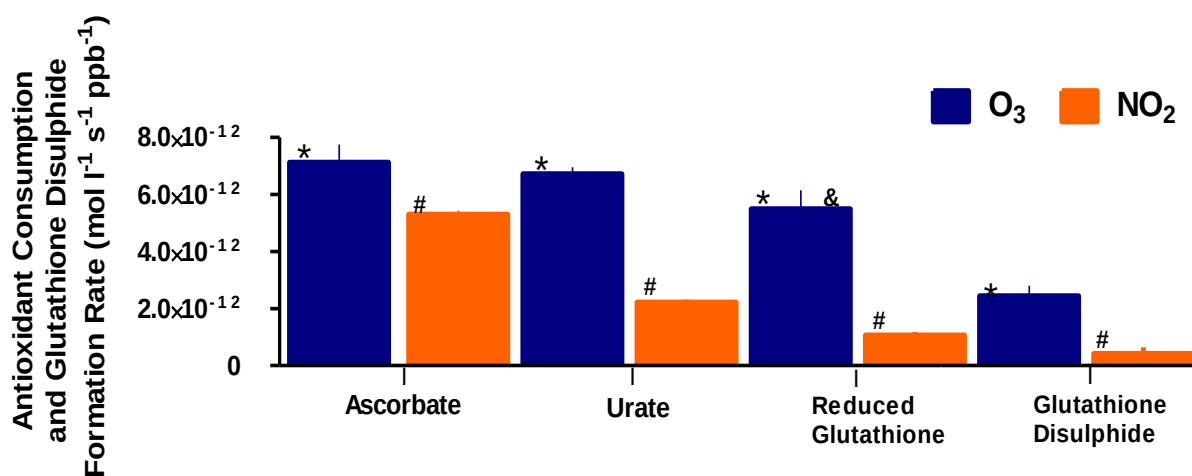


**Figure 27: Overall Antioxidant Depletion and Glutathione Disulphide Formation Rates.**

Values represent mean  $\pm$  SD ( $n = 3$ ). The overall consumption rates were calculated from the gradient of the consumption rates vs. the concentrations. \* indicates a significant difference between O<sub>3</sub> and NO<sub>2</sub>. & indicates a significant difference between AH<sub>2</sub> and UA vs. GSH for O<sub>3</sub>. # indicates that for NO<sub>2</sub> there is a significant hierarchy in antioxidant depletion, depicted as; AH<sub>2</sub> >> UA > GSH.

These rates are indicative of the substrate reactivity towards O<sub>3</sub> or NO<sub>2</sub> on a unitary basis as the same value of ppb by volume for gases is equivalent to the same number of moles for each gas. The rates are depicted in **Figure 27** and aim to facilitate direct comparisons between NO<sub>2</sub> and O<sub>3</sub> and allow the construction of reactivity hierarchies. Significant differences were observed in the overall consumption rates within and between exposure groups. Specifically, in the case of O<sub>3</sub> ascorbate and urate rates were very similar, but both were significantly higher than the overall antioxidant depletion capacity of reduced glutathione ( $p = 0.02$ ). While for NO<sub>2</sub>, all rate comparisons were significantly different ( $p < 0.0001$ ); ascorbate presented the highest reactivity and reduced glutathione the lowest. Thus, the overall reactivity hierarchies can be denoted as  $AH_2 \geq UA > GSH$  for O<sub>3</sub> and  $AH_2 \gg UA > GSH$  for NO<sub>2</sub>. In accordance with my initial hypothesis, O<sub>3</sub> presented significantly higher antioxidant depletion capacity than NO<sub>2</sub> ( $p < 0.0001$ ). Based on these results the two hierarchies can be merged as follows:

$$AH_2(O_3) \geq UA(O_3) > GSH(O_3) \geq AH_2(NO_2) \gg UA(NO_2) > GSH(NO_2).$$

Formation of oxidation products was followed only with respect to the reduced glutathione O<sub>3</sub> and NO<sub>2</sub> challenges. The rates of consumption were calculated from the gradients of the regression lines, similarly to the antioxidant depletion rates. O<sub>3</sub> resulted in a significantly higher rate of glutathione disulphide formation ( $p = 0.0006$ ). Taking into account the average and standard deviation of the reduced and oxidised glutathione rates, it can be concluded that the classical 2:1 GSH:GSSG reaction stoichiometry was occurring upon reaction with O<sub>3</sub> or NO<sub>2</sub> even under the most extreme exposure conditions.

### 3.6 Discussion

O<sub>3</sub> and NO<sub>2</sub> are the two major components of photochemical air pollution and their inhalation is associated with negative effects in the respiratory system [73, 391]. Their toxicity is thought to be mediated through the generation of radical and non radical cytotoxic products derived from their ability to rapidly oxidise biological target molecules [267, 275, 392-394]. ROS can react with proteins directly by attacking the protein backbone or by modifying the side chain amino acid residues, while indirect effects may include the crosslinking of oxidised proteins into protein aggregates by lipid peroxidation products [166]. These changes can lead to dysfunction of enzymes/proteins and ultimately breakdown of cell function [166]. In accordance with

this premise, animals exposed to O<sub>3</sub> presented decreased activity of histone deacetylase 2 (HDAC-2), likely due to protein modification and activation of the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), resulting in increased inflammatory response and emphysema [395]. Similarly, direct evidence of NO<sub>2</sub>-mediated peptide degradation has been shown *in vitro* [396]. The ozonation and nitration of lipids by these air pollutants [125, 397, 398] can further exacerbate their oxidative effects as the lipid oxidation products can induce inflammatory pathways [399, 400]. This direct and indirect activation of the inflammatory defences can lead to oxidative stress if the antioxidant defences are overwhelmed and ultimately chronic inflammatory diseases can be presented.

The first point of contact between the inhaled oxidants and the airways is the RTLF which is well invested with antioxidant defences, including small molecular electron donors [74]. In this study the aim was to characterize and directly compare the reaction of NO<sub>2</sub> and O<sub>3</sub> with ascorbate, urate and reduced glutathione via the use of a mixed interfacial exposure model. The concentration of the antioxidants used was based on published values reported in BAL studies that reflected the undiluted human RTLF antioxidant concentrations [401]. In addition, the chosen gaseous levels represent everyday (50ppb), elevated (150ppb) and extreme (400, 1000ppb) exposures but certainly remain within a realistic framework. A similar methodological approach has been adopted previously in studies examining the antioxidant depletion capacity of NO<sub>2</sub> and O<sub>3</sub> [268, 269]. However differences in experimental conditions such as the synRTLF composition complicate direct comparisons. Indicatively, an examination of the published antioxidant rate constants in studies examining either O<sub>3</sub> [286, 371, 387, 389, 390] or NO<sub>2</sub> [91, 268, 402, 403] reveals conflicting reaction rate calculations that most probably reflect significant differences in the exposure parameters. Indeed, a surprisingly small number of studies have compared the effects of the aforementioned pollutants within the same biological model. For this reason, great emphasis was placed in ensuring identical conditions in the exposure of synRTLF to O<sub>3</sub> or NO<sub>2</sub>, parameters that were considered including; temperature, gas flow, time points, pH, antioxidant quantification protocols, exposure chamber, interfacial mixing, and synRTLF batch, a more detailed presentation can be found in the methods section (Chapter 2). It should be noted that the model that was used constitutes undoubtedly an oversimplified version of the complex respiratory tract lining fluid presented in the introductory chapter. However, the antioxidant concentrations used were at physiologically relevant levels [73, 74] and the chosen air pollutant concentrations were in the lowest range of those

presented in the literature so far in order to attain an environmentally relevant perspective. Ultimately this model allowed clear examination of causal relationships and offered direct comparisons with the other air pollutants examined in the project.

O<sub>3</sub> and NO<sub>2</sub> are both oxidizing agents and therefore in many respects their effects on the respiratory system are similar [118]. Therefore, exposure of RTL models to NO<sub>2</sub> or O<sub>3</sub> has been shown to lead to depletion of ascorbate, urate and reduced glutathione [53, 268, 269, 404-406]. In accordance with these observations both gases presented antioxidant depletion capacity in this study which was time and concentration dependent (**Table 9**). NO<sub>2</sub> likely dissolved to produce nitrite and nitrous acids (  $3\text{NO}_2 + \text{H}_2\text{O} \rightarrow 2\text{HNO}_3 + \text{NO}$ ,  $\text{HNO}_3 + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \text{NO}_3^-$  ) while the reaction of the hydroxide anion with O<sub>3</sub> is thought to form hydroxyl radicals, via a cyclic reaction chain process that generates the superoxide anion and the perhydroxyl radical via several aqueous free radical intermediates ( $\text{O}_3 + \text{OH}^- \rightarrow \text{O}_2^{\cdot-} + \text{HO}_2^{\cdot}$ ) [407, 408]. Important differences were observed in the antioxidant depletion capacity of the two air pollutants, O<sub>3</sub> exposures resulted in significant antioxidant consumption at all tested concentrations while NO<sub>2</sub> exposures presented a significant effect at concentrations above 50ppb. This higher O<sub>3</sub>-induced depletion can be attributed to its redox potential. Specifically, O<sub>3</sub>, a powerful oxidant has a very high redox potential at 2.07V and NO<sub>2</sub>, an acid anhydride, has a more modest value at 0.75V [409, 410]. According to the premise of reactive absorption, the driving force for the net flux of O<sub>3</sub> and NO<sub>2</sub> is dictated by chemical reactions between the gaseous pollutants and chemical constituents of the RTL, especially the water soluble antioxidants. These interactions are thought to be the driving force maintaining the transfer of NO<sub>2</sub> and O<sub>3</sub> from the gaseous to the aqueous phase [52, 411]. This assertion could explain why O<sub>3</sub> resulted in higher oxidizing effects in the synRTL than NO<sub>2</sub>, despite O<sub>3</sub> having a lower solubility in biological fluids.

The antioxidant depletion rates were plotted against their respective O<sub>3</sub> and NO<sub>2</sub> concentrations once the 0ppb depletion was subtracted (**Figure 26**). Including the background control would not affect the overall regression analyses, however, given the lower reduced glutathione autooxidation this approach offered a clearer representation of the effects. It could be observed that O<sub>3</sub> presented higher antioxidant depletion rates than NO<sub>2</sub>, and both groups presented linear relationship between reactivity rates and gaseous concentration ( $r^2$ :0.88-0.99). Overall, reduced glutathione was the least reactive substrate for the O<sub>3</sub> and NO<sub>2</sub> exposures, and this observation could be partly

attributed to its pKa value which is lower than the other two antioxidants, and thus it is expected to be less reactive in non acidic conditions [91]. In addition, reduced glutathione presented a plateau-like effect at the two highest O<sub>3</sub> concentrations. This observation could be attributed to the very high depletion of urate and ascorbate when exposed to O<sub>3</sub>. Thus, based on the theory of reactive absorption it can be hypothesised that this significant decrease of the two aforementioned antioxidants resulted in a decrease of O<sub>3</sub> absorbed in the solution and hence a lower depletion of reduced glutathione. In addition, the recycling of reduced glutathione by urate and ascorbate may have enhanced this plateau effect.

Quantitative comparisons of the temporal antioxidant depletion across the different gaseous concentrations were achieved by calculating the slope of the linear regressions. The results revealed that O<sub>3</sub> exhibited similar affinity towards ascorbate and urate and the reduced glutathione depletion rate was significantly lower (**Figure 27**). This order of significance is in agreement with kinetic studies at physiological pH values [269, 389, 412, 413]. Exposure of synRTLTF to NO<sub>2</sub> resulted in significantly lower oxidation effects compared to the equivalent O<sub>3</sub> values. Also, NO<sub>2</sub> exhibited a more defined hierarchy; ascorbate was the primary target, urate the secondary and reduced glutathione was quite unreactive. This reactivity order is in agreement with previously published antioxidant rate constants towards NO<sub>2</sub> [91, 402, 414].

The only further study in the literature to have exposed an RTLTF model to various NO<sub>2</sub> concentrations in order to quantify antioxidant depletion rates is that of Kelly and Tetley [268]. Their study and the one presented in this chapter shared the same NO<sub>2</sub> concentrations, flow rate and very similar exposure chamber. The antioxidant depletion rates for ascorbate, urate and reduced glutathione given by Kelly and Tetley [268] were  $1.94 \pm 0.53$ ,  $2.13 \pm 0.64$  and  $1.17 \pm 0.64 \text{ nmol}^{-1} \text{ h}^{-1} \text{ ppb}^{-1}$ . These rates for comparison purposes were converted to  $\text{m l}^{-1} \text{ s}^{-1} \text{ ppb}^{-1}$  by multiplying these rates by  $10^{-9}$  ( $\text{nmol} \rightarrow \text{mol}$ ) and then by 3600 ( $\text{hours} \rightarrow \text{seconds}$ ). The resulting rates for ascorbate, urate and reduced glutathione were  $6.98 \pm 1.91$ ,  $7.67 \pm 2.30$ , and  $4.21 \pm 1.66 \times 10^{-6} \text{ mol l}^{-1} \text{ s}^{-1} \text{ ppb}^{-1}$ . Thus, the difference with my results is that ascorbate and urate did not differ significantly while these rates are strikingly higher ( $10^6$  difference) than my NO<sub>2</sub> and O<sub>3</sub> results.

There were however major differences in the composition of the synRTLTF between the two studies. Firstly, Kelly and Tetley [268] utilised a significantly higher concentration of reduced glutathione (412 compared to 200  $\mu\text{M}$ ) that could have accelerated the

depletion kinetics by increasing the reactive absorption [415] and hence potentially accelerated the depletion rates. In addition, the authors used saline as a solvent, while I used Chelex treated water, sodium chloride however-a major component of saline- can result in higher oxidation rates [416, 417]. In addition, in that study there was EDTA in the synRTLF, however, in the presence of trace metals like iron, ascorbate reduces ferric EDTA to ferrous EDTA and gets depleted in the process [418], this mechanism may be presented by the other antioxidants as well.

A further and potentially more important difference between our synRTLF composition and their model, is that their model contained deferoxamine (DFO) to minimize autoxidation [268], while this study used Chelex. However a recent study by Adgent and colleagues [419] suggested that DFO can potentially influence multiple biochemical functionalities when exposed to NO<sub>2</sub> including radical repair and reactive absorption. These main differences highlight the difficulties in directly comparing the findings of the two studies while explain why their rates are a million times higher than the ones presented in this chapter and the rates of more oxidising air pollutants like ozone [269]. These differences further highlight that the biochemical composition can greatly influence the kinetics and ultimately the calculations.

Overall, the calculated hierarchy is consistent with the hierarchy resulting from previously published antioxidant rate constants towards NO<sub>2</sub>. According to the literature, ascorbate is the most reactive with a rate constant of  $3.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  at pH 6.7 [402], while the same value was calculated to be  $1.9 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  for urate and  $1.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$  for reduced glutathione at pH 7 [91]. It can be hypothesised that ascorbate could have an even higher rate at pH 7, due to its dissociation constant; however, it is not known if that potential positive trend would be statistically significant. Also, it should be noted that the aforementioned studies generated NO<sub>2</sub> through precursors, via pulse radiolysis, though this approach may result in the generation of other confounding reactive species in the process [419].

The very high reactivity of ascorbate towards NO<sub>2</sub> is in accordance with previous studies showing that this moiety was the most effective aqueous-phase antioxidant in human blood plasma [414]. In support of its significantly protective actions against NO<sub>2</sub>, animal studies have shown that ascorbic acid deficiency enhanced the toxic pulmonary effects of NO<sub>2</sub> [257, 258]. Similarly, pre-treatment with ascorbic acid (500mg/d for 3d) prevented an increased airway responsiveness to methacholine aerosol in humans challenged with NO<sub>2</sub> (2ppm, 1h) [380]. However, in that study RTLF

samples were not investigated, thus it is not clear whether RTL F ascorbic acid levels were altered, but from animal studies it can be hypothesised that there was an elevation in that region [420]. Further support of ascorbates's superior antioxidant depletion capacity can be derived from *in vitro* systems comparing the antioxidant consumption capacity of different antioxidants. Indicatively, ascorbate was shown to be offering superior protection than vitamin E against NO<sub>2</sub> radical-like species induced lipid peroxidation [421]. Also, ascorbate presented higher scavenging abilities than urate and alpha-tocopherol in human plasma exposed to NO<sub>2</sub> [360]. This could be due to a greater affinity for NO<sub>2</sub> and also due to its ability to 'recycle' the other two antioxidants [360] as it is discussed further on.

In the process of comparing my findings with previous *in vitro* studies focusing on the antioxidant depletion rates of the antioxidants in question by O<sub>3</sub> I constructed a comprehensive literature summary (**Table 9**) a similar table was not constructed for NO<sub>2</sub> as studies investigating its antioxidant depletion capacity in RTL F models are scarce. It should be noted that for two of the studies considered the rates were not provided in the original publications and thus I have extrapolated them from the graphical representations (**Appendix A**), while for the purely kinetic models the rates expressed as mol l<sup>-1</sup>s<sup>-1</sup> were divided by the O<sub>3</sub> concentration used in those experiments in order to get an approximate estimate of the equivalent mol l<sup>-1</sup>s<sup>-1</sup>ppb<sup>-1</sup> value. Therefore, for those references the value is an approximation but nevertheless the antioxidant depletion hierarchy presented is identical to the original published values. The last four studies presented in the table represent rate constants, Kermani *et al.*, (2006) [390] compared their results with those of Mudway and Kelly (1998) [269] by a series of calculations and regression equations using selected data points from the Mudway and Kelly (1998) paper. The rate constants that they derive from Mudway and Kelly (1998) are about four times smaller for ascorbate and urate and 10 times smaller for reduced glutathione than their work. Given that the Mudway and Kelly (1998) results were very similar to the current project, it can be postulated that the same difference applies between Kermani *et al.*, (2006) and this work (see below).

Only nine studies have been conducted, and there is a great variation in the chosen O<sub>3</sub> concentrations with my experiments belonging in the lowest spectrum. In fact, there is no study that has used lower O<sub>3</sub> concentrations than the ones I used, and very rarely is there justification of the chosen concentrations. In a closer inspection of **Table 9** it can be seen that there is also great variation in the substrate composition, including;

**Table 9: Summary of Studies Investigating Ozone's Rate of Ascorbate, Urate and Reduced Glutathione Depletion in *in vitro* models**

| Reference                       | O <sub>3</sub> (ppb) | Antioxidant Concentration (μM)                                                                                                 | Exposure Conditions                                                                                                                                                                                                                      | Antioxidant Consumption Rates (mol l <sup>-1</sup> s <sup>-1</sup> ppb <sup>-1</sup> ) |                                                              |                                                                                      |
|---------------------------------|----------------------|--------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------|--------------------------------------------------------------|--------------------------------------------------------------------------------------|
|                                 |                      |                                                                                                                                |                                                                                                                                                                                                                                          | AH <sub>2</sub>                                                                        | UA                                                           | GSH                                                                                  |
| Current study                   | 50-1000              | AH <sub>2</sub> , UA, GSH each 200 in composite biochemical model                                                              | mixed interfacial exposure system: 2 ml in multiwell plate in a 5.6L chamber on an orbital shaker with 3L <sup>-1</sup> m <sup>-1</sup> flow of ozonated air, 37°C pH7, up to 120mins.                                                   | 7.12 ± 0.63 x10 <sup>-12</sup>                                                         | 6.72 ±0.24 x10 <sup>-12</sup>                                | 5.49 ±0.60 x10 <sup>-12</sup>                                                        |
| Mudway & Kelly 1998 [241]       | 100 -1500            | AH <sub>2</sub> and UA: 200 and GSH: 412 in composite biochemical model – further models were used                             | mixed interfacial exposure system: 1 ml in multiwell plate in a 5.6L chamber on an orbital shaker with 3L <sup>-1</sup> m <sup>-1</sup> flow of ozonated air, 37°C pH7.4, up to 720 mins.                                                | 5.39 ±1.04 x10 <sup>-12</sup>                                                          | 6.73±1.03 x10 <sup>-12</sup>                                 | 4.17±0.56 x10 <sup>-12</sup>                                                         |
| Mudway et al., 1996 [375]       | 50-1000              | AH <sub>2</sub> : 0.01-1.77, UA: 0.8-3.3 GSH; 1.9-4.1 from human BALF                                                          | mixed interfacial exposure system: 1 ml in multiwell plate in a 5.6L chamber on an orbital shaker with 3L <sup>-1</sup> m <sup>-1</sup> flow of ozonated air, 37°C pH7.4, up to 720 min.                                                 | 1.70 ± 0.8 x10 <sup>-12</sup>                                                          | 1.00 ± 0.5 x10 <sup>-12</sup>                                | Depletion was O <sub>3</sub> concentration independent                               |
| Housley et al., 1995 [258]      | 50-1000              | UA; 8.6 from human nasal lavage; pooled sample- AH <sub>2</sub> and GSH were mostly not detected                               | mixed interfacial exposure system: 1 ml in multiwell plate in a 5.6L chamber on an orbital shaker with 3L <sup>-1</sup> m <sup>-1</sup> flow of ozonated air, 37°C, pH7.4, up to 240 mins                                                | -                                                                                      | 6.48±1.03 x10 <sup>-5</sup>                                  | -                                                                                    |
| Cross et al., 1992 [381]        | 16000                | AH <sub>2</sub> : 59-84, UA: 317-445 from human blood plasma                                                                   | mixed interfacial exposure system: 5 ml on surface of a rotating culture dish inside a closed container in 5% CO <sub>2</sub> and 95% O <sub>2</sub> , 2L <sup>-1</sup> .m <sup>-1</sup> flow of ozonated air, 37°C, pH7., up to 240min. | 2.96 x 10 <sup>-13</sup> **                                                            | 1.64 x 10 <sup>-12</sup> **                                  | -                                                                                    |
| Vander Vliet et al., 1995 [382] | 2000-16000           | AH <sub>2</sub> : 48-93, UA: 225-350 from human blood plasma. GSH was investigated separately by addition of 1000μM in plasma. | 4ml placed in petri dishes inside 6L chamber in 5% CO <sub>2</sub> and 95% O <sub>2</sub> with 3L <sup>-1</sup> .m <sup>-1</sup> flow of ozonated air, pH7.4, up to 120mins.                                                             | 1.79 x 10 <sup>-13</sup> **                                                            | 1.32 x 10 <sup>-12</sup> **                                  | 1.74 x 10 <sup>-11</sup> **                                                          |
| Kermani et al., 2006 [376]      | 3000                 | 200 in single antioxidant solutions                                                                                            | glass diffuser: 3ml stirred liquid placed in glass diffuser and contacted with ozonated air 25°C, pH 7.4, 15mins.                                                                                                                        | 5.83 x 10 <sup>4</sup> mol l <sup>-1</sup> s <sup>-1</sup> &                           | 5.50 x 10 <sup>4</sup> mol l <sup>-1</sup> s <sup>-1</sup> & | 57.5 mol l <sup>-0.75</sup> s <sup>-1</sup> &                                        |
| Pryor et al., 1984 [372]        | Not Given            | Exact concentration not given, postulated very high                                                                            | stop flow spectrometer system in a range of acidic pHs, and then data extrapolated to pH7.                                                                                                                                               | -                                                                                      | -                                                            | 7.0 x 10 <sup>8</sup> mol l <sup>-1</sup> s <sup>-1</sup>                            |
| Giamalva et al., 1995 [373]     | Not Given            | Exact concentration not given, postulated very high, single antioxidant solutions                                              | stop flow spectrometer system in a range of acidic pHs, and then extrapolated to pH7.                                                                                                                                                    | 6.0 x 10 <sup>7</sup> mol l <sup>-1</sup> s <sup>-1</sup>                              | 1.4 x 10 <sup>6</sup> mol l <sup>-1</sup> s <sup>-1</sup>    | -                                                                                    |
| Kanofsky & Sima, 1995 [374]     | 30,000               | 100,000 (Biochemical model also containing methionine thiosulfate, cysteine)                                                   | static solution:, ozonated air bubbled through an impinging jet at 1.25 ml/sec for 2min, pH7.                                                                                                                                            | 1.60 x 10 <sup>3</sup> *<br>4.8 x 10 <sup>7</sup> mol l <sup>-1</sup> s <sup>-1</sup>  | -                                                            | 8.33x 10 <sup>1</sup> *<br>2.5 x 10 <sup>6</sup> mol l <sup>-1</sup> s <sup>-1</sup> |

A brief summary of the experimental conditions and results of similar studies are presented in the table above. In order to facilitate direct comparisons all results have the same units \* indicates that the original rates (presented in blue color) were divided by the single exposure concentration in order to get a per ppb estimate\*\*indicates that the values were estimated from graphs, & indicates that the authors compared their findings with Mudway et al., 1996 in the Appendix of the publications and found results that were larger but in the same order of magnitude.

biochemical solutions, blood plasma, nasal and bronchoalveolar lavage. Also, exposure protocols vary, including a glass diffuser, flow stop reaction set ups, static biochemical solutions and exposure chambers using a mixed interfacial exposure system like the one I used. In this study, O<sub>3</sub> was streamed rather than bubbled as constant bubbling is unlikely *in vivo* due to the surfactant providing stable surface tension, in addition, pilots showed that it greatly increased evaporation. These important methodological differences may partly explain the contrasting hierarchies of the ascorbate, urate and reduced glutathione reactivities towards O<sub>3</sub>.

Pryor's group utilised a stopped flow reactor and reported the following reaction rates;  $7 \times 10^8 \text{ mol}^{-1} \text{ s}^{-1} \text{ ppb}^{-1}$  for reduced glutathione [388] and  $6 \times 10^6 \text{ mol}^{-1} \text{ s}^{-1} \text{ ppb}^{-1}$  for ascorbate and  $1.4 \times 10^6 \text{ mol}^{-1} \text{ s}^{-1} \text{ ppb}^{-1}$  for urate [387]. Therefore, suggesting a predicted hierarchy; GSH > AH<sub>2</sub> > UA. The authors investigated the kinetics in single antioxidant solutions rather than a mixture, and this factor may possibly influence the magnitude and potentially the order of the oxidative potential due to interaction effects such as antioxidant recycling mentioned previously. In addition, the authors used acidic pHs and hypothesized that the latter is not an important factor on antioxidant consumption, an argument that has been challenged as previous studies have shown that the rates of reaction between O<sub>3</sub> and a range of substrates is significantly influenced by the pH of the exposed solution [304]. The antioxidant depletion hierarchy presented by Pryor has not been replicated by any further studies. For example, Kanofsky and Sima [389] utilized a needle that bubbled 1.25 ml/sec of ozonated air in single antioxidant solutions. There reported rates present a hierarchy of AH<sub>2</sub> > GSH. The direction of their results is in accordance with my findings; however, they have a much higher magnitude. There are a lot of potential explanations for this including the extremely high concentration of O<sub>3</sub> that was used in their study and the gaseous impingement approach used which could result in an increase of the O<sub>3</sub> diffusion and hence kinetics. Also, a significant factor to consider when interpreting these results is that the authors used reactive rate constants that were mathematically estimated based on literature values of the methionine reaction rate with O<sub>3</sub>, thus their design can be described as a quasi experimental mathematical modelling approach.

My results are in closer agreement, overall, with studies that employed a streaming method in a composite solution. For example, my O<sub>3</sub> depletion rates and resulting hierarchy are in close agreement with the ones presented by Mudway and Kelly [269], differences in our rates are not statistically significant. My study and the one conducted

15 years ago had a lot of methodological similarities but also a few differences; as Mudway and Kelly used a longer exposure period, a different O<sub>3</sub> concentration range and a slightly higher pH. The latter has been shown to be an important factor, influencing depletion rate of thiol containing compounds, including glutathione [304]. Acidic pH is associated with higher rates for glutathione, while ascorbate and urate rates are not thought to change significantly for a pH range between 5 and 7.4 [304]. It can be assumed; that the 0.4pH difference between our studies would be unlikely to cause a marked effect and any way it is within the pH instrument reading error. The good agreement in results signifies the repeatability of the research findings and the robustness of the experimental methodology. There has been a very careful consideration of potential confounders that were controlled for and these include; system sealing, flow, concentration and temperature stability, also, evaporation and pH consistency. All these parameters are discussed in detail in the methodological controls subsections (2.2.1).

Our antioxidant depletion rates were also comparable with *in vitro* exposure of bronchoalveolar lavage (BAL) samples to O<sub>3</sub> by Mudway and colleagues [371]. The authors utilized a similar set up to the one that I used and reported a similar antioxidant depletion hierarchy. However, in that study reduced glutathione was largely unreactive, as it presented the same rate of depletion for all ozonated air exposures. This contrast between the BALF and biochemical medium results can be attributed to the compositional complexity of the former. Potentially, its multiple components lead to differential kinetics. To complicate matters further, lavage samples from different parts of the respiratory tract exposed to O<sub>3</sub> have been shown to present very different antioxidant depletion rates. Housley and colleagues (1995) reported a very high urate depletion rate in nasal lavage samples exposed to O<sub>3</sub> and a near complete absence of ascorbate and reduced glutathione [286]. Therefore, the very high urate depletion rate reported in that study may be partly attributed to urate being the sole target, and thus not having to antagonize the scavenging abilities of other antioxidant moieties.

All studies presented in **Table 9**, had a higher depletion rate of ascorbate than urate when these antioxidants were tested in a composite solution. However, only three presented error estimates besides the current [269, 286, 371] and for those studies the difference between the two antioxidants is statistically insignificant, for the remaining studies, it is unknown if this difference is important. Also, all studies that included reduced glutathione in a composite system, presented it as the least reactive substrate,

but the magnitude of that difference varied greatly across studies. It can thus be postulated, that all previous studies point towards the direction that ascorbate and urate have probably quite similar scavenging rates and reduced glutathione is significantly less affected by an O<sub>3</sub> challenge. It is also true that human samples presented a higher complexity, with the rate of antioxidant depletion being dependent on the exact physiological origin of the sample (nasal vs. bronchoalveolar lavage). Taken together, I believe that it was the right decision to perform the O<sub>3</sub> experiments presented in this chapter rather than base my NO<sub>2</sub> and PM comparisons (chapter 5) on theoretical calculations and historical values.

The observed differences in the antioxidant depletion hierarchies of O<sub>3</sub> and NO<sub>2</sub> may imply differences in substrate affinities towards the gaseous pollutants but also different substrate interactions within each exposure condition. Specifically, the urate radical is thought to be able to be converted back to its reduced form by ascorbate in the presence of NO<sub>2</sub> [89, 90] but not in the presence of O<sub>3</sub> [422]. O'Neill and colleagues (1995) demonstrated that a) addition of ascorbate prior to the NO<sub>2</sub> exposure diminished urate oxidation, b) depletion of ascorbate by ascorbate oxidase accelerated urate's depletion and importantly these effects were not observed for O<sub>3</sub> [422]. This observation implies mechanistic differences in the reaction of the two gases with urate which still have not been fully revealed. It was suggested that the urate radical can be reduced by ascorbate upon NO<sub>2</sub> oxidation [422] and therefore the experimentally calculated rates in composite antioxidant solutions can differ from theoretical calculations and from single antioxidant solutions (underestimation of ascorbate reactivity and overestimation of urate reactivity as they would not consider this 'recycling' process). Differences in antioxidant interactions following O<sub>3</sub>/NO<sub>2</sub> exposures have also been demonstrated in other *in vitro* studies (**Figure 28**).

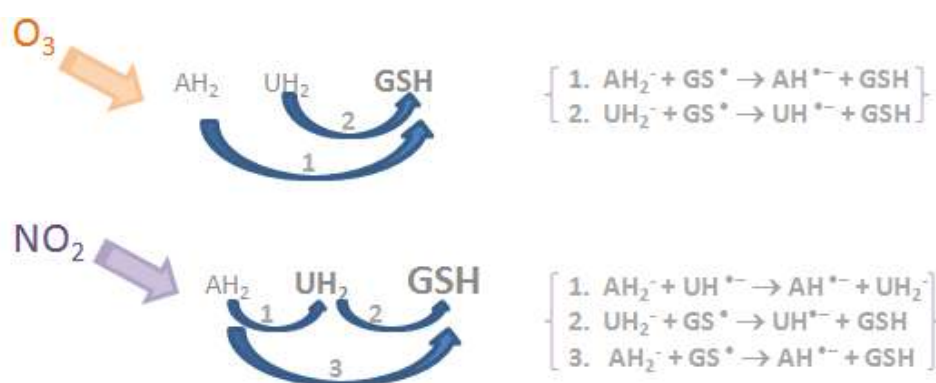


**Figure 28: Schematic of Potential Antioxidant Electron Transfer (Repair) Mechanisms upon  $O_3$  or  $NO_2$  Exposure.**

Based on the results of this study and previous ones [89, 90, 422] it is hypothesised that the antioxidant interactions are oxidant specific. Exposure of RTLFL to  $NO_2$  may result in urate radicals that can be more readily converted back to their reduced form by ascorbate. This observation along with  $O_3$ 's higher redox potential may explain differences in the magnitude and hierarchy of the antioxidant consumption.

Indicatively, glutathione depletion resulted in increased sensitivity in the  $O_3$  exposures compared to  $NO_2$ , and ascorbate preincubation was more beneficial in cells exposed to  $NO_2$  [382]. Taken together, these findings along with the results of this work, suggest that the antioxidant interactions within an *in vitro* exposure system are different upon exposure to  $O_3$  or  $NO_2$ , a theory that however awaits *in vivo* confirmation. Therefore, it can be suggested that upon  $O_3$  or  $NO_2$  exposure some of the antioxidant radicals can be reduced, and this electron transfer mechanism may be more extensive for  $NO_2$  (**Figure 28**).

However, in human exposures the antioxidant depletion hierarchy and time dependent changes appear to be more complex. Indicatively, a human chamber study changes in the antioxidant levels of BL and BAL samples were investigated 1.5, 6 and 24h post a 2ppm  $NO_2$  exposure [128]. The investigators demonstrated that these responses varied depending on the time that had elapsed post exposure; ascorbate and urate levels decreased 1.5 hours post exposure. Afterwards, ascorbate levels returned at the baseline values, but surprisingly urate levels increased above the control values at 6h and returned to normal values at 24h [128]. In the same study reduced glutathione levels were significantly higher than the control for the first two time points and concentrations returned to normal at the last sample collection [128]. This chamber study is the only human exposure to  $NO_2$  that has measured changes in ascorbate, urate

and reduced glutathione levels at multiple time points, the increase in the glutathione levels could be reflecting active secretion to the oxidant insult, while the increase in urate levels still has not been fully explained.

To complicate matters further, it has been shown that after four sequential exposures to 2ppm NO<sub>2</sub>, there was no change in the BL or BAL antioxidant status [240]. These results generate a lot of questions that have not been answered, such as, is this accommodated antioxidant function response true only for high NO<sub>2</sub> concentrations? Is there an unidentified threshold effect? Could one argue that brief exposure to high NO<sub>2</sub> concentrations has a protective effect? How is the antioxidant accommodation signal transmitted, is there a potential pharmacological target? Why did the concentration of urate increase after 6h, and not ascorbate that is, what are the differences between the two antioxidant secretion pathways? [128] Is there an equivalent response in individuals with pre-existing respiratory illness? Future *in vivo* studies examining multiple environmentally relevant NO<sub>2</sub> concentrations and time points, in healthy and respiratory challenged individuals, in combination with isotopically labelled antioxidants could delineate some of these questions. Given the *in vitro* set up of the current study, these questions could not be addressed, however, the kinetic data and direct air pollutant comparisons in further chapters aimed to assist the field in moving in a forward direction.

In the process of comparing the NO<sub>2</sub> and O<sub>3</sub> effects it is important to consider their site of action in the respiratory tract as this can influence differences in the antioxidant availability and hence the reaction kinetics. Both O<sub>3</sub> and NO<sub>2</sub> (0.1-0.5ppm) are thought to be absorbed primarily in the lower respiratory tract [54, 56]. In the upper respiratory system, the uptake efficiency has been calculated to be around 40% for both pollutants [54, 423] and in the lower respiratory tract the values range between 60% and near 100% depending on ventilation rates [54, 423]. These study results utilizing concentrations in the range of 0.1-0.4ppm are in accordance with animal morphological studies demonstrating that O<sub>3</sub> and NO<sub>2</sub> uptake occurs throughout the lower respiratory tract and the principal site is the centriacinar pulmonary region but O<sub>3</sub> effects extend also more peripherally towards neighbouring alveolar walls and ducts potentially due to higher toxicity [424].

Now looking at the antioxidant's concentrations at the suggested sites of NO<sub>2</sub> and O<sub>3</sub> action, urate is the principal antioxidant present in the proximal and distal airways; its concentration is thought to be double the reduced glutathione levels and triple the

ascorbate concentration [73, 74]. It should be noted that despite this variation in human RTLF composition, the synRTLF concentration of 200 $\mu$ M was chosen because it constitutes a representative concentration of the entire RTLF. Also, there are large error estimates in studies that have measured antioxidant concentration in the human RTLF, this deviation is potentially reflective of intersubject variability *in vivo* and/or represents an artifact of the sample preparation associated with human lavages. Given the antioxidant's relative concentrations *in vivo* and the pollutant's physiological sites of action it is hypothesised, based on the theory of reactive absorption, that urate would exhibit the highest depletion rates when exposed to either O<sub>3</sub> or NO<sub>2</sub>. In addition, it is predicted that reduced glutathione, the least reactive substrate, would confer insignificant reducing abilities when exposed to NO<sub>2</sub> *in vivo*. Indeed, Postlethwait's group have demonstrated in rat BAL and pure biochemical solutions of GSH that the greater the initial antioxidant concentration the greater the fractional uptake of these gaseous pollutants during the exposure period, and hence the greater the rate of reactive absorption [53, 267, 411]. However, it is fully acknowledged that such predictions are rather simplistic as inter-individual differences in antioxidant concentration, potential adaptation effects, the presence of other components such as proteins and lipids, and mechanistic/metabolic changes construct a more complex *in vivo* reality. Nonetheless, the purpose of this exercise is to highlight the intricacy in establishing the importance of different antioxidant substrates.

A further issue to be considered is whether combined exposure to O<sub>3</sub> and NO<sub>2</sub> would result in different effects. This study focused on the independent effects of these two air pollutants partly because they present opposing concentrations in the environment (Chapter 5). However, some work was conducted in order to investigate this issue. In the literature, a few studies explored the effects of combined O<sub>3</sub> and NO<sub>2</sub> concentrations, often however individual effects were not tested within the same experimental system, and therefore the relative effects were obscured. If one considers animal studies that included such controls, NO<sub>2</sub> and O<sub>3</sub> appear to synergistically cause lethality and biochemical changes [245, 425-428]. However, it should be noted that such interactions are more prominent at concentrations that exceed realistic environmental or occupational scenarios [427]. Human chamber studies, however, have not replicated the animal findings as the doses used would be unethical and often lethal, also, species dependent differences and lower duration of exposure are further factors that may explain this inconsistency [429-432]. Similarly, the only study to have

examined the effects of concomitant O<sub>3</sub> and NO<sub>2</sub> exposure in an RTLTF model found no synergistic effects; in fact, there were antagonistic actions [422]. Specifically, exposure to either 6ppm NO<sub>2</sub> and 10ppm O<sub>3</sub> or 14.4ppm NO<sub>2</sub> and 0.8ppm O<sub>3</sub> (the opposite combinations were not examined i.e. 0.8ppm NO<sub>2</sub> and 14.4ppm O<sub>3</sub>) resulted in significantly lower antioxidant depletion than the independent exposures. In accord with these findings, in the current study, exposure of synRTLTF to O<sub>3</sub> and NO<sub>2</sub> resulted in effects that, depending on the concentration combinations, did not differ either from the independent gaseous exposures or air controls. Initially, equal concentrations of NO<sub>2</sub> and O<sub>3</sub> were mixed, this created very significant antagonistic effects (i.e. decrease in oxidative damage) that are in accordance with previous studies [422] and as a result the end points considered did not differ from the control group. There was a very important, immediate O<sub>3</sub> disappearance (8±5ppb), while the NO<sub>2</sub> changes could not be monitored accurately due to the inability to distinguish between NO<sub>2</sub> and N<sub>2</sub>O<sub>5</sub>. In contrast, this antagonistic effect was not significant when either air pollutant's concentration was notably higher. Specifically the antioxidant depletion of synRTLTF exposed to 1000ppb O<sub>3</sub> and 100ppb NO<sub>2</sub> concomitantly did not differ significantly from a single 1000ppb O<sub>3</sub> exposure and *vice versa*.

Thus, these findings agree with the contention that the product of the reaction between NO<sub>2</sub> and O<sub>3</sub>; dinitrogen pentoxide, does not have a significant toxicity *in vitro* and may even act as a scavenger of O<sub>3</sub> derived radicals but form cytotoxic products [422]. Though the possibility of encountering equally high concentrations of these gases in real life, like the ones used in the current experiment is extremely unlikely as the aforementioned pollutants follow an opposite concentration pattern. Also, the effects of combined exposures in humans are complicated by the fact that continuous exposure to NO<sub>2</sub> can result in increased inhaled bolus O<sub>3</sub> absorption in healthy humans compared to continuous O<sub>3</sub> exposure in a single pollutant exposure system [433]. It has been suggested that *in vivo* exposure to either pollutant may lead to the remaining gas penetrating more deeply in the respiratory tract and hence causing a greater toxicological response [422]. Future studies should measure in detail the reaction products formed during the combined exposure of O<sub>3</sub> and NO<sub>2</sub> in different RTLTF models and include further oxidative markers. Given the conflicting results presented in *in vivo*, *in vitro* and chamber studies the public health significance of this interaction remains to be determined.

A potential limitation of the model that I used is its lack of lipids and proteins. Specifically,  $O_3$  can readily react with unsaturated fatty acids and these ozonated products can then diffuse through the cell membrane [385, 393]. Indeed, studies have shown strong similarities between the inflammatory responses caused by direct  $O_3$  exposure and that caused by direct administration of the lipid ozonation products, thus supporting the contention of lipid reaction product driven  $O_3$  toxicity [399]. Similarly,  $NO_2$  –lipid protein reaction- The presence of lipids in my model would probably cause a decrease in the antioxidant consumption rates, as in studies where lipid containing samples were utilised, such as BAL samples, lower antioxidant depletion rates were observed [371] compared with similar studies using simpler biochemical models but the hierarchy was the same. In contrast, I would hypothesise a different hierarchy in the presence of protein. It has been shown previously in human albumin models that urate reactivity is greater than that of ascorbate and reduced glutathione combined, potentially because the intrinsic reactivities of the two aforementioned substrates towards  $O_3$  are more in line with those of the reactive amino acids [269]. This limitation of my model is also a potential advantage, as it allows me to focus on the antioxidant depletion kinetics of  $O_3$  and attribute direct causation.

Last, but not least, in this Chapter  $O_3$ 's and  $NO_2$ 's oxidising effects were considered under identical concentrations, but their real life ratios will be considered as well. Certainly  $O_3$  presented higher antioxidant depletion rates than  $NO_2$ , however, one has to wonder whether that hierarchy would be maintained once their relative concentrations in different areas, and in London especially, are taken into account. This topic will be considered in detail in chapter 5 along with the PM rates.

## Conclusion

Modelling the interaction of gaseous pollutants will always be challenging, this study is limited by the simplification of the synRTLTF composition. Indeed, *in vivo* the RTLTF is well invested with further antioxidant defences such as metal binding proteins, enzymes, and other antioxidant compounds that can be replenished [80, 81]. However, the water soluble antioxidants that were examined have no major stores of constant supply within the body. In addition, while there was a lack of protein and lipid moieties,  $O_3$  and  $NO_2$  antioxidant depletion is thought to be influenced in a similar way by the model complexity, that is, both air pollutants exhibited lower rates in studies that utilized different models of complexity, but differences were within the same order of

magnitude [268, 269]. This could be because water-soluble antioxidants are believed to be the principal drivers of NO<sub>2</sub> and O<sub>3</sub> absorption [53, 267].

The major concern of this project was to quantitatively compare the antioxidant depletion capacities of NO<sub>2</sub> and O<sub>3</sub>, the major gaseous air pollutants, due to a lack of studies comparing them under identical control conditions. This RTLTF model may be considered appropriate as inferences about causal relationships are simplified. In summary, this study demonstrated that when synRTLTF was exposed to environmentally relevant concentrations of O<sub>3</sub> or NO<sub>2</sub>, antioxidant depletion occurred. Therefore, these findings are in agreement with previous research suggesting that antioxidants in the respiratory system are probably playing a protective role. In addition, NO<sub>2</sub> presented a lower oxidizing potential than O<sub>3</sub> and there were also differences in the pollutant's antioxidant depletion hierarchies. However, the relative O<sub>3</sub> and NO<sub>2</sub> concentrations at each environmental scenario should be taken into account before presuming their oxidative impact.

## 4 Exposure of a Human Respiratory Tract Lining Fluid Model to Particulate Matter

### 4.1 Introduction

Numerous epidemiological studies have found positive associations between exposure to particulate matter (PM) with aerodynamic diameter  $\leq 10\mu\text{m}$  ( $\text{PM}_{10}$ ) -also known as the ‘thoracic particles’- and adverse health effects, including respiratory morbidity and mortality [434-437]. These findings are in accordance with controlled human exposures, animal and *in vitro* studies [438, 439]. The toxicological basis of these health effects has not been fully delineated; however accumulating evidence suggests that the capacity of inhaled PM to induce respiratory and systemic inflammatory toxicity is a function of its ability to generate reactive oxidative species in the lung versus the robustness of antioxidant defences in the lung [247, 440-442]. Furthermore, spatial formation of these species is believed to be focused in the respiratory tract lining fluid (RTLFL) where there is a network of antioxidants [79]. These are thought to interact with oxidizing species located on the particle surface such as transition metals and this results in the scavenging of the RTLFL antioxidants and particles reaching the surface of the lung in a less active form [79].

The challenge in establishing PM’s biological effects partly lies in its diverse chemical and physical characteristics [243, 443, 444]. This heterogeneity can be addressed in toxicological studies by measuring the capacity of PM to oxidise physiological targets, that is, an integrated sum of its oxidant components can be a biologically relevant metric of its toxicity [79, 281, 445]. A small number of studies have systematically assessed the PM oxidative potential (OP) of different locations [280, 446-448] and some have included in their comparison roadside locations and those situated away from major roads [113, 114, 449-451]. There are important methodological differences among these studies which result in varied interassay correlations [452]. Overall however these field studies have elucidated the spatial variation of PM characteristics, and from the outdoor samples tested roadside PM had a higher oxidative activity than the background location. Yet, the aforementioned studies have not a) conducted oxidative analyses from concurrently collected filters b) utilised 24h PM deposition on filters c) compared more than 40 samples per location.

In addition, increasing interest has developed in assessing the relative contribution of different experimental methodologies on redox or oxidant measurements. Some

researchers have postulated that the physical and chemical properties of particles may be critically dependent on the extraction and suspension protocols employed [295-298] as well as the field collection procedures [453]. For this reason, this study employed a novel *in vitro* protocol that quantified directly the antioxidant depletion rate of PM filter disc cuts under physiologically relevant conditions. The aim of this study was to quantify the antioxidant depletion capacity of PM in the synRTLTF so that its effects could be compared in the following chapter with those of NO<sub>2</sub> and O<sub>3</sub>. Given the spatial and temporal heterogeneity of PM's composition it was decided to utilize multiple samples from two contrasting locations. Therefore a major aim of this chapter was to quantitatively compare the oxidative burden associated with matched daily PM filters from a busy roadside and a background site by utilising a synthetic human RTLTF model. It was hypothesised that roadside samples would exhibit significantly higher antioxidant consumption ability than the background samples.

## 4.2 Experimental Design

In this model system, RTLTF was represented by a composite solution of its three major water soluble antioxidants; ascorbate, urate and reduced glutathione, as described in chapter 2.1.3. PM<sub>10</sub> samples were collected daily on teflon coated glass fiber filters (Emfab) at the Marylebone Road and North Kensington air quality monitoring stations, but I was blinded about the identity of the filters in order to eliminate bias. Simultaneous collection (i.e. the same dates for the two sites) occurred by utilizing the Partisol-Plus 2025 sequential air samplers at a 24m<sup>3</sup>d<sup>-1</sup> flow rate. The antioxidant depletion capacity of the different filters was assessed by exposing samples to synRTLTF as the standardised protocol describes in 2.3. Briefly, disc cuts (5mm) were performed on 44 matched roadside (Marylebone Road) and urban background (North Kensington) filters, these were then incubated in synRTLTF for 120 minutes and quantification of antioxidant concentrations and glutathione disulphide formation occurred at 0, 30, 60, 90 and 120 minute as outlined in 2.1.4 and 2.1.5. This model differs from non-RTLTF methods because it incorporates the direct antioxidant consumption by PM, also the antioxidant adsorption on particles and the antioxidant oxidation by secondary oxidative reactions. Therefore it integrates the oxidant components of PM while avoiding potential drawbacks associated with extraction protocols such as loss of oxidants.

### 4.3 Results

A total of 88 daily PM<sub>10</sub> filters were collected in London in 2010, the samples originated from two locations; background and roadside and were collected simultaneously. A synRTLF model was utilised in order to assess the particle antioxidant depletion capacity at five time points. All tested PM<sub>10</sub> samples resulted in ascorbate and reduced glutathione consumption as well as glutathione disulphide formation. Urate levels however, showed no signs of depletion (**Figure 30**) and for this reason urate is not depicted in any further figures in this chapter.

Exposure of PM<sub>10</sub> disc filter cuts to synRTLF resulted in a constant antioxidant depletion that was linear with time ( $r^2$ : 0.79-0.99). This consistency of response with time showed that the rate of depletion was independent of the antioxidant concentration, that is, the reaction was zero order with respect to the antioxidant concentration. Therefore, a simple linear regression model was used in order to calculate the rate of antioxidant consumption and glutathione disulphide formation. For each filter this rate was equivalent to the slope of the linear regression ( $y = a + bx$ ;  $b = ((n\sum Xy - (\sum x)(\sum y)) / (n\sum x^2 - (\sum x)^2))$  where the dependent variable was concentration ( $\text{mol l}^{-1}$ ) and the independent variable was time (seconds).

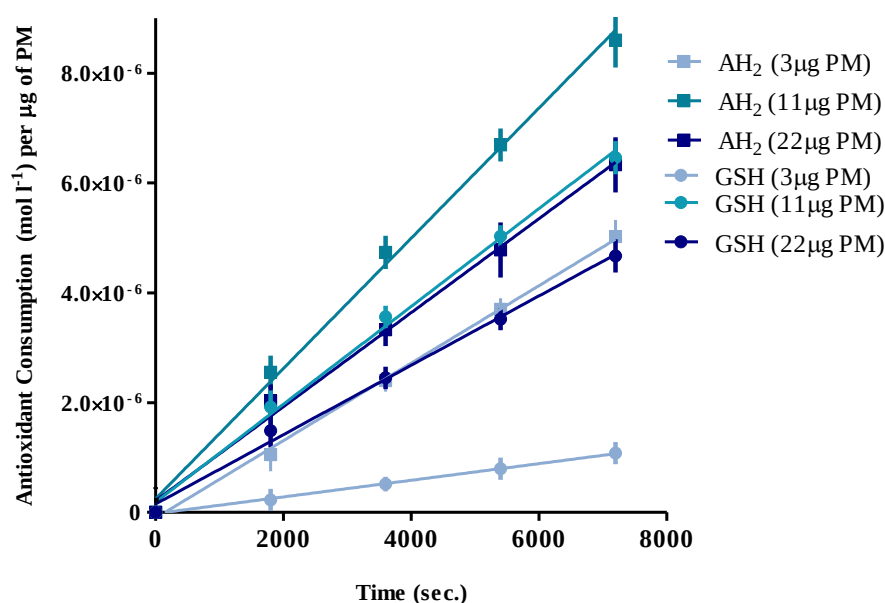


**Figure 29: Relationship between the Rate of Antioxidant Consumption and Glutathione Disulphide Formation with PM Filter Disc Cuts having different Weight.** Each point represents average  $\pm$  SD ( $n=3$ ).

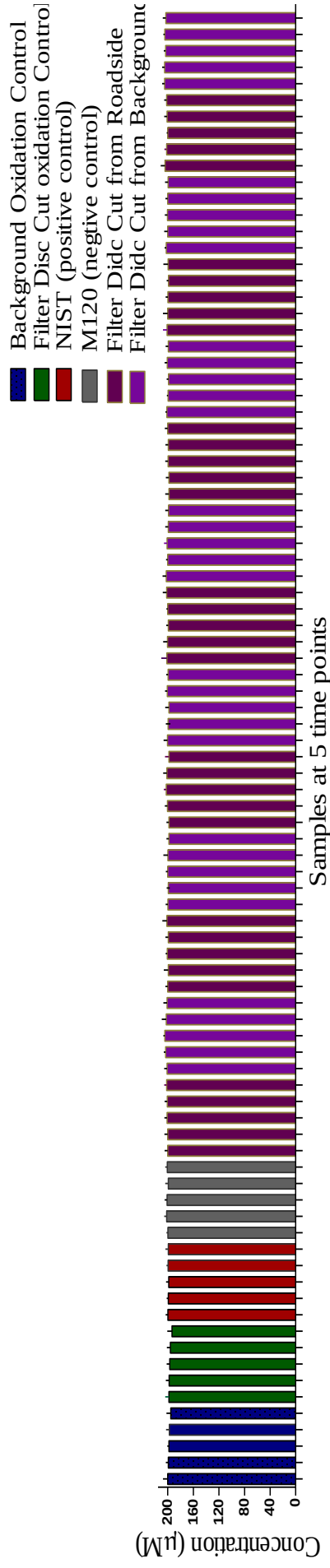


**Figure 30: Lack of Significant Urate Depletion in synRTL F Exposed to PM**

Each point represents the average concentration  $\pm$  SD ( $n=3$  for roadside and background samples and  $n=42$  for the controls). The concentration changes of the controls and 14 out of the 88 filters are presented for the 5 successive collection time points. The results of each roadside sample are followed by its matched (i.e. collected on the same day) background filter. The results of these filters are highly representative of the remaining filters, they were chosen randomly as none of the remaining filters presented a significantly different result, and thus there was uniformly a lack of consumption. The reason for presenting 14 out of the 88 filters is because illustrating clearly 460 data points on the same graph was not possible. In contrast to both the ascorbate and reduced glutathione data, no losses were observed for urate in the composite antioxidant solution exposed to PM filter disc cuts or particulate controls (NIST and M120). Similarly, no losses were seen in the particle free control. In addition UA appeared stable in the particle free controls over the duration of the incubation period (a marginal average loss of 3.5% after 2h).

All filters presented linear antioxidant consumption over time irrespective of PM mass on the filter disc cuts. **Figure 29** presents the antioxidant depletion ( $\text{mol l}^{-1}$  per  $\mu\text{g}$  of PM over time) of typical randomly selected samples derived from low, medium and high filter disc cut weight bands. The graph illustrates that the same linear pattern was observed for PM samples originating from 3, 11 and 22  $\mu\text{g}$  disc cuts. Two further points can be observed, a lack of association between PM mass and antioxidant depletion, and also ascorbate exhibiting higher consumption than reduced glutathione; these last two findings are elucidated in more detail further on. The averaged points in **Figure 29** denote greater standard deviations compared to the gaseous experiments, potentially due to the increased complexity of the experimental procedure, though the data quality remained high.

A further point that is elucidated on this figure is that there was variability in antioxidant consumption effects, unlike the gaseous exposures due to PM's compositional heterogeneity. This finding led to the hypothesis that it would be more appropriate to report separately the oxidative effects of the two locations, as the latter influences PM's composition. The hypothesis was that roadside samples would result in higher antioxidant depletion than their matched background filters due to the chemical composition of the former being characterized by a higher oxidative potential.

The resulting depletion rate for each concentration could be denoted as  $\text{mol l}^{-1}\text{s}^{-1}$  and was then divided by each of the respective 5mm disc cut's weight ( $\mu\text{g}$ ) in order to present the final expression:  $\text{mol l}^{-1}\text{s}^{-1}$  per  $\mu\text{g}$  of PM. This calculation allowed direct comparisons between samples of different filter weight (**Figure 31**) and thus a quantitative evaluation of the overall oxidative activity at five different exposure time points was achieved.

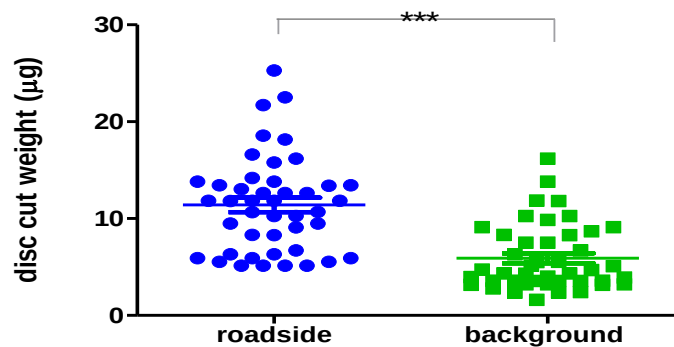


**Figure 31: Filter disc cut weight of simultaneously collected roadside (n=44) and background (n=44) samples.**

The weight of each 5mm disc cut was relative to the weight of the 39mm filter, pilot work established the uniformity of the sample deposition (**Chapter 2, section 2.3.3.4**). There was a larger mass on the roadside collected filters, the rate calculation was expressed per µg of PM so that it equalized differences in disc cut weight, \*\*\* indicates  $p < 0.001$ .

An analysis of the distribution of the disc cut weights from the roadside and background locations revealed a wider distribution of high weight bands at the roadside location. The frequency distributions were not normal (parametric) as the value density was not higher in the middle point of each distribution (**Figure 32**). However, the background derived distribution was noticeably skewed towards the left side of the graph, as 28 out of the 44 PM filters had disc cuts that weighed up to a maximum of 5µg. In contrast, only 9 roadside filter disc cuts weighed 5µg and this was the minimum value for this group.

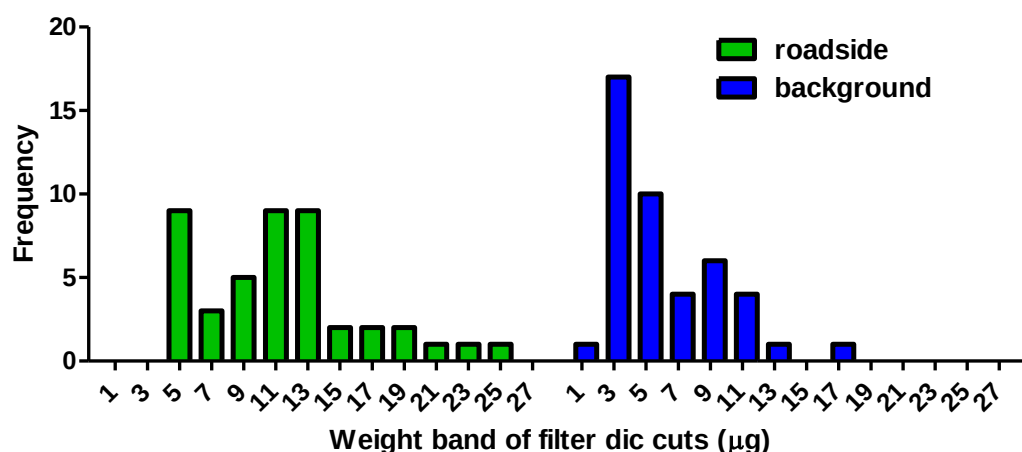


**Figure 32: Frequency Distribution of PM Disc Cut Weight Bands**

The frequency distribution of different filter weight bands is presented for the total 88 filters, this analysis is subcategorized into roadside and background filters. Roadside filters presented overall a greater PM mass dispersion and higher values, while background derived PM mass distribution was skewed towards lower values.

All background and roadside samples resulted in significantly larger antioxidant consumption and glutathione disulphide formation rates than the blank control disc cuts, therefore the hypothesis was supported by the empirical findings. Antioxidant consumption presented a maximum 60% depletion at the two hour time point, while there were no instances of complete antioxidant depletion due to prior pilot work which aimed at determining an appropriate disc cut diameter. A non parametric one-way ANOVA (Kruskal-Wallis with Dunn's post hoc) was employed in order to compare substrate reactivity at the contrasting locations.

The results revealed that within each location ascorbate was the most reactive substrate. In addition, both antioxidants exhibited significantly higher antioxidant consumption rates at roadside compared to background (**Figure 33**).

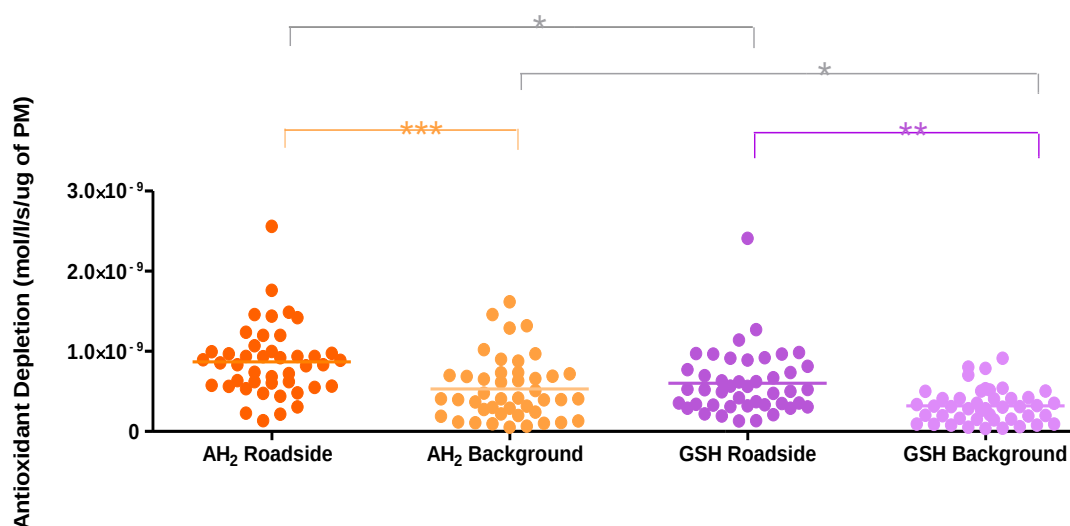


**Figure 33: Oxidative consumption of ascorbate and reduced glutathione by roadside or background collected PM<sub>10</sub> in an RTLF model.**

Each point represents the mean  $\pm$ SD) rate of depletion of a daily PM<sub>10</sub> filter (n=3). Samples were collected simultaneously on both locations and results of the comparison between different groups is denotes as; \*  $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

Specifically, ascorbate exposed to roadside PM was oxidised on average  $2.6 \pm 2.4$  (median: 1.4 range: 9.4) times more than the simultaneously collected background PM, similarly reduced glutathione was depleted  $2.6 \pm 2.5$  (median: 1.7 range: 12.3) times more when it derived from Marylebone road than when it originated from North Kensington filters. Moreover, ascorbate presented oxidation rates that were on average 1.6 times higher than the equivalent reduced glutathione values (at roadside: AA>GSH;

average 1.6, median 1.5, range 1.3, at background: AA>GSH; average 1.6, median 1.7, range 1.4)

The calculated antioxidant depletion rates following exposure of synRTLF to PM from roadside and background locations were also summarised in a frequency distribution histogram. The results did not present a normal distribution (**Figure 34**).

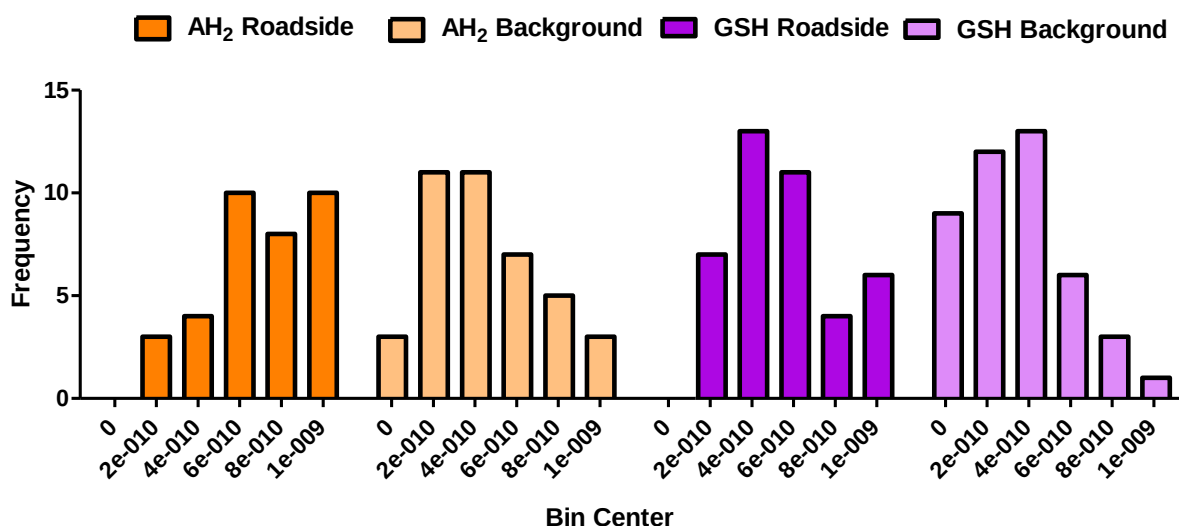


**Figure 34: Frequency distribution of PM antioxidant depletion rates**

The calculated antioxidant depletion rates are based on the incubation of 44 roadside and 44 matched background filters in synRTLF. The frequency distribution was not normal for any of the graphed groups. Ascorbate from roadside PM was the only group to have a frequency distribution that was skewed towards the right side, that is, the higher values. This observation is in agreement with the significantly higher consumption of ascorbate by roadside PM (**Figure 33**).

The reduced glutathione rates of the background filters were skewed towards lower reactivities, a similar but less prominent pattern was observed for their roadside rates and ascorbate reactivity following incubation with background PM filters. In contrast, roadside ascorbate consumption rates were skewed towards higher values in the presence of roadside PM, thus it is not surprising that a statistical comparison of the different antioxidant reactivities showed that ascorbate exposed to roadside PM presented the highest depletion (**Figure 33**).

As stated previously, the roadside derived PM<sub>10</sub> samples weighed significantly more than the background derived filters (**Figure 31**) and even though the antioxidant depletion rate ( $\text{mol l}^{-1}\text{s}^{-1}$  per  $\mu\text{g}$  of PM) expression takes into account differences in disc cut weight it was important to confirm whether the original PM<sub>10</sub> mass on the filter

skewed the results in either direction. One could argue that the presence of a larger mass could thwart the interaction of PM with the antioxidants thus resulting in a lower antioxidant depletion per  $\mu\text{g}$  of PM. Conversely, it could be argued that a larger mass could be potentially associated with a higher concentration of redox active components such as metals; hence heavier filters could result in higher depletion rates. For this reason, roadside and background rates were plotted against their respective disc cut weights in order to elucidate whether PM mass acted as a confounding/predictive factor (Figure 35).

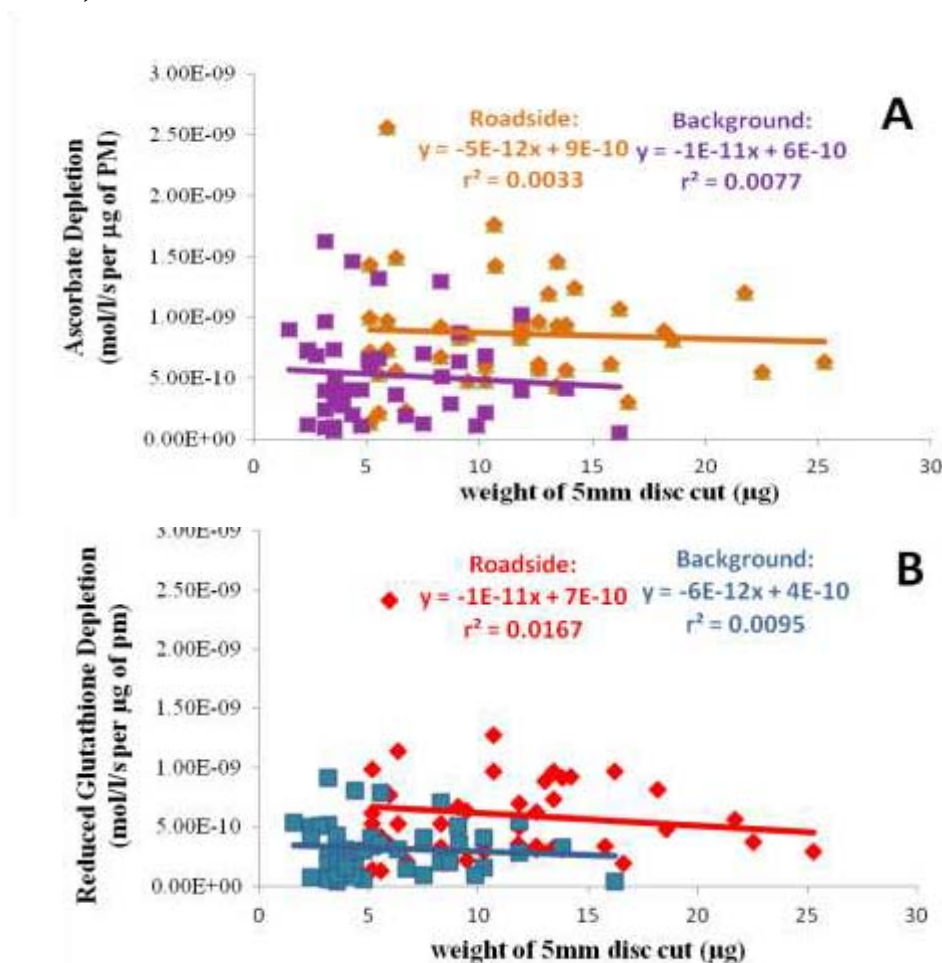


**Figure 35: Association between antioxidant depletion rates and  $\text{PM}_{10}$  mass on the 5mm disc cut**

The average ascorbate (graph A) and reduced glutathione (graph B) depletion rates (mol/l/s per  $\mu\text{g}$  of PM) at both locations were plotted against their respective disc cut weight in order to investigate whether initial weight differences resulted in confounding effects. No association was found ( $r^2 = 0$ ). Therefore, it can be concluded that differences in disc cut weight were not a confounding factor, as sample weight did not skew the results. In addition, these findings imply that weight on its own is not predictive of antioxidant depletion capacity. This can be attributed to compounds such as metals that do not have a high atomic weight but still carry a high redox potential.

The results confirmed that there was no correlation between PM<sub>10</sub> mass on the disc cuts and substrate depletion rate at either location ( $r^2 = 0$ , **Figure 35**). Therefore, it is believed that differences in redox activity resulted from the differences in PM<sub>10</sub> source and their associated compositional differences.

A grouped graph of the calculated antioxidant depletion rates following the exposure of synRTLF in 88 PM filters from roadside and background location can offer a more detailed depiction of the daily variations and the relationship between locations and antioxidants (**Figure 36**).

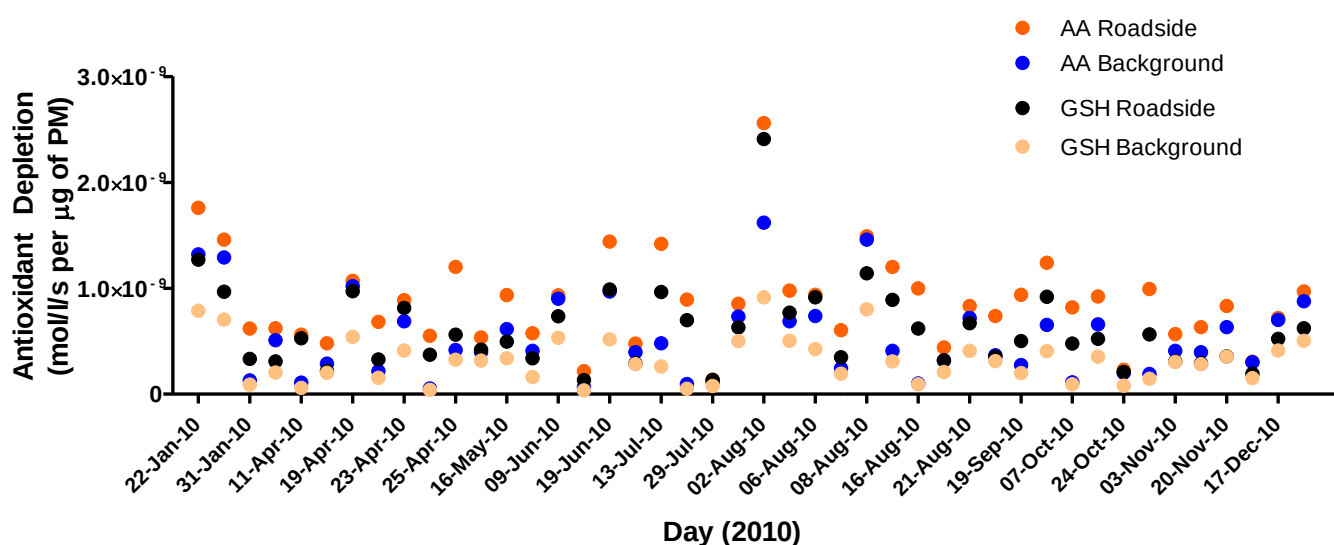


**Figure 36: Grouped graph of antioxidant depletion by PM**

The antioxidant depletion rates are depicted against the filter collection days. Overall, ascorbate and reduced glutathione appear to be varying in parallel, especially within the same location. It is interesting to note that the rates vary from day to day, however the roadside samples always presented higher antioxidant depletion values than the background location. There was no statistically significant variation. Roadside samples presented higher antioxidant depletion than their roadside pairs and ascorbate depletion was always higher than reduced glutathione, though the magnitude of this difference was day specific ( $p < 0.05$ ).

Plotting the calculated rates against each respective collection day clearly showed that ascorbate depletion rates were higher than the reduced glutathione rates for all tested days. Also, ascorbate and reduced glutathione varied in parallel across time and space.

In other words, on a day that ascorbate depletion is high, reduced glutathione depletion is also high and both ascorbate and reduced glutathione depletion are higher at the roadside than the background location. These observations along with the glutathione disulphide values fitting the classical 2:1 stoichiometry suggested a close correlation between the different antioxidant moieties and oxidative degradation products. A Spearman rank-order correlation was employed in order to quantitatively report the statistical dependence between the full data set of the empirically calculated substrate reactivities and locations. The specific test was chosen due to the fact that the data were non parametric (the results of the normality test are presented in **Appendix B**). The association analysis presented very high antioxidant and glutathione disulphide correlations (**Table 10**) within each location (rho= 0.94-0.99) and also high associations between locations (rho= 0.77-0.79). Therefore, these findings suggest that quantification of just one antioxidant depletion rate can have a high predictive value of the other antioxidant reactivities at either site.

|                              | GSH road.   | GSSG road.  | AH <sub>2</sub> backg. | GSH backg.  | GSSG backg. |
|------------------------------|-------------|-------------|------------------------|-------------|-------------|
| <b>AH<sub>2</sub> road.</b>  | <b>0.94</b> | <b>0.91</b> | <b>0.77</b>            | <b>0.78</b> | <b>0.77</b> |
|                              | 2.622E-21   | 2.006E-17   | 9.92E-10               | 5.63E-10    | 7.94E-10    |
|                              |             |             |                        |             |             |
| <b>GSH road.</b>             |             | <b>0.99</b> | <b>0.79</b>            | <b>0.78</b> | <b>0.77</b> |
|                              |             | 3.35E-10    | 2.92E-10               | 3.47E-10    | 9.81E-10    |
|                              |             |             |                        |             |             |
| <b>GSSG road.</b>            |             |             | <b>0.79</b>            | <b>0.79</b> | <b>0.77</b> |
|                              |             |             | 2.26E-10               | 2.23E-10    | 7.24E-10    |
|                              |             |             |                        |             |             |
| <b>AH<sub>2</sub> backg.</b> |             |             |                        | <b>0.98</b> | <b>0.96</b> |
|                              |             |             |                        | 5.865E-31   | 8.021E-24   |
|                              |             |             |                        |             |             |
| <b>GSH backg.</b>            |             |             |                        |             | <b>0.98</b> |
|                              |             |             |                        |             | 4.79E-30    |
|                              |             |             |                        |             |             |

**Table 10: Correlation between ascorbate, reduced glutathione and glutathione disulphide rates when exposed to PM<sub>10</sub> from roadside or background location.**

The statistical dependence of the antioxidant depletion and glutathione disulphide formation rates were assessed using a spearman correlation, the rank correlation coefficients (rho; in bold) and *p* values are provided.

The delta increment (i.e. roadside minus background) was calculated in order to further delineate the effect of date and location on substrate reactivity. The results depicted in **Figure 37** clearly show that the increment was always positive, thus demonstrating that the North Kensington samples did not surpass the antioxidant depletion of the Marylebone samples on any day. In addition, ascorbate presented overall higher increments compared to reduced glutathione, but the difference was borderline significant ( $p=0.06$ ). Therefore, potentially ascorbate's depletion could be more sensitive to the sample origin and associated components. Also, the difference between background and roadside followed a similar pattern for ascorbate and reduced glutathione ( $\rho: 0.69$ ,  $p = 2.24\text{E-}07$ ), this correlation could reflect a similar impact of temporal collection characteristics on PM oxidative potential at both sites.

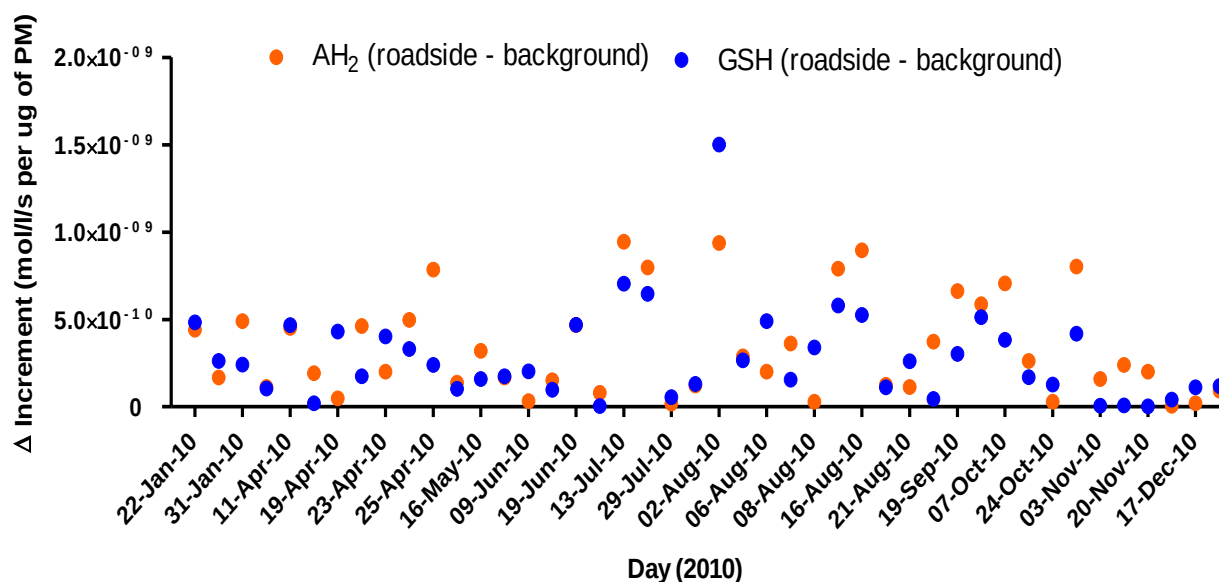


**Figure 37:  $\Delta$  Increment of ascorbate and reduced glutathione throughout the collection period.**

The difference between roadside and background is depicted for ascorbate and reduced glutathione. Positive values denote that roadside derived filters resulted in higher depletion rates than the background filter of the pair.

#### 4.3.1 An Indirect Analysis of the PM Filter Metal Content

The variability of antioxidant depletion rates was reduced once the samples considered separately based on the location of their collection location, though there was still a large range of calculated values within each group. For this reason a statistical model was employed in order to investigate potential associations between antioxidant

depletion and the metal content of PM, the latter was estimated based on the predominant wind direction on different days. The analyses used were derived from a theoretical model developed by Dr Barratt from the environmental research group, King's College London. Dr Barratt found significant associations between metal content (PM<sub>10</sub>, 2008, ICP-MS) and wind direction (personal communication). In the current study it was hypothesised that wind directions indicating a PM source rich in highly redox active metals would be associated positively with higher antioxidant consumption.

In order to test this hypothesis the wind directions present on each PM<sub>10</sub> collection day were converted to a percentage term based on hours of the day from a defined wind direction related to a source/source mixture so that all wind directions added up to 100% (**Appendix C**). Therefore, it was aimed to quantify the antioxidant reactivity of samples on days that a particular wind direction dominated (~80- 100%), as this approach minimised the noise in the data. An ordinary least squares regression analysis was utilised in order to investigate whether the ascorbate and reduced glutathione depletion rates were associated with the wind directions. Thus the model was written as  $y = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3$ , where  $y$  was the antioxidant depletion rate and  $x$  were the different wind directions, and  $\beta$  was the regression coefficient. In addition, the least squares estimates of the coefficients were given by:  $\beta = (X'X)^{-1}X'Y$ , where  $Y$  was the dependent variable (antioxidant rate, 44x1 column vector) and  $X$  were the explanatory variables (wind direction, 44x4 matrix). Note that the number 44 signifies the total number of available filters per location. Samples collected at Marylebone road presented significant associations between ascorbate depletion and winds coming from the roadside ( $p=0.002$ ) as well as ascorbate consumption and long range winds ( $p=0.039$ ). Similarly, samples collected from North Kensington exhibited a significant association between ascorbate reactivity and winds coming from the roadside ( $p=0.007$ ). Indeed, Ba, Cu, and Fe (associated with the roadside cluster) presented the strongest associations with antioxidant depletion at both sites, but it is interesting to note that NO<sub>x</sub> levels were highly correlated with the local (roadside) PM<sub>10</sub> levels (**Appendix C**). The difficulty in confidently distinguishing between the independent effects of NO<sub>x</sub> and PM<sub>10</sub> was discussed in the introductory chapter and certainly is reflected in this quasi experimental application. Reduced glutathione presented a similar pattern, however the associations were not significant potentially because the

metals most important for GSH depletion could be less bioavailable than those important for ascorbate depletion .

#### **4.3.2 Investigating the Potential Modification of PM's Oxidative Potential Following Exposure to Ozone or Nitrogen Dioxide**

A further question that was explored was the possibility of interaction effects between gases and PM<sub>10</sub>. A potential relationship between PM's empirically calculated antioxidant depletion rates and the levels of air pollution on the days of PM collection was tested by using a Spearman rank correlation coefficient. The statistical dependence between NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> concentrations (monitoring data downloaded from LAQN website) on the days of collection and the ascorbate and reduced glutathione data set were investigated (all correlations can be found in **Appendix D**). The results of this analysis revealed a very weak but positive trend between ascorbate consumption by roadside PM filters and NO<sub>2</sub> (rho: 0.28,  $p=0.062$ ) and PM<sub>10</sub> (rho: 0.29,  $p=0.056$ ) levels at the same location. A similar pattern was observed in the case of reduced glutathione at roadside location, while there was not an obvious trend for either pollutant at background location. In contrast, the association between O<sub>3</sub> concentration and ascorbate consumption exhibited a negative trend at roadside (rho: -0.29,  $p=0.058$ ) and no trend was observed for reduced glutathione. While background O<sub>3</sub> levels were negatively associated with depletion by PM of ascorbate (rho: -0.33,  $p=0.029$ ) and reduced glutathione (rho: -0.28,  $p=0.067$ ) rates derived from the same location. These results faintly support that NO<sub>2</sub> could possibly enhance the PM OP potentially by nitration reactions and/or by enhancing the bioavailability of oxidizing species. The independent effects of each air pollutant are impossible to disentangle since NO<sub>2</sub> and PM<sub>10</sub> presented a highly significant positive correlation at both sites, while O<sub>3</sub> levels were negatively correlated with these pollutants at either location O<sub>3</sub> (more details about the levels of these air pollutants at the two sites and their associations are provided in the following chapter.)

Therefore, the possibility of effect modification was investigated under controlled experimental conditions. For this purpose disc cuts were performed on 40 PM<sub>10</sub> filters and each filter was exposed to the following conditions prior to incubation in synRTLF; a) standard exposure protocol b) air c) 1000ppb O<sub>3</sub> d) 1000ppb NO<sub>2</sub>. The conditions employed for the gaseous exposures were identical to the ones used for the gaseous

studies except that there were PM filter disc cuts rather than synRTLTF in the wells. Following these gaseous exposure, the antioxidant depletion capacity of the PM filter disc cuts was tested by utilising the same protocol that was followed for the standard PM exposures. However, the results revealed no significant differences in the ascorbate ( $p=0.997$ ) and reduced glutathione depletion rates ( $p= 0.858$ ) of the three groups. The exact results of the statistical comparisons are presented in **Table 11**. Similarly to the non exposed filter disc cuts, ascorbate was more reactive than reduced glutathione and urate did not present any measurable oxidation.

| Group                                                                                                                                         | N  | Missing | Median   | 25 <sup>th</sup><br>percentile | 75 <sup>th</sup><br>percentile |
|-----------------------------------------------------------------------------------------------------------------------------------------------|----|---------|----------|--------------------------------|--------------------------------|
| (mol l <sup>-1</sup> s <sup>-1</sup> per µg of PM)                                                                                            |    |         |          |                                |                                |
| Ascorbate - Air                                                                                                                               | 40 | 0       | 6.08E-10 | 4.09E-10                       | 8.79E-10                       |
| Ascorbate - O <sub>3</sub>                                                                                                                    | 40 | 0       | 6.14E-10 | 4.02E-10                       | 8.62E-10                       |
| Ascorbate - NO <sub>2</sub>                                                                                                                   | 40 | 0       | 6.13E-10 | 4.21E-10                       | 8.55E-10                       |
| Reduced glutathione – Air                                                                                                                     | 40 | 0       | 3.47E-10 | 2.41E-10                       | 6.26E-10                       |
| Reduced glutathione – O <sub>3</sub>                                                                                                          | 40 | 0       | 3.75E-10 | 2.42E-10                       | 6.54E-10                       |
| Reduced glutathione – NO <sub>2</sub>                                                                                                         | 40 | 0       | 3.92E-10 | 2.55E-10                       | 6.2E-10                        |
| Ascorbate; H = 0.00609 with 2 degrees of freedom ( $p = 0.997$ ),<br>Reduced Glutathione; H = 0.306 with 2 degrees of freedom ( $p = 0.858$ ) |    |         |          |                                |                                |

**Table 11: Antioxidant Depletion Capacity of PM Disc Cuts Exposed to Ozone or Nitrogen Dioxide or Air prior to SynRTLTF Incubation**

PM disc cuts derived from 20 roadside and 20 background filters were exposed to air or O<sub>3</sub> or NO<sub>2</sub>. Each filter was exposed to all three conditions in order to limit variation. The exposed disc cuts were then incubated in synRTLTF and their antioxidant depletion capacity was quantified. However, the antioxidant depletion capacity of the three groups did not differ. The descriptive and inferential statistics are provided in the table (non parametric one way ANOVA-Kruskal Wallis, ascorbate  $p=0.997$ , reduced glutathione  $p= 0.858$ )

The possibility of interaction effects was further tested by exposing 12 PM filters to O<sub>3</sub> and NO<sub>2</sub> concomitantly (1000ppb) or air. In addition, part of the same filters was not exposed to any gas in order to investigate whether gas flow was confounding the results by blowing away particles. The results from the three groups did not differ significantly (**Figure 38**).

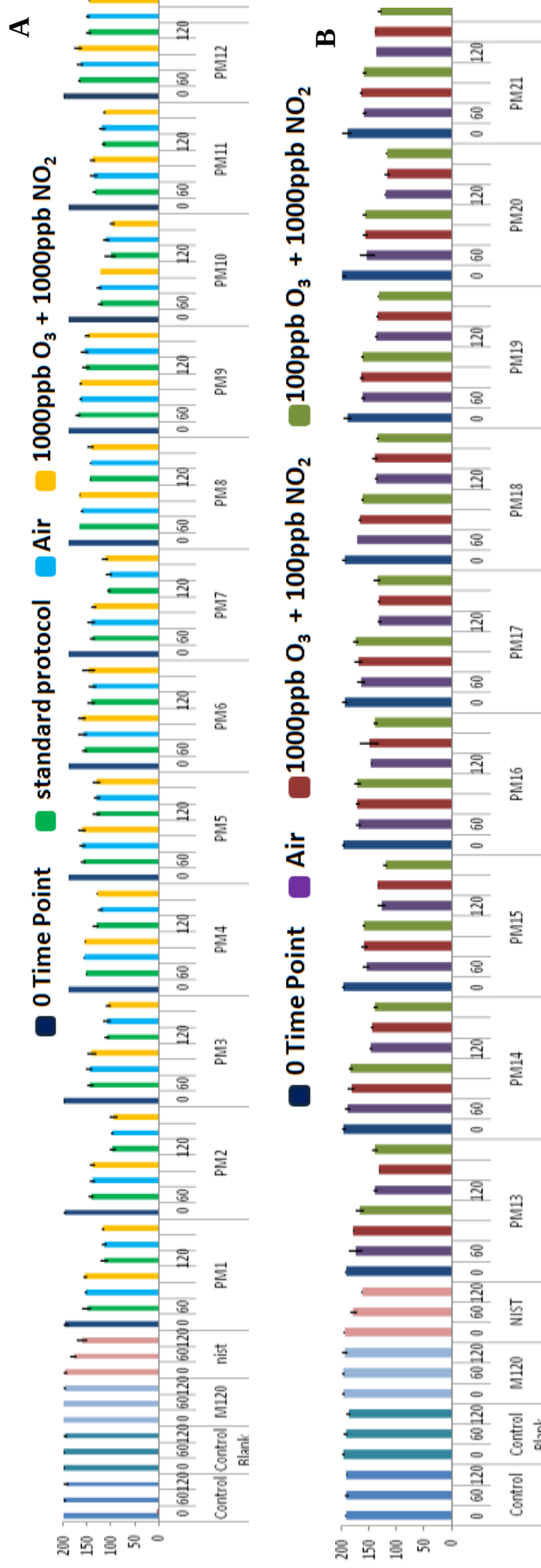


**Figure 38: Investigating potential modification of PM antioxidant depletion capacity in synRTLF following separate or concomitant exposure to ozone and nitrogen dioxide**

**A:** The hypothesis that concurrent O<sub>3</sub> and NO<sub>2</sub> exposures could alter PM antioxidant depletion capacity was tested by exposing PM disc cuts to a) standard exposure (i.e. no air stream) b) air c) 1000ppb O<sub>3</sub> and 1000ppb NO<sub>2</sub>. PM disc cuts exposed concurrently to the highest O<sub>3</sub> and NO<sub>2</sub> concentrations did not differ significantly from the control exposures in their synRTLF ascorbate depletion capacity, the same observations hold true for reduced glutathione. In addition, the standard protocol and air exposure (same flow as NO<sub>2</sub> and O<sub>3</sub>) did not differ, thus the experimental system was not confounding the disc cut oxidative potential. Please note that fewer time points were tested (60 and 120 minutes) in these experimental series as there was not enough material, for the same reason there was one 0 time point used for each filter.

**B:** PM disc cuts were exposed to O<sub>3</sub> and NO<sub>2</sub> concomitantly using unequal concentrations (1in10 dilution) in order to limit potential antagonistic effects. 9 filters were selected randomly for this experiment and each filter was exposed to all experimental conditions. As the figure illustrates here were no differences in ascorbate depletion of different treatment groups, again the same findings hold true for reduced glutathione, and urate levels remained unchanged. The results of both experiments support the contention that there were no synergistic or interaction effects in this experimental set up, however, this is an *in vitro* study and thus real life interactions cannot be excluded.

A further 9 PM filters from roadside and background location were exposed to O<sub>3</sub> and NO<sub>2</sub> concomitantly but in this set of experiments unequal concentrations were used. Specifically a 1 in 10 dilution was implemented (100ppb O<sub>3</sub> and 1000ppb NO<sub>2</sub>, or 1000ppb O<sub>3</sub> and 100ppb NO<sub>2</sub>) in order to investigate potential antagonistic effects. However, the simultaneous exposure of PM to the gaseous air pollutants did not differ from the control exposures (**Figure 38**). Therefore, it was concluded that in the current *in vitro* model, PM<sub>10</sub> redox activity was not affected by exposure to O<sub>3</sub> or NO<sub>2</sub>.

#### 4.4 Discussion

Particles can exert their adverse effects only once they have been inhaled, and the first physiochemical barrier that they encounter is the RTLF [79].

The present study investigated differences in the antioxidant depletion capacity of daily PM<sub>10</sub> filters collected concurrently from a roadside and a background location, utilising a human RTLF model [269]. The results showed that roadside samples had a significantly higher antioxidant depletion activity than the background site (**Figure 33**). These results are in agreement with previous studies comparing roadside and background locations (**Table 12**) demonstrating that from the outdoor sites investigated PM filters from roadsides had the highest oxidative capacity [113, 114, 449-452]. This conformity exists even though each study has utilised different particle collection methods and different oxidative potential (OP) assays which in turn are thought to have different sensitivities [281]. A comparison of the roadside to background ratios for oxidative potential reveals fairly similar results. Specifically, in this project antioxidant depletion rates within an RTLF model were 2.6 times higher at roadside than at background. Similarly, other Dutch studies showed that PM OP at roadside was about 2 times higher than the background samples, using the DTT assay [113] and OP AA [114]. Also, Boogaard and colleagues (2012) found that roadside OP PM<sub>10</sub> in the Netherlands was on average 3.6 times higher than at background by using the ESR assay, this factor may be slightly higher however, it is within an overlapping range [450]. The only study not to have found a significant OP difference between the aforementioned sites is that of Godri *et al.*, 2011, though in this study roadside and background samples were not collected concurrently, while the high temporal variability, and limited number of samples (one week campaign/site) further impeded the comparison of samples collected on different occasions [112].

**Table 12: Summary of Studies that have Conducted Oxidative Potential Analyses on PM Collected from Outdoor Locations, including Roadside and Background Sites**

| References                                                               | PM Collection                                                                                                                                                                                                                                                                                                                               | Sites                                                                                                                                                                                                                          | OP Assays                                                                                                                                                                      | Main results                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
|--------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Current</b>                                                           | <ul style="list-style-type: none"> <li>PM<sub>10</sub> collected on daily Emfab filters (Partisol 2025)</li> <li>No extraction, direct exposure of disc cuts</li> </ul>                                                                                                                                                                     | <ul style="list-style-type: none"> <li>London, two sites: roadside and background</li> <li>PM collected simultaneously</li> </ul>                                                                                              | <ul style="list-style-type: none"> <li>AA, GSH, (and UA) depletion rates in the RTLF model</li> </ul>                                                                          | <ul style="list-style-type: none"> <li>AA road:&gt;GSH backg.&gt;AA backg. &gt;GSH backg.&gt;&gt;UA</li> <li>Roadside samples had on average a 2.6 times higher antioxidant depletion capacity than background.</li> <li>AA was on average 1.6 times more reactive than GSH.</li> </ul>                                                                                                                                                                                                                                                            |
| <b>Janssen et al., 2014 (RAPTES project-intercomparison study) [450]</b> | <ul style="list-style-type: none"> <li>PM<sub>2.5</sub> and PM<sub>10</sub> mass - Harvard Impactors (HI).</li> <li>PM<sub>2.5</sub> and PM<sub>10</sub> mass - Harvard Impactors (HI).</li> <li>PM collection for OP analyses- Micro-Orifice Impactor (MOI) -6h/ day sampling</li> <li>Evaporation, extraction and resuspension</li> </ul> | <ul style="list-style-type: none"> <li>Different cities in Netherlands, 5 sites: underground platform, 2 traffic sites, urban background</li> <li>PM was not collected simultaneously</li> </ul>                               | <ul style="list-style-type: none"> <li>Dithiothreitol (OP<sup>DTT</sup>)</li> <li>Electron spin resonance (OP<sup>ESR</sup>)</li> <li>Ascorbate - (OP<sup>AA</sup>)</li> </ul> | <ul style="list-style-type: none"> <li><b>For outdoor sites: PM from continuous traffic site had the highest OP for all methods</b></li> <li>PM<sub>2.5</sub>- OP<sup>DTT</sup>/μg&gt; PM<sub>10</sub> OP<sup>DTT</sup>/μg</li> <li>PM<sub>2.5</sub>- OP<sup>ESR</sup> &lt; PM<sub>10</sub> OP<sup>ESR</sup></li> <li><b>OP metrics highly correlated (r<sup>2</sup> 0.79-0.97)</b></li> <li><b>OP<sup>AA</sup> and OP<sup>ESR</sup> – high correlation with traffic components, while OP<sup>DTT</sup> - high correlation for mass</b></li> </ul> |
| <b>Boogaard et al., 2012 [448]</b>                                       | <ul style="list-style-type: none"> <li>PM<sub>10</sub> and PM<sub>2.5</sub> -gravimetrically on Teflon filters</li> <li>7 day sampling period - collection lasted 5min every 20 min</li> <li>Evaporation, extraction and resuspension</li> </ul>                                                                                            | <ul style="list-style-type: none"> <li>Different cities in Netherlands, 18 sites: 10 traffic sites, five urban streets and five suburban background sites.</li> <li>PM collection were not conducted simultaneously</li> </ul> | <ul style="list-style-type: none"> <li>ESR assay</li> </ul>                                                                                                                    | <ul style="list-style-type: none"> <li><b>OP<sup>ESR</sup> of PM<sub>10</sub> on average 3.6 times higher at traffic than at urban background</b> and 6.5 times higher than suburban,</li> <li><b>OP of PM<sub>10</sub> was 4.6 higher than OP of PM<sub>2.5</sub></b> (per volume unit)</li> <li>OP of PM<sub>10</sub> was 3.1 higher than OP of PM<sub>2.5</sub> (per mass)</li> </ul>                                                                                                                                                           |
| <b>Strak et al., 2012 (RAPTES project) [113]</b>                         | <ul style="list-style-type: none"> <li>PM<sub>2.5</sub> and PM<sub>10</sub> mass - Harvard Impactors (HI).</li> <li>PM for OP - Micro-Orifice Impactor -5-6h per day</li> <li>Extraction, evaporation resuspension</li> </ul>                                                                                                               | <ul style="list-style-type: none"> <li>Different cities in Netherlands, 5 sites: underground platform, 2 traffic sites, urban background, farm,</li> <li>Measurements were not conducted simultaneously</li> </ul>             | <ul style="list-style-type: none"> <li>OP<sup>AA</sup> –RTLF model</li> <li>OP<sup>GSH</sup> – RTLF model</li> <li>OP<sup>TOTAL</sup></li> </ul>                               | <ul style="list-style-type: none"> <li><b>OP<sup>AA</sup> and OP<sup>GSH</sup> were 1.5-2 times higher at roadside than at background</b></li> <li>Overall, <b>OP<sup>AA</sup> was 1.5 times higher than OP<sup>GSH</sup></b></li> </ul>                                                                                                                                                                                                                                                                                                           |

A brief summary of the PM collection sites, methods and main findings is provided for each study.

**Table 12: Summary of Studies that have Conducted Oxidative Potential Analyses on PM Collected from Outdoor Locations, including Roadside and Background Sites (*contd.*)**

| References                                   | PM Collection                                                                                                                                                                                                                                                | Sites                                                                                                                                                                                                                                      | OP Assays                                                                                             | Main results                                                                                                                                                                                                                                                                                                                                                                  |
|----------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Godri et al., 2011[111]                      | <ul style="list-style-type: none"> <li>PM<sub>1,9-10,2</sub> - 7 day MOUDI filters</li> <li>Evaporation, extraction and resuspension</li> </ul>                                                                                                              | <ul style="list-style-type: none"> <li>7 London primary school sites: 4 at background and 3 at roadside</li> <li>PM collections were not conducted simultaneously</li> </ul>                                                               | OP <sup>AA</sup> , OP <sup>GSH</sup> in RTLF model                                                    | <ul style="list-style-type: none"> <li>No significant differences between OP and location</li> </ul>                                                                                                                                                                                                                                                                          |
| Steenhof et al., 2011 (RAPTES project) [112] | <ul style="list-style-type: none"> <li>PM for OP- Versatile Aerosol Concentration Enrichment System (VACES), coarse, fine and ultra fine PM, 6h per day</li> <li>Samples diluted or concentrated and resuspended</li> </ul>                                  | <ul style="list-style-type: none"> <li>Different cities in Netherlands, 8 sites: underground platform, 3 traffic sites, urban background, farm, harbour, steel mill</li> <li>Measurements were not conducted simultaneously</li> </ul>     | DTT consumption rate RAW 264.7 cell line exposed to PM fractions (MTT, TNF- $\alpha$ and IL-6 assays) | <ul style="list-style-type: none"> <li>The DTT consumption rate was overall ~2 times higher at traffic sites than at background</li> <li>From outdoor locations traffic sites had the highest OP, followed by steelworks, then background and finally farm.</li> <li>highest pro-inflammatory response by traffic and highest MTT increase by underground samples.</li> </ul> |
| Wessels et al., 2010 [447]                   | <ul style="list-style-type: none"> <li>PM samples of different fractions (3-7, 5-3, 0.95-1.5, 0.5-0.95, and &lt;0.5 <math>\mu</math>m) - high volume cascade impactor.</li> <li>Extraction, evaporation resuspension</li> </ul>                              | <ul style="list-style-type: none"> <li>4 Different sites in Birmingham and Ireland: Birmingham - urban background, roadside, and road tunnel. Ireland – remote location</li> <li>Measurements were not conducted simultaneously</li> </ul> | ESR A549 cells: LDH assay, IL-8 release, comet assay                                                  | <ul style="list-style-type: none"> <li>OP<sup>ESR</sup> road tunnel&gt;roadside&gt;background&gt;remote background. For the smallest fraction, results non significant.</li> <li>Location more important than PM size</li> </ul>                                                                                                                                              |
| Shi et al., 2006 [446]                       | <ul style="list-style-type: none"> <li>Coarse and fine PM<sub>10</sub> fractions- on weekly Teflon filters using Graseby-Anderson dichotomous low volume samplers.</li> <li>Extraction, concentration estimate via standard curve, and adjustment</li> </ul> | <ul style="list-style-type: none"> <li>4 Different cities in Germany: 1 rural and 3 urban/industrialised</li> <li>Measurements were not conducted simultaneously</li> </ul>                                                                | ESR 8-OHdG- in isolated calf thymus DNA A549 : comet assay for fine PM exposure                       | <ul style="list-style-type: none"> <li>hydroxyl radical generation: urban PM&gt;rural PM</li> <li>DNA strand breakage correlated with hydroxyl radical generating capacity, but differences between groups were not significant.</li> <li>The OP of both fractions varied with time and location</li> </ul>                                                                   |

A brief summary of the PM collection sites, methods and main findings is provided for each study.

An additional aim of this project was to compare the antioxidant depletion capacity of three major water soluble antioxidants in the RTLF; urate, ascorbate and reduced glutathione [73, 74]. Urate did not present any signs of depletion; this finding is in accordance with previously published studies exposing synRTLF to particles [234, 277, 280, 454]. It should be noted that urate is a strong scavenger of other air pollutants including NO<sub>2</sub> and O<sub>3</sub> (Chapters 3). This lack of quantifiable reactivity towards particles *in vitro* may be attributed to complex ascorbate-urate interactions in the presence of metals. It is hypothesised that urate binded to PM metals and in this process it did not get oxidised but indirectly protected ascorbate from oxidation, while urate's HPLC quantification was possible potentially due to unbinding of the metal complex. *In vitro* studies supporting this theory have shown that the rate of ascorbate oxidation by copper is significantly reduced in the presence of urate. Specifically, ascorbate transfers one electron to the cupric ion (Cu<sup>+</sup>) and this results in semidehydroascorbic acid and cuprous ion (Cu<sup>++</sup>) formation, the latter would autooxidise and form cupric ions, however urate is thought to bind to it and thus limits further oxidation of ascorbate [455]. In this process, urate itself gets oxidised in a very slow process, which becomes even less significant as the urate concentration increases [456]. Though this study utilized a urate concentration range that was below the physiological range (2.5–60µM) and a Cu concentration of 1µg which would be highly unlikely to be found in my samples given their weight (some weighed 1µg) these differences imply an even slower reaction process in my project. Other studies have demonstrated that iron mediated ascorbate depletion was inhibited in the presence of urate, and again this process does not involve urate's oxidation [457]. The urate-Fe<sup>3+</sup> complex forms in a 2:1 ratio of urate molecules to Fe<sup>3+</sup>, and this complex results in a decrease of the Fe reduction potential from -0.77 V to -0.67V [457]. In addition, urate can form ligands with further metals such as sodium, potassium, nickel, and manganese, though there is limited evidence about how these complexes could affect the reduction potential of the antioxidants [458]. Thus, it can be hypothesized that due to the presence of metals in PM, urate is indirectly acting as a free radical scavenger by acting as a metal chelator but does not show any signs of depletion in this model due to its high concentration and relatively brief time of exposure. There is some uncertainty as to whether a urate-metal complex would interfere with the quantification method employed in this project, though previous studies have found no difference in the quantification of urate exposed

to metals via HPLC or spectrophotometrically [457]. In the possibility that this antioxidant-metal interaction is present, one cannot exclude an interaction with reduced glutathione. Therefore, the role of small molecular weight antioxidants within the RTLRF is likely to be more complex than possessing radical scavenging abilities

Contrary to urate's lack of depletion, ascorbate and reduced glutathione were consumed when exposed to any of the PM samples; however the magnitude of the depletion was day and site specific. Overall ascorbate was 1.6 times more reactive than reduced glutathione which is in accordance with previously published studies [114]. Despite differences in the magnitude of the response of ascorbate and reduced glutathione, they were highly correlated within each location (**Table 10**: rho: 0.94-0.99). Thus implying that ascorbate and reduced glutathione depletion rates were driven by the same factors. These results though contrast two earlier studies that found no significant correlations between the aforementioned antioxidants [111, 112]. It should be noted however that important methodological differences exist; two seven day micro-orifice uniform deposited impactor (MOUDI) filters were used at two school sites [112] and tapered element oscillating micro-balance (TEOM) filters were repeatedly exposed close to waste transfer stations [111] therefore different PM physiochemical characteristics may have resulted in different antioxidant specific effects.

In addition, the ascorbate and reduced glutathione values were significantly correlated between the different locations, these associations were more modest than the within location comparisons (rho: 0.77-0.78). Therefore, this lowering of the association suggests that there is some spatial variation. This could be attributed to location specific sources at the roadside, such as brake and tyre wear and their associated metals [459], but also greater vegetation at the background site [460]. Taken together, the correlations overall imply that a single antioxidant depletion rate could be predictive of all the other antioxidant consumption rates.

An association between antioxidant depletion capacity and mass on each disc cut was examined, but no association was found. Therefore this result highlights the inadequacy of PM mass on its own as a predictor of oxidative burden in this study. Indeed, the mass and chemical composition of particles are not related since the amount of different components can vary, therefore, filters with the same particle mass could have very different composition and hence different toxicity. Thus, these results suggest that the ambient concentration of PM, which has been nearly universally employed in the

epidemiological literature, may not be the best measure of the biological activity of inhaled particles.

A compositional characterisation of each filter was beyond the scope of this project, however an indirect statistical model was used in order to obtain some insight into their effects. Specifically, the different wind directions at the day of PM<sub>10</sub> collection were identified and were clustered into 3-4 groups. The relationship between these wind clusters and the antioxidant depletion rates was then investigated by using a least squares regression, this analysis presented a significant association between ascorbate antioxidant depletion rates at the roadside and wind coming from the roadside and to a smaller extent the long range cluster, similarly at the background site ascorbate depletion was associated with wind coming from the roadside. Thus, the results suggested that iron, copper and barium from roadside winds were the metals most strongly associated with ascorbate depletion, this significant association between OP and the aforementioned roadside metals has also been confirmed by a recent field study [450]. This approach certainly requires validation and a larger number of samples, however it supports the feasibility of predicting PM OP and associated characteristics based on modelling approaches [459].

An additional question that was investigated was the possibility of PM<sub>10</sub> to have additive or synergistic interactions when exposed to O<sub>3</sub> and NO<sub>2</sub>, a question that can be difficult to address in field studies given the high association between different air pollutants. For this purpose PM<sub>10</sub> disc cuts were exposed to a single gas concentration (O<sub>3</sub> or NO<sub>2</sub>) and also to mixtures (O<sub>3</sub> + NO<sub>2</sub>); prior to synRTLF incubation, but there were no signs of altered antioxidant depletion compared with disc cuts from the same filter that has not been exposed to the gases. However, in the literature there is not an established perspective about the toxicity of the aforementioned copollutant model. Some positive findings have been presented in animal models, though usually DEP is used which complicates direct comparisons. It appears that rodents exposed to concomitantly to O<sub>3</sub> and PM have increased respiratory inflammation [461, 462] while equivalent *in vivo* results were reported when the ozonation of DEP preceded the exposure [463], a methodology which is equivalent to the one I used. However, there is lack of *in vitro* studies delineating the biochemical basis of such effects. In addition, epidemiological studies have presented conflicting results, the APHEA2 results suggested that PM associations with mortality were increased in the presence of high

NO<sub>2</sub> concentrations [464] however this evidence of effect modification was not replicated in later epidemiological studies [465]. An Occam's razor approach to addressing this limited evidence would imply that there was no interaction observed in the current project, because this phenomenon did not occur. However, the fact that aged PM was used in our experiments is likely to have resulted in saturation of the nitration and ozonation targets and hence this may have blocked any potential further effects. Future *in vitro* studies utilizing diverse exposure protocols and more 'fresh' PM may delineate these potentially complex interactions.

The results of this study suggest that the PM exposure protocol developed can offer robust results. In addition, the method that was employed allowed Emfab filters of different weight to be directly exposed in the synRTLTF (**Figure 31**). It was demonstrated that differences in the PM mass did not skew the final rate calculations (**Figure 35**) while blank controls were always included to establish that the filter material was not acting as a confounding factor. Therefore, this approach could eliminate the need of repeated or extended PM collection time periods in order to increase filter mass loading [111, 280]. Thus, this approach could facilitate the collection of daily samples, reduce cost, and also potentially protect the biological activity of PM [453]. In addition, a further difference of this protocol to some others that have been presented before is that a water solvent (synRTLTF) was used and no alcohol was applied at any stage of the process. This approach may have limited the metal availability, but is in line with the physiological conditions and focuses on the bioavailable metal content [466].

#### 4.5 Conclusion

The results of this study advance our understanding of the antioxidant screen within the respiratory tract and its response to inhalation of urban PM from different locations. All tested samples resulted in significant antioxidant depletion, thus highlighting the potential of these moieties to act as protective sacrificial substrates. In terms of redox differences at each location, the results suggest that roadside samples consistently exhibited higher reactivity than their background pairs. A principal aim was to characterise the reaction of PM<sub>10</sub> with ascorbate, urate and reduced glutathione within the RTLTF model. Ascorbate presented the highest relative importance and urate the lowest. The antioxidants consumption rates of ascorbate and reduced glutathione correlated highly and this response may have been enhanced by the simultaneous

sample collection. Urate was not depleted. In contrast PM mass provided a poor predictor of oxidative effects. This precludes giving one result for the antioxidant depletion rate of PM for each antioxidant but this chapter has explored ways to categorise PM to give a range of results for the antioxidant depletion rates related to different locations and sources. The exposure conditions utilised were identical to the ones used in gaseous exposures in order to facilitate direct comparisons in the following chapter.

## **5 Nitrogen Dioxide, Ozone and Particulate Matter; a Direct Comparison of their Antioxidant Depletion Capacity in a Human Respiratory Tract Lining Fluid Model**

### **5.1 Introduction**

There is no doubt that poor air quality results in ill health, indicatively it has been estimated that around 7million deaths each year, that is, 1 in 8 deaths worldwide are attributed to air pollution [467]. To put this figure into perspective, the number of deaths from indoor; 4.3mil [468] and/or outdoor air pollution; 3.7mil [469] is larger than the Malaria and HIV fatalities combined; 2.36 mil [470, 471]. Air pollution is a complex mixture of thousands of pollutants including; ozone (O<sub>3</sub>), particulate matter (PM), and nitrogen dioxide (NO<sub>2</sub>). The aforementioned pollutants are among the most commonly found ambient air pollutants in the developed world and are considered the most important following the remarkable decrease in sulphur dioxide [472]. Epidemiological and toxicological studies have identified strong associations between exposure to these pollutants and adverse health outcomes including respiratory function [473].

However, in epidemiological studies it is rather challenging, if not impossible, to confidently disentangle the independent effects of each air pollutant [474]. This is particularly true for NO<sub>2</sub> which tends to be highly correlated with primary and secondary combustion products and thus it is difficult to ascertain if it is a surrogate of traffic related pollution or a pollutant in its own right [174, 475-477]. In addition, the heterogeneity observed in the estimated health effects, and the potential occurrence of effect modification interactions between NO<sub>2</sub> and PM<sub>10</sub> [464] as well as the difficulty in complying with the legally binding outdoor NO<sub>2</sub> limits, constitute the main reasons why there is an increased interest in unravelling the independent effects of NO<sub>2</sub> [4].

Indeed, multipollutant models have provided inconsistent evidence about the independent health effects of NO<sub>2</sub>, risk estimates of the single pollutant models were largely reduced or became insignificant after adjusting for copollutants in some research papers [182, 183, 185, 351, 478-482], whereas in other studies the effects persisted or even increased in the multipollutant models [174, 337, 483-488].

This challenge in distinguishing the independent effects of each air pollutant and particularly NO<sub>2</sub> has given a renewed impetus to controlled laboratory studies. Toxicological studies comparing the potency of the major air pollutants under the same controlled conditions represent a scientific approach that could potentially assist relevant policy developments [4]. In the process of selecting an appropriate experimental model, the respiratory tract lining fluid presents an attractive option. This aqueous hydrophase is rich in antioxidant moieties and represents the ‘first line of defence’ encountered by inhaled air pollutants [80, 286, 489]. Atmospheric pollutants are thought to exert their pathophysiological actions, at least in part, through oxidation of this antioxidant defensive screen in the lung [79, 81, 490]. Therefore, quantification of the ambient pollutant’s antioxidant depletion capacity in a respiratory tract lining fluid (RTLFL) model can serve as an *in vitro* toxicological indicator. Despite the extensive literature investigating the oxidative potential of O<sub>3</sub>, NO<sub>2</sub>, and PM<sub>10</sub>, no experimental study thus far has compared the antioxidant depletion capacity of these air pollutants under the same controlled conditions.

The aim of this project was to quantitatively compare the antioxidant consumption capacity of the aforementioned pollutants in a human RTLFL model under identical experimental conditions. Differences in antioxidant depletion rates and hierarchies were investigated. The antioxidant consumption resulting from exposure of synRTLFL to the gaseous pollutants was presented and compared on the same unitary basis (mol l<sup>-1</sup> s<sup>-1</sup> ppb<sup>-1</sup>) in chapter 5, whereas this section includes PM<sub>10</sub> and the pollutant’s real life concentrations form the basis of these analyses. Given, the compositional diversity of PM<sub>10</sub> and the obvious presence of a solid state that is different from the gaseous state of O<sub>3</sub> and NO<sub>2</sub>, a major aim of this project was to develop a methodology that allowed quantitative comparisons among the three different air pollutants.

## 5.2 Experimental Design

Physiologically relevant concentrations of the three major water soluble antioxidants present in the respiratory tract lining fluid; ascorbate, urate and reduced glutathione were prepared as described in 2.1.4 and 2.1.5. This human RTLFL model was exposed to O<sub>3</sub> or NO<sub>2</sub> or PM<sub>10</sub> using the detailed conditions outlined in 2.2. Differences in the *in vivo* respiratory deposition mechanisms between the gaseous pollutants and PM<sub>10</sub> were modelled *in vitro* by utilizing a mixed interfacial exposure system for the gases, that is,

a gas stream of O<sub>3</sub> or NO<sub>2</sub> was blown inside a chamber containing synRTLF whereas PM<sub>10</sub> disc cuts were incubated in synRTLF.

Exposure of the RTLF model to the different pollutants followed the same time points. Specifically, exposures lasted 120 minutes and samples were collected at 0, 30, 60, 90 and 120 minutes, identical quantification protocols of antioxidant depletion and glutathione disulphide formation were followed. While the chemical composition of O<sub>3</sub> and NO<sub>2</sub> in controlled experiments does not vary, different concentrations can be chosen, five environmentally relevant and high but attainable concentrations (0, 50, 150, 400, 1000ppb) were tested. These concentrations were chosen on the basis of being realistic and also in order to replicate previous relevant studies [268, 269]. By contrast, PM<sub>10</sub> is characterized by compositional diversity [491], for this reason 88 daily PM<sub>10</sub> filters of known weight were tested. These were collected in London in 2010, and samples were concurrently collected from two locations; urban background (North Kensington) and roadside (Marylebone road).

As discussed in the relevant chapters an overall antioxidant depletion rate was calculated for the gases based on the antioxidant consumption rate at the different concentrations (mol l<sup>-1</sup> s<sup>-1</sup> ppb<sup>-1</sup>) and similarly a quantitative evaluation of the overall oxidative activity at the five different time points was achieved for the PM<sub>10</sub> filters (mol l<sup>-1</sup> s<sup>-1</sup> per µg of PM). A principal aim was to directly compare the antioxidant depletion capacity of the gases and PM, a straightforward comparison would ideally occur on a molar basis. This direct comparative evaluation was possible for the gaseous pollutants tested, as they are characterized by a definite chemical composition, but was not possible for PM since the latter is characterized by compositional complexity (mixture of components) and heterogeneity (composition varies spatially and temporally). Thus, comparisons were based on the pollutant's real life concentrations, this method may be more complicated but presents a more relevant and realistic approach. For this reason for the days that PM<sub>10</sub> filters had been exposed to synRTLF, the daily average concentrations of O<sub>3</sub>, NO<sub>2</sub> and PM<sub>10</sub> were downloaded from the London air quality network website (<http://www.londonair.org.uk/london/asp/datadownload.asp>, note that the gaseous pollutants were converted from µg/m<sup>3</sup> to ppb for the calculations as the latter expression was used for the laboratory experiments). The antioxidant depletion rates of each air pollutant were then multiplied by their respective daily concentration (i.e. antioxidant depletion rate \* daily concentration\* 86400 seconds). Thus, ultimately O<sub>3</sub>, NO<sub>2</sub> and PM<sub>10</sub> antioxidant depletion was

uniformly expressed as mol antioxidant depleted per litre per day and direct comparisons could be performed.

### 5.3 Results

Differences in the airshed of roadside and background locations were observed, the concentrations ( $\mu\text{g}/\text{m}^3$ ) of the pollutants investigated are presented in **Figure 39**. Given that  $\text{PM}_{10}$  composition may vary from day to day, it was decided to focus only on the 44 days where  $\text{PM}_{10}$  samples were simultaneously collected at the two London sites.

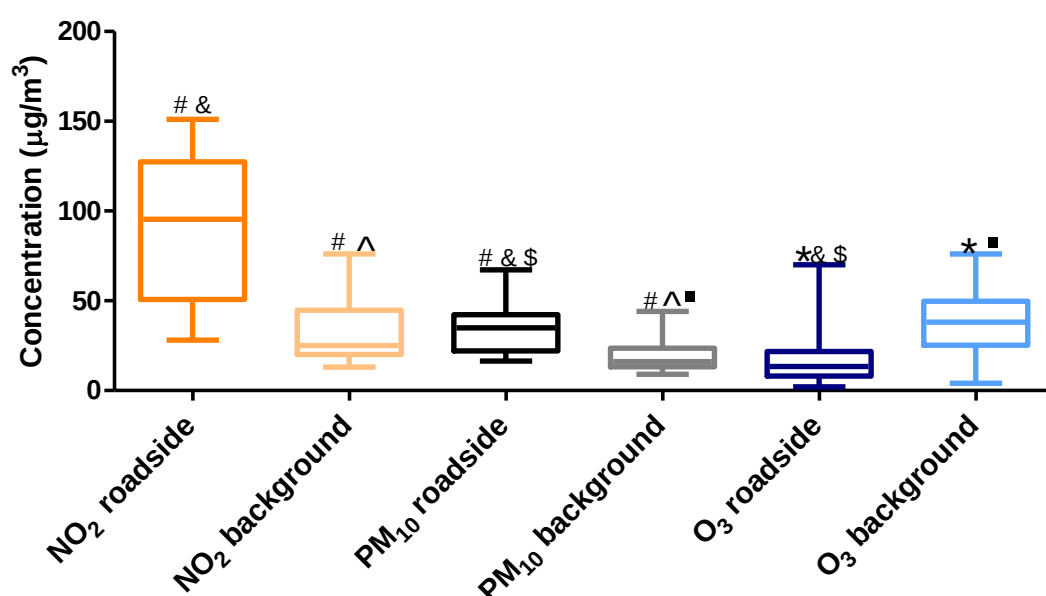


**Figure 39: Summary Air Pollutant Concentrations at Background and Roadside Locations.**

The whisker plots summarise the  $\text{NO}_2$ ,  $\text{PM}_{10}$  and  $\text{O}_3$  levels at the roadside (Marylebone road) and background (North Kensington) London locations. & depicts that  $\text{NO}_2$  roadside levels were significantly higher than the  $\text{PM}_{10}$  and  $\text{O}_3$  levels at the same location. Also, \$ indicates that  $\text{PM}_{10}$  levels were significantly higher than the  $\text{O}_3$  levels at Marylebone road. # shows that roadside concentrations were significantly higher than the background pair. \* symbolises that  $\text{O}_3$  background concentration was significantly higher than at roadside location ^ indicates that  $\text{NO}_2$  was significantly higher than  $\text{PM}_{10}$  at the North Kensington site. Also, ■ indicates that  $\text{O}_3$  background levels were higher than those of  $\text{PM}_{10}$ .

For the days and air pollutants considered,  $\text{NO}_2$  at roadside exhibited the highest airshed contribution and was significantly higher than the  $\text{PM}_{10}$  and  $\text{O}_3$  levels at Marylebone

road ( $p < 0.001$ ). In addition, roadside  $\text{NO}_2$  and  $\text{PM}_{10}$  presented significantly higher concentration than their background pair. In contrast,  $\text{O}_3$  exhibited higher concentrations at background than at roadside. Thus,  $\text{O}_3$  had a higher median concentration than  $\text{NO}_2$  at background (North Kensington) while  $\text{PM}_{10}$  had the lowest representation in the background airshed site ( $p < 0.01$ ). It should be noted, that the gases and  $\text{PM}_{10}$  are *de facto* measured using different equipment, chemiluminescence was used for the gases and the TEOM VCM method for  $\text{PM}_{10}$  [492]. The VCM correction takes measurements of volatile PM from different instruments and adds them back onto the TEOM measurements to provide a mass that is approximately equal to the mass deposited on Partisol filters, this correction method is applied on all LAQN PM values as volatile PM is present on all ambient collected samples and thus is thought to be the best available approach for Emfab filters (personal communication with Dr David Green). Though all measurements are rigorously calibrated against standards and all equipment undergoes regular quality assurance and control to ensure stability and value equivalence.

|                           | $\text{O}_3$<br>road. | $\text{PM}_{10}$<br>road. | $\text{NO}_2$<br>back. | $\text{O}_3$<br>back. | $\text{PM}_{10}$<br>back. |
|---------------------------|-----------------------|---------------------------|------------------------|-----------------------|---------------------------|
| $\text{NO}_2$<br>road.    | -0.55                 | 0.93                      | 0.45                   | -0.29                 | 0.40                      |
|                           | 1.42E-04              | 2.00E-07                  | 2.17E-03               | 5.40E-02              | 8.02E-03                  |
| $\text{O}_3$<br>road.     |                       | -0.52                     | -0.37                  | 0.68                  | -0.13                     |
|                           |                       | 3.59E-04                  | 1.27E-02               | 2.00E-07              | 4.06E-01                  |
| $\text{PM}_{10}$<br>road. |                       |                           | 0.44                   | -0.22                 | 0.52                      |
|                           |                       |                           | 2.82E-03               | 1.52E-01              | 3.75E-04                  |
| $\text{NO}_2$<br>back.    |                       |                           |                        | -0.76                 | 0.74                      |
|                           |                       |                           |                        | 2.00E-07              | 2.00E-07                  |
| $\text{O}_3$<br>back.     |                       |                           |                        |                       | -0.34                     |
|                           |                       |                           |                        |                       | 2.64E-02                  |

**Table 13: Correlation between  $\text{NO}_2$ ,  $\text{PM}_{10}$  and  $\text{O}_3$  Levels at Background and Roadside Locations.**

The statistical relationship between selected air pollutants at two London locations were assessed using a spearman correlation, the coefficient of determination and  $p$  value is provided.

Potential air pollutant correlations within and between locations were explored by employing a Spearman correlation (**Table 13**). The highest positive correlations were found between NO<sub>2</sub> and PM<sub>10</sub> at roadside (rho= 0.93) and background (rho=0.74). O<sub>3</sub> exhibited significant negative correlations with NO<sub>2</sub> and PM<sub>10</sub> at roadside (rho = -0.55 and -0.52 respectively) and background (rho = -0.76 for NO<sub>2</sub> and -0.34 for PM<sub>10</sub>). In addition, each air pollutant's levels were correlated between sites (e.g. NO<sub>2</sub>'s concentration at Marylebone Road was predictive of the NO<sub>2</sub>'s concentration at North Kensington and *vice versa*).

The relative rates of consumption of each of the antioxidant moieties by NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> were presented in Chapters; 3 and 4 respectively. Each of the experimental series was carefully designed in order to allow ultimately direct comparisons. For the gaseous exposures the slope of the linear regression analysis of the antioxidant depletion rate vs. the gaseous concentration (expressed as mol l<sup>-1</sup> s<sup>-1</sup> ppb<sup>-1</sup>) was calculated. Similarly, for PM<sub>10</sub> a linear regression analysis of the antioxidant concentration over time followed by a disc cut weight division resulted in a final expression; mol l<sup>-1</sup>s<sup>-1</sup> per µg of PM. Therefore, the gaseous and PM<sub>10</sub> rates can be compared using the same units (mol antioxidant depleted l<sup>-1</sup>) by multiplying these rates by the pollutant's concentration (expressed per µg/m<sup>3</sup>) and time over a day (\*86400s). This application was only carried out for days that PM<sub>10</sub> filters had been analysed and daily average concentrations were chosen because 24h PM<sub>10</sub> filters had been analysed *in vitro*. This direct comparison method was developed in order to quantitatively compare the ascorbate depletion capacity of the three air pollutants.

The daily ascorbate depletion of PM<sub>10</sub> and the gaseous pollutants is presented in **Figure 40**. Given that PM concentration can vary temporally and spatially, all presented PM values are based on experimentally calculated filters, while the gaseous results in this chapter have been extrapolated from laboratory studies (chapter 3) of set gaseous concentrations. The ascorbate results denoted that PM caused the greatest antioxidant consumption followed by NO<sub>2</sub> at roadside and O<sub>3</sub> at background site. Though, the greater ability of PM to deplete ascorbate may be partly attributed to methodological differences between the PM and gaseous exposure protocols (large PM mass incubated in synRTLF). The results of this grouped graph also demonstrate that NO<sub>2</sub> and PM at both sites presented strong positive correlations and both were negatively associated with O<sub>3</sub>, thus these results exemplify the difficulty in disentangling their effects.

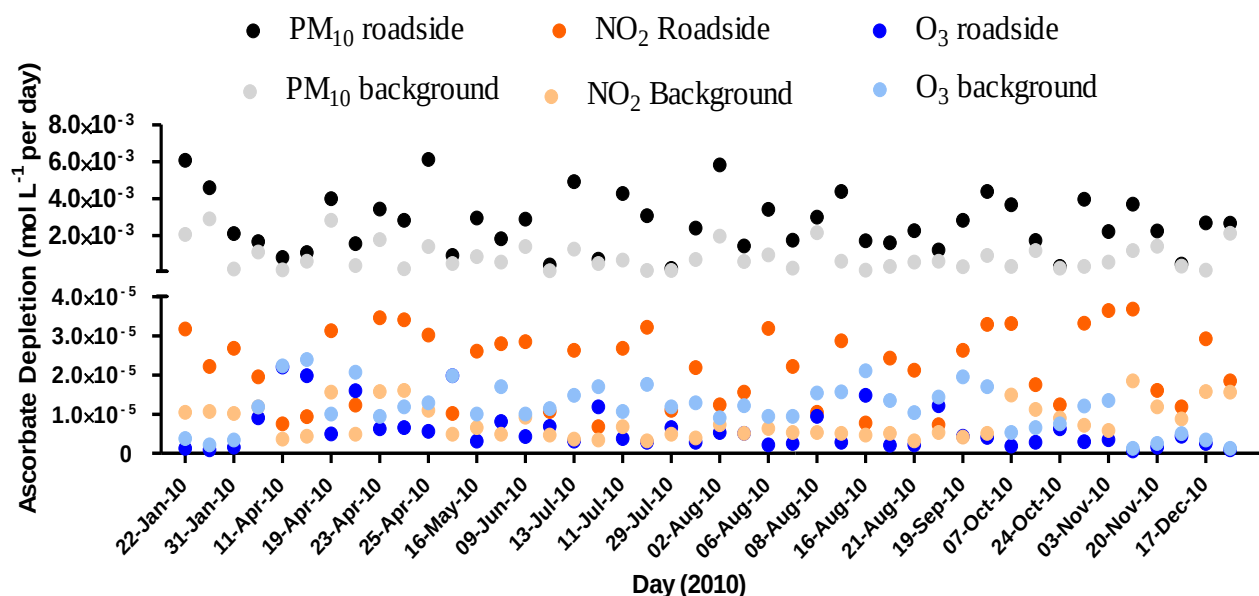


**Figure 40: Grouped Graph of Ascorbate Depletion by the Air Pollutants Examined**

The ascorbate depletion rates of PM, O<sub>3</sub> and NO<sub>2</sub> are plotted for all days considered (mean±SD, n=3). All graphed PM<sub>10</sub> values represent the experimentally calculated ascorbate depletion capacity of 24h PM filters, while the gaseous results are based on the extrapolation of the experimentally calculated antioxidant depletion capacity of set NO<sub>2</sub> and O<sub>3</sub> concentrations. Identical experimental conditions were used for the gaseous studies and equivalent for PM. Ascorbate depletion was higher at roadside for PM and NO<sub>2</sub>, and the opposite was true for O<sub>3</sub> ( $p < 0.001$ ). PM<sub>10</sub> exposure resulted in higher depletion than the gaseous pollutants at either location, while NO<sub>2</sub> was more important at roadside and O<sub>3</sub> at background locations ( $p < 0.001$ ). However, caution should be exercised when comparing the effects of PM and those of the gaseous pollutants as synRTLF was exposed to a high PM mass (~10µg) and this methodological aspect may be driving the large difference observed between the antioxidant depletion capacity of the gaseous pollutants and PM.

The daily ascorbate depletion findings were summarised in box and whisker plots denoting the median, minimum and maximum values of each air pollutant (**Figure 41**). Location was certainly a principal factor determining the antioxidant depletion capacity of the pollutants. The results indicated that PM<sub>10</sub> derived from either location resulted in markedly higher ascorbate oxidation than the gaseous pollutants. Also, PM<sub>10</sub> and NO<sub>2</sub> presented a higher affinity for ascorbate than their background pair and in contrast ascorbate depletion by O<sub>3</sub> at background was significantly higher than ascorbate consumption at roadside. Specifically, NO<sub>2</sub> had a significantly higher ascorbate consumption capacity than O<sub>3</sub> at roadside, while the exact opposite observation was made for the background location. This finding illustrates that real-life pollutant

concentrations can result in NO<sub>2</sub> being a more reactive gaseous pollutant, despite having a lower oxidative potential than O<sub>3</sub> in a laboratory setting.

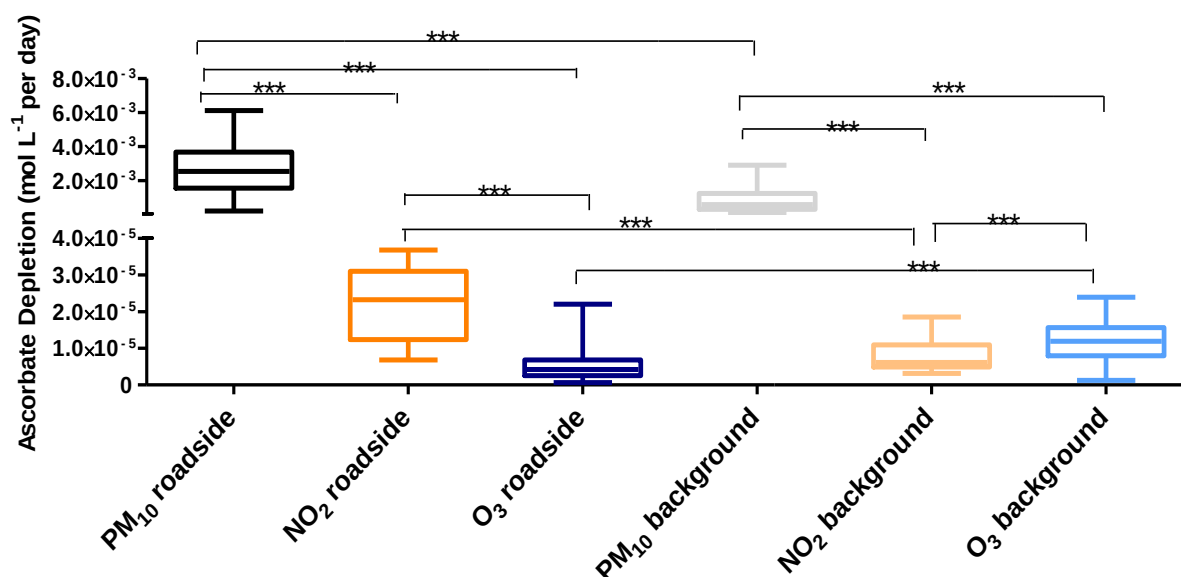


**Figure 41: Ascorbate Depletion by the Air Pollutants Examined at Roadside and Background Location.**

The ascorbate depletion rates of PM (disc cuts run in triplicate), O<sub>3</sub> and NO<sub>2</sub> (three independent experiments) were calculated under equivalent experimental conditions and their relative daily mean concentration was applied in order to achieve direct comparisons among different air pollutants. PM values represent experimentally calculated rates, while the gaseous results were extrapolated from laboratory studies utilising specific gaseous concentrations. All comparisons have been drawn on the graph, \*\*\* indicates that  $p < 0.001$ . The greater antioxidant depletion capacity of PM should be interpreted with caution given methodological differences between the gaseous and PM experimental protocols.

In addition, daily urate depletion presented the same gaseous hierarchy as ascorbate, though the antioxidant depletion effects were lower (the same axis were used for all antioxidant depictions in order to facilitate visual comparisons; **Figure 42**). A major difference between the ascorbate and urate depletion calculations is that PM resulted in no measurable urate consumption. Also, the daily urate depletion by NO<sub>2</sub> is significantly lower than ascorbate consumption. These theoretical calculations clearly reflect the antioxidant depletion hierarchies observed in the laboratory studies (chapters 3 and 4).

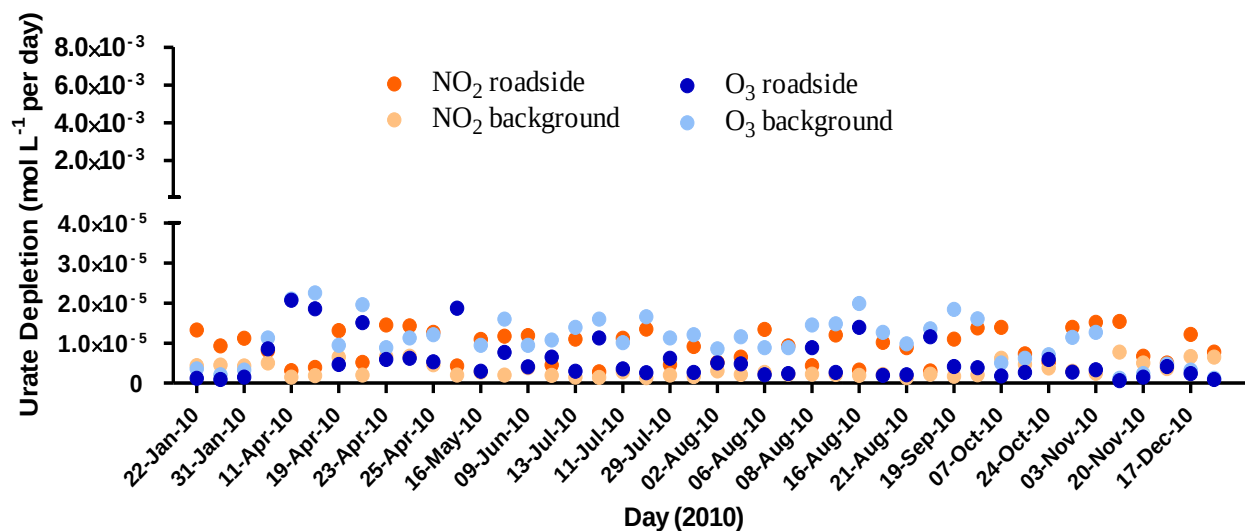


**Figure 42: Grouped Graph of Urate Depletion by NO<sub>2</sub> and O<sub>3</sub> at the Background and Roadside Locations**

The urate depletion rates of O<sub>3</sub> and NO<sub>2</sub> have been extrapolated from laboratory studies (mean  $\pm$  SD, three independent experiments) with identical experimental conditions. The daily mean concentration of each air pollutant at roadside and background location was applied on the laboratory rates in order to achieve direct comparisons. PM did not cause any measurable depletion of urate, a finding which is in accordance with synRTLF studies mentioned in chapter 5 and for this reason PM results are not present in this graph. Urate depletion was higher at roadside than at background location for NO<sub>2</sub> and the opposite was true for O<sub>3</sub> ( $p < 0.001$ ). Also, NO<sub>2</sub> resulted in significantly higher urate consumption than O<sub>3</sub> at roadside and this pattern was reversed at the background site ( $p < 0.001$ ).

Similarly to the ascorbate results, urate antioxidant consumption was positively associated at the two locations when either gaseous pollutant was considered, for example, on days that NO<sub>2</sub> levels resulted in high urate consumption at the North Kensington site, a high oxidative effect was also expected at Marylebone road. In addition, as expected from their photochemical characteristics, NO<sub>2</sub> and O<sub>3</sub> levels exhibited opposite patterns and hence antioxidant consumption also exhibited opposing patterns. Therefore, on days that NO<sub>2</sub> presented low urate consumption, O<sub>3</sub> exhibited high urate depletion and *vice versa*.

The urate values were also summarised in box and whisker plots presented in **Figure 43**. It should be noted that the O<sub>3</sub> and NO<sub>2</sub> concentrations of any day or even shorter time period could have been applied on the experimentally calculated rates. However, this application focused only on the daily NO<sub>2</sub> and O<sub>3</sub> levels of 44 days, as on those

days daily matched PM filters were collected and their antioxidant depletion capacity in the synRTLTF was quantified.

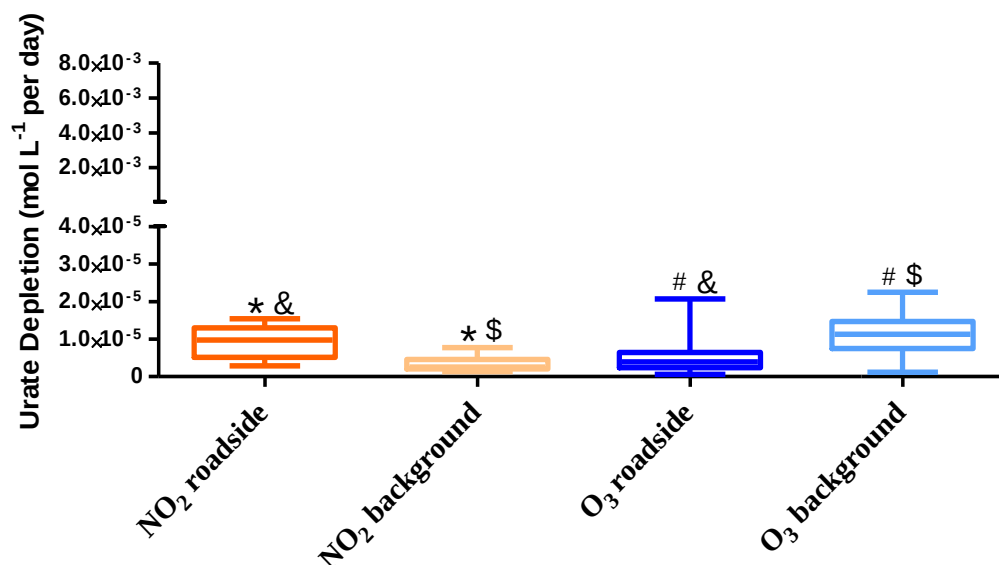


**Figure 43: Summary of Urate Depletion by NO<sub>2</sub> and O<sub>3</sub>**

The urate depletion rates of O<sub>3</sub> and NO<sub>2</sub> (three independent experiments) were calculated under identical experimental conditions and their relative daily mean concentrations were applied in order to take into account their real life concentrations at roadside and background locations. & depicts that roadside NO<sub>2</sub> resulted in significantly higher urate depletion than O<sub>3</sub> at the same location. Also, \* indicates that NO<sub>2</sub> had a higher oxidative potential at roadside than at background, # shows that the opposite effect was true for O<sub>3</sub>, and \$ depicts that O<sub>3</sub> antioxidant consumption at background was significantly higher than NO<sub>2</sub>'s effects at the same site.

The summary urate graph depicts the average, minimum and maximum antioxidant depletion by NO<sub>2</sub> and O<sub>3</sub> at the two locations (**Figure 43**). This graph interestingly shows that despite O<sub>3</sub> exhibiting a significantly higher urate consumption rate than NO<sub>2</sub> in the controlled laboratory studies (chapter 3), once their real life concentrations were applied NO<sub>2</sub> resulted in a significantly higher oxidative potential at roadside ( $p < 0.001$ ). However, at the background site this hierarchy is reversed, as discussed previously ( $p < 0.001$ ).

Furthermore, the real life concentrations of the air pollutants were applied in order to investigate their reactivity towards -a further RTLTF antioxidant- reduced glutathione (**Figure 44**). As expected, PM caused greater antioxidant depletion than the gaseous pollutants. An important difference with the ascorbate and urate findings is that O<sub>3</sub> presented higher reduced glutathione depletion than NO<sub>2</sub> at both sites; this result reflects the significantly higher O<sub>3</sub> reactivity towards reduced glutathione in the controlled

exposures. The contrast between the two gaseous pollutants was even more prominent at the background site, as O<sub>3</sub> levels were higher at North Kensington.

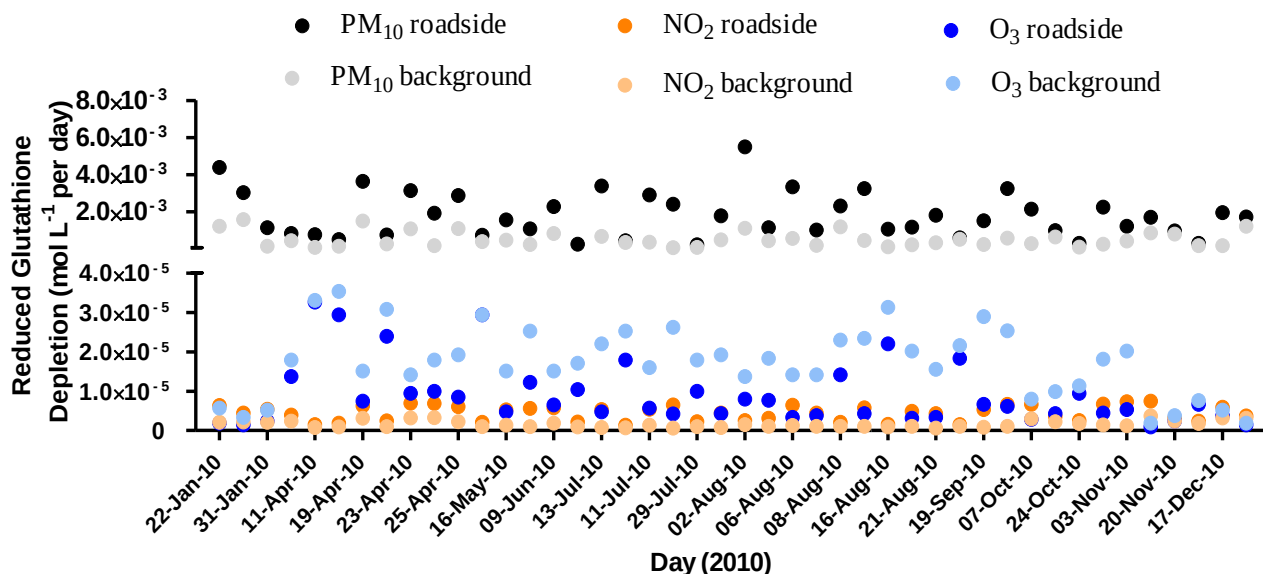


**Figure 44: Grouped Graph of Reduced Glutathione Depletion by the Air Pollutants Examined**

The reduced glutathione depletion rates of PM, O<sub>3</sub> and NO<sub>2</sub> are plotted for all days considered (mean  $\pm$  SD, n=3). All graphed PM<sub>10</sub> values represent the experimentally calculated reduced glutathione depletion capacity of 24h PM filters, while the gaseous results are based on the extrapolation of the experimentally calculated antioxidant depletion capacity of set NO<sub>2</sub> and O<sub>3</sub> concentrations. Identical experimental conditions were used for the gaseous studies and equivalent for PM. Reduced glutathione depletion was higher at roadside for PM and NO<sub>2</sub>, and the opposite was true for O<sub>3</sub> ( $p < 0.001$ ). PM<sub>10</sub> exposure resulted in higher antioxidant depletion than the gaseous pollutants at either location, while O<sub>3</sub> resulted in higher oxidative effects than NO<sub>2</sub> at both locations considered ( $p < 0.001$ ). However, caution should be exercised when comparing the effects of PM and the gaseous pollutants as synRTLF was exposed to a high PM mass ( $\sim 10\mu\text{g}$ ) and this methodological aspect may be driving the large difference observed between the antioxidant depletion capacity of the gaseous pollutants and PM.

The boxplots in **Figure 45** present the summary reduced glutathione depletion by the air pollutants examined. A consistent finding for all antioxidants considered was the outstanding antioxidant depletion capacity of PM, indicatively; in the reduced glutathione summary graph PM has overall a 100 times higher antioxidant depletion capacity than the gaseous pollutants. Though experimental differences between the gaseous exposures and PM cast doubt on the validity on this finding. In addition, this graph clearly shows that despite the very high NO<sub>2</sub> levels in London, reduced glutathione was depleted more by O<sub>3</sub> at both sites ( $p < 0.001$ ). This finding reflects the experimentally calculated NO<sub>2</sub> antioxidant depletion hierarchy (Chapter 3).

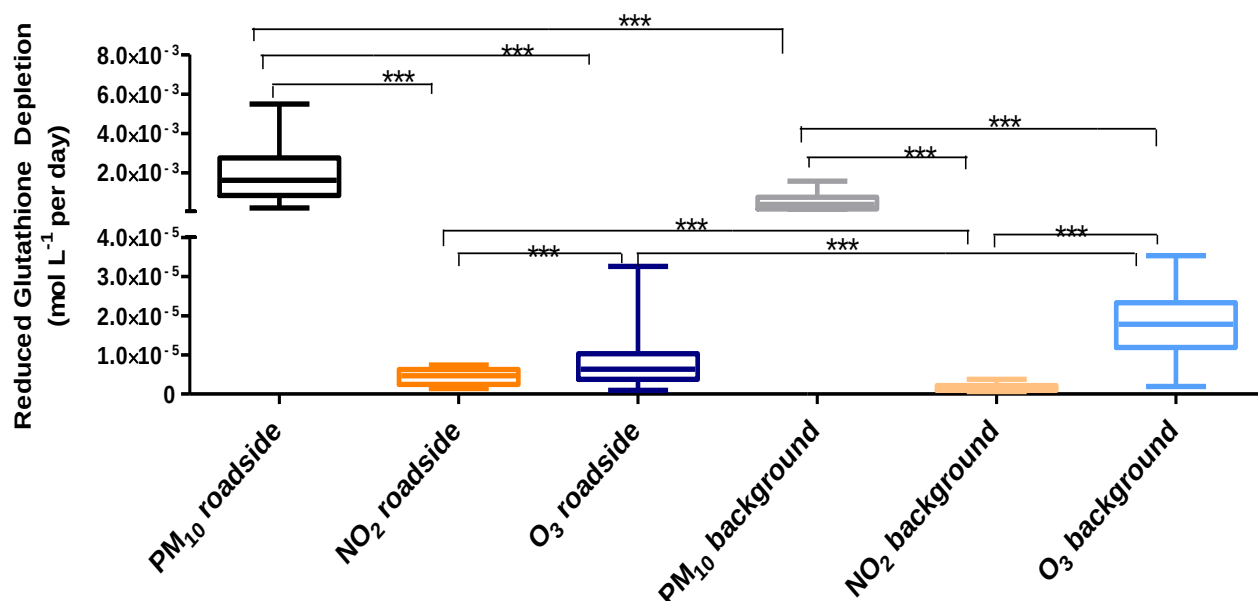


**Figure 45: Reduced Glutathione Depletion by the Air Pollutants Examined at Roadside and Background Location.**

The reduced glutathione depletion rates of PM (disc cuts run in triplicate), O<sub>3</sub> and NO<sub>2</sub> (three independent experiments) were calculated under equivalent experimental conditions and their relative daily mean concentration was applied in order to achieve direct comparisons among air pollutants. The graphed PM results summarise the oxidative effects of 44 roadside and 44 matched background 24h PM filters, while the gaseous results have been extrapolated from experimental studies utilising set O<sub>3</sub> and NO<sub>2</sub> concentrations. All comparisons have been drawn on the graph, \*\*\* indicates that  $p < 0.001$ . The greater antioxidant depletion capacity of PM should be interpreted with caution given methodological differences between the gaseous and PM experimental protocols. Reduced glutathione was the only antioxidant to be depleted more by O<sub>3</sub> than NO<sub>2</sub> at the roadside location, a finding which reflects the experimentally calculated antioxidant depletion capacity of these air pollutants in the svnRTLF.

It is noteworthy that the air pollutant correlations presented in **Table 13** were maintained once the real life concentrations were applied to the laboratory calculated rates. This positive association of NO<sub>2</sub> and PM<sub>10</sub> rates, and their inverse relationship with O<sub>3</sub> and air pollutant associations between sites can be observed in all grouped graphs. This information has also been summarized in **Table 14** for convenience. The table shows that for the days considered the highest NO<sub>2</sub> driven antioxidant depletion was observed on November 19<sup>th</sup> 2010 at the background and roadside location and on the same day the lowest O<sub>3</sub> driven antioxidant depletion was calculated. This same day presents one of the highest PM<sub>10</sub> antioxidant consumption effects, but not the maximum value. In addition, this table clearly shows the contrast in antioxidant depletion between

roadside and background location with the former always presenting lower values than the roadside pair and also it highlights overall the role of ascorbate as the primary target.

| Daily Antioxidant Depletion mol l <sup>-1</sup> * 10 <sup>-5</sup> |                 |          |            |                |                |                |                |
|--------------------------------------------------------------------|-----------------|----------|------------|----------------|----------------|----------------|----------------|
| Pollutant                                                          |                 | Median   |            | Maximum        |                | Minimum        |                |
|                                                                    |                 | Roadside | Background | Roadside       | Background     | Roadside       | Background     |
| NO <sub>2</sub>                                                    | AH <sub>2</sub> | 2.32     | 0.61       | 2.67 (19 Nov.) | 1.85 (19 Nov.) | 0.68 (19 Jun.) | 0.32 (21 Aug.) |
|                                                                    | UA              | 0.97     | 0.26       | 1.54 (19 Nov.) | 0.78 (19 Nov.) | 0.29 (19 Jun.) | 0.13 (21 Aug.) |
|                                                                    | GSH             | 0.47     | 0.12       | 0.74 (19 Nov.) | 0.37 (19 Nov.) | 0.14 (19 Jun.) | 0.06 (21 Aug.) |
| O <sub>3</sub>                                                     | AH <sub>2</sub> | 0.42     | 1.19       | 2.20 (11 Apr.) | 2.38 (15 Apr.) | 0.06 (19 Nov.) | 0.13 (19 Nov.) |
|                                                                    | UA              | 0.39     | 1.13       | 2.07 (11 Apr.) | 2.25 (15 Apr.) | 0.06 (19 Nov.) | 0.12 (19 Nov.) |
|                                                                    | GSH             | 0.32     | 0.92       | 3.26 (11 Apr)  | 3.53 (15 Apr.) | 0.10 (19 Nov.) | 0.19 (19 Nov.) |
| PM <sub>10</sub>                                                   | AH <sub>2</sub> | 254      | 59.8       | 612 (25 Apr.)  | 290 (24 Jan.)  | 21.5 (29 Jul.) | 8.82 (18 Jun.) |
|                                                                    | UA              | -        | -          | -              | -              | -              | -              |
|                                                                    | GSH             | 163      | 40.2       | 549 (2 Aug.)   | 158 (24 Jan.)  | 21.3 (29 Jul.) | 4.90 (18 Jun.) |

**Table 14: Daily Antioxidant Depletion Rate.**

The table presents the descriptive statistics of the calculated daily antioxidant depletion (PM samples were run in triplicate, and three separate experiments were performed for O<sub>3</sub> and NO<sub>2</sub>). For the extreme observations the exact day is given inside the parentheses.

Furthermore, the total antioxidant depletion resulting from each air pollutant was considered. This calculation, aimed to investigate whether the pollutant's oxidative hierarchy would change once the entire ambient pollutant reactivity was considered (**Figure 46**). For the gaseous pollutants this calculation was performed by adding the ascorbate, urate and reduced glutathione consumption values, while for PM<sub>10</sub> there were no urate values to consider. Also, there was increased interest in clarifying the gaseous pollutant hierarchy at the roadside location. This is because when ascorbate and urate were considered NO<sub>2</sub> presented significantly higher depletion than O<sub>3</sub> at Marylebone road, whereas the oxidative potential of the two gases did not differ significantly once reduced glutathione was considered. Interestingly, the results of the total antioxidant depletion calculation showed that PM<sub>10</sub> was responsible for the most significant part of antioxidant depletion (**Figure 46**). In addition, location was a determining factor of O<sub>3</sub> and NO<sub>2</sub> oxidative capacity, overall, NO<sub>2</sub> was more important at roadside and O<sub>3</sub> more significant at background.

Following the examination of the total antioxidant depletion capacity of each air pollutant, this calculation was further expanded by adding these values at each site in

order to calculate the overall daily antioxidant depletion at roadside or background location (**Figure 47**).

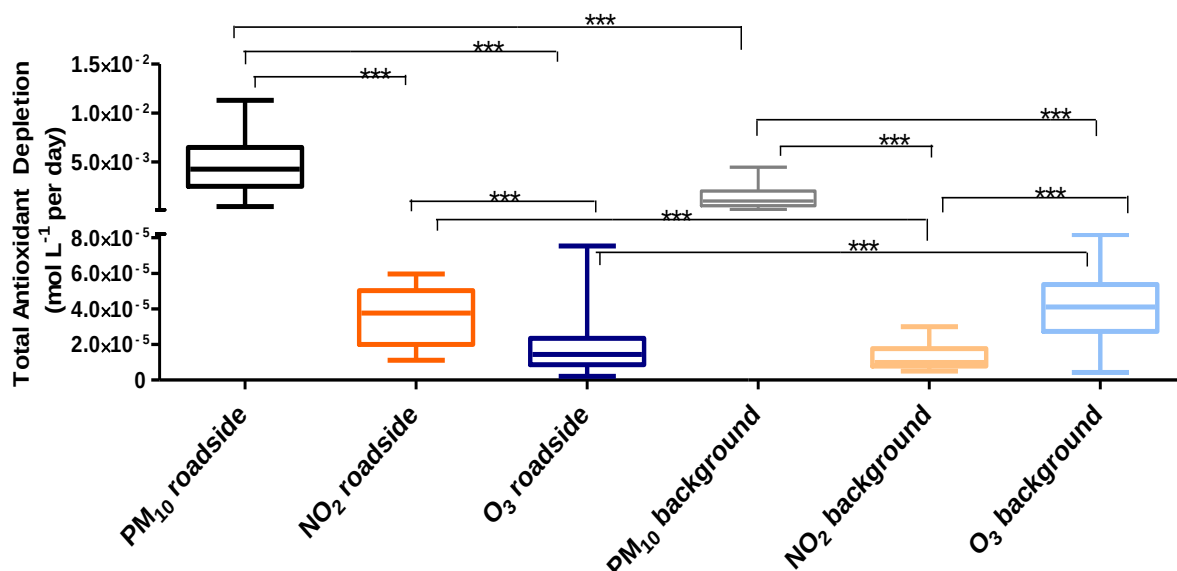


**Figure 46: Total antioxidant Depletion by the Air Pollutants Examined at Roadside and Background Location.**

The total antioxidant depletion of each air pollutant was calculated by adding the total antioxidant consumption within the synRTLF. All comparisons have been drawn on the graph, \*\*\* indicates that  $p < 0.001$ . The graphed PM results summarise the oxidative effects of 44 roadside and 44 matched background 24h PM filters, while the gaseous results have been extrapolated from experimental studies utilising set O<sub>3</sub> and NO<sub>2</sub> concentrations. The greater antioxidant depletion capacity of PM should be interpreted with caution given methodological differences between the gaseous and PM experimental protocols. Also, it should be noted that the total antioxidant depletion calculation has been performed under the assumption that the total antioxidant consumption equals the sum of the individual effects.

Thus, this value represented the overall antioxidant depletion associated with the real life concentrations of NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> following a 24h exposure and assuming again that these effects would occur in an additive manner (**Figure 47**). This calculation highlighted the considerable endogenous oxidant activity of these air pollutants, which was as discussed previously more prominent at the roadside location. This graph shows the detailed temporal profile of the antioxidant consumption results which were highly variable. Indicatively, the overall antioxidant depletion of the roadside and background samples collected on April 19<sup>th</sup> 2010 belonged on the higher end of all antioxidant depletion rates, while the next day the estimated antioxidant consumption represented

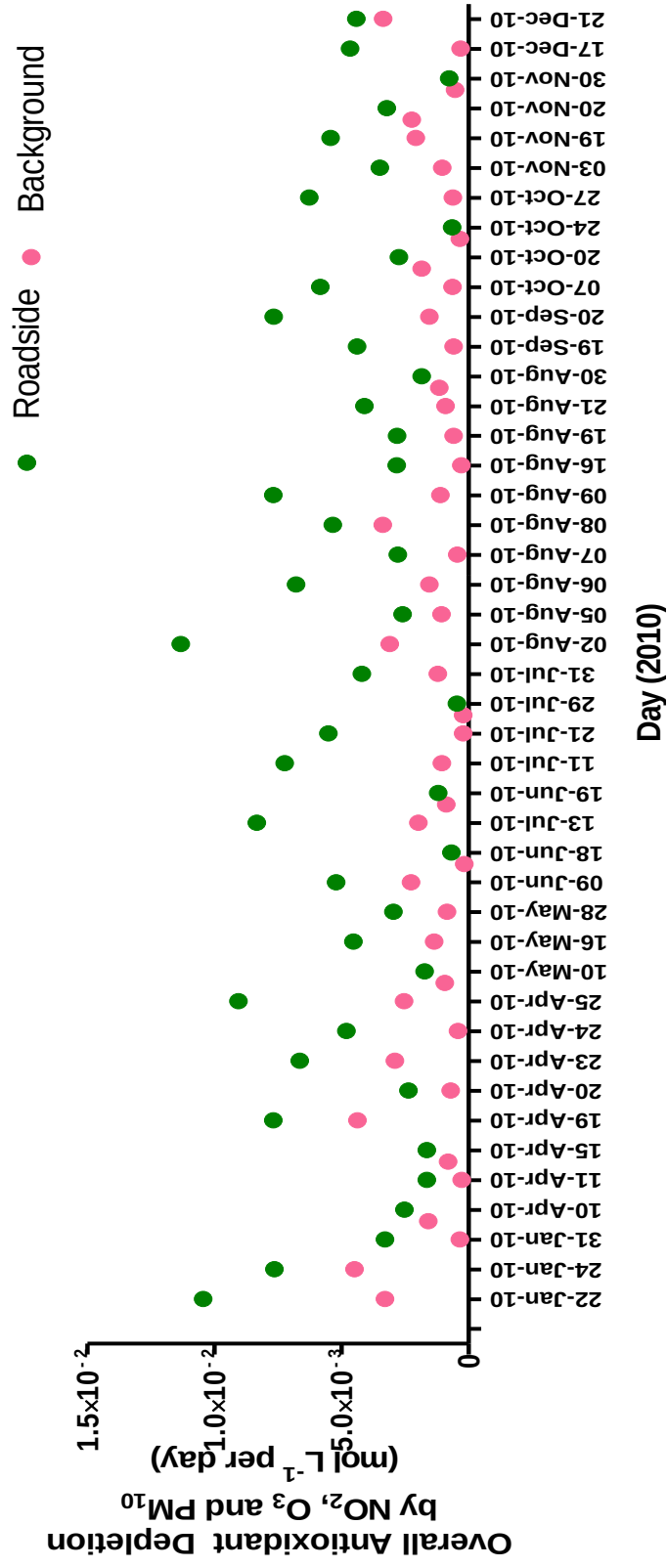


**Figure 47: Overall Antioxidant Depletion by  $\text{NO}_2$ ,  $\text{O}_3$  and  $\text{PM}_{10}$  at Roadside and Background Location**

The daily overall antioxidant depletion resulting by exposure of all three air pollutants at each site pollutant was calculated by adding their daily total antioxidant consumption within the synRTLF. The overall antioxidant depletion calculation has been performed under the assumption that the total antioxidant consumption equals the sum of the individual effects.

one of the lowest effects. It can be concluded that the highest antioxidant depletion effects were transient but recurring, and possibly this high temporal variation resulted in non significant differences across seasons.

#### 5.4 Discussion

As has been mentioned earlier in the thesis,  $\text{NO}_2$ ,  $\text{O}_3$  and  $\text{PM}_{10}$  are thought to mediate their pathophysiological actions through the generation of radical and non radical cytotoxic products derived from their ability to rapidly oxidise biological target molecules [114, 267, 275, 277, 392-394]. The spatial formation of these reactive species is thought to be occurring in the RTLF [51, 288, 415, 440]. The latter contains a range of protective biochemical species, including water soluble antioxidants [276, 493] and these are believed to be acting as sacrificial substrates to conserve more sensitive biomolecules [79]. The aim of the current project was to directly compare the interaction of  $\text{NO}_2$ ,  $\text{O}_3$  and  $\text{PM}_{10}$  with the water soluble antioxidants present in the RTLF.

The controlled experiments focused on defining the relative rates of consumption of each of these antioxidant species by  $\text{NO}_2$ ,  $\text{O}_3$  and  $\text{PM}_{10}$ . A comparison of the  $\text{NO}_2$  and  $\text{O}_3$  antioxidant depletion capacity was presented in chapter 5, and the results pointed towards  $\text{O}_3$  exhibiting a significantly higher oxidative potential than  $\text{NO}_2$ . In the current section the pollutant's ambient or real life concentrations were applied to the laboratory calculated rates in order to a) compare the gaseous pollutants with -the more chemically varied-  $\text{PM}_{10}$  and b) investigate whether the antioxidant depletion contrast between the two gaseous pollutants persisted following this application.

A direct comparison of the aforementioned pollutants under equivalent controlled conditions was deemed of interest given the difficulty in disentangling their independent effects and particularly those of  $\text{NO}_2$  [476, 477]. Also, this is the first study of its kind and placing the  $\text{O}_3$  vs.  $\text{NO}_2$  comparison within a realistic framework was compelling. The application of the outdoor concentrations to the calculated rates was feasible because the same conditions including; time points, temperature, RTLF, and antioxidant quantification methods were utilised for the three air pollutants. It should be noted that the gaseous rates could have been applied on any day as their chemical composition is standard however given that the  $\text{PM}_{10}$  calculation was based on daily collected samples the analysis focused on days that the oxidative potential of PM samples had been

calculated experimentally. The resolution used was 24h, reflecting the total duration of the PM<sub>10</sub> collection.

For the days considered, the airshed composition summary revealed that at roadside NO<sub>2</sub> had the highest concentration, followed by PM<sub>10</sub> and then O<sub>3</sub> (**Figure 39**). Also, NO<sub>2</sub> and PM<sub>10</sub> exhibited significantly higher levels at roadside compared to their background levels. In contrast, O<sub>3</sub> exhibited higher levels at the North Kensington site and had in fact the highest median value at that location followed by NO<sub>2</sub> and finally PM<sub>10</sub> which had a significantly lower concentration (**Figure 39**). These findings are in agreement with the literature, specifically, the 2010 European Environment Agency (EEA) report stated that the yearly average of the NO<sub>2</sub> concentration at the Marylebone road was the highest roadside in Europe, while PM<sub>10</sub> at the same location was the worst in the UK [494]. Though Putney High Street in Wandsworth appears to have even higher NO<sub>2</sub> concentrations than areas in the ‘official network’, indicatively, it has unofficially been characterized as the ‘worst site in Europe for NO<sub>x</sub>’ in a local website by a TFL environmental manager [495], similarly it has been recently suggested that a central London location, Oxford street, may potentially present the highest NO<sub>2</sub> concentrations in the world [496]. The persistence of high NO<sub>2</sub> and PM<sub>10</sub> concentrations despite more optimistic expectations has been highlighted in the literature [497]. Increased road traffic emissions appear to be partly responsible for high PM<sub>10</sub> levels [50], while the persistently high NO<sub>2</sub> levels can be attributed at least in part to increasing numbers of new diesel cars utilising catalytic diesel particulate filters which result in direct emission of NO<sub>2</sub> [46]. Given the higher traffic flow and congestion presented at roadside locations [477], the contrast between the two sites was expected, in general the concentration of these pollutants declines with distance from roadside. In addition, the O<sub>3</sub> levels were significantly higher at background compared to the Marylebone road site, and this phenomenon can be attributed to the higher levels of fresh NO<sub>x</sub> at the roadside [498]. Specifically, in the presence of low NO<sub>x</sub> levels, O<sub>3</sub> is the major component of OX (i.e. NO<sub>2</sub> and O<sub>3</sub>), while NO<sub>2</sub> is the dominant pollutant at high levels [499, 500]. This occurs because a great abundance of NO<sub>x</sub> results in high NO concentrations destroying O<sub>3</sub> faster than NO<sub>2</sub> can create it [501].

The correlation analysis of these air pollutants resulted in high correlation coefficients (**Table 13**) and therefore, by retrieving the concentration of just one of the aforementioned pollutants at either site, one could predict with relative confidence the concentrations of the remaining pollutants. These results highlighted the difficulty in

distinguishing between NO<sub>2</sub> and PM<sub>10</sub>, this high correlation has been attributed to the fact that they originate from the same combustion sources [477]. Also, the negative correlation between NO<sub>2</sub> and O<sub>3</sub> was expected as for decades it has been known that the latter is formed by the photolysis of the former [7]. This inverse relationship was somewhat larger at the background site and may be attributed to meteorological and spatial differences (e.g. building arrangement) affecting flow and turbulence and ultimately local concentrations [502, 503]. Despite, these differences, overall the air pollution levels between the two sites were significantly correlated, this may be a result of similar patterns of air pollution sources such as traffic activity as well as similar weather conditions and especially winds blowing from Marylebone Road towards North Kensington and *vice versa*, or alternatively air pollution driven from another ‘hot spot’ (or even country) but affecting both of these locations.

The high positive correlation between NO<sub>2</sub> and PM<sub>10</sub> and the inverse with O<sub>3</sub> was also reflected once the real life concentrations were applied to the rates. Indeed, high NO<sub>2</sub> and PM<sub>10</sub> antioxidant depletion was associated with low O<sub>3</sub> oxidative potential and *vice versa*, this does not mean however that the maximum NO<sub>2</sub> or PM<sub>10</sub> values were always associated with the lowest O<sub>3</sub> value (**Table 14**). The concentration application also revealed that PM<sub>10</sub> carries a greater antioxidant depletion than the gaseous pollutants examined, as it resulted in significantly higher antioxidant consumption of ascorbate and reduced glutathione (**Figure 45**). However, it did not show any signs of urate depletion and thus the total antioxidant depletion of each air pollutant was investigated by adding their individual antioxidant consumption effects (**Figure 46**). The results showed that PM<sub>10</sub> still possessed the highest redox activity and thus support the hypothesis that PM may be the principal driver of the pathophysiological effects observed in epidemiological studies. Also, the high levels of NO<sub>2</sub> dependent antioxidant depletion at roadside highlight that it can be powerful oxidant in its own right, in fact more so than O<sub>3</sub> at certain locations. It is appreciated of course that this is a simplistic mathematical operation and potentially more complex interactions may be present *in vivo*.

PM<sub>10</sub> depletion exhibited day and site dependent heterogeneity as it was expected [243], but importantly for all days considered PM associated activity surpassed the gaseous effects. This very high oxidative potential observed for PM<sub>10</sub> is thought to be mainly attributed to its bioavailable metal content. It may be surprising that O<sub>3</sub> which has a higher redox potential (2.07V) than metals found in PM such as iron and copper

(0.769V and 0.518V respectively) presented a lower antioxidant depletion capacity. Though, these PM constituents are able to form hydroxyl radicals through Fenton and Fenton like reactions [448, 504-506], importantly this heterogeneous oxidizing pool is also enriched with further oxidizing agents such as quinones [161, 507] . In addition, PM can come in direct contact with the RTLF, while O<sub>3</sub> has a very limited solubility which renders its absorption dependent on its interaction with RTLF moieties [53].

The current study focused on the water soluble fraction of PM<sub>10</sub> as this is the most relevant to living organisms and has been shown to carry the greatest oxidative damage in biological models [508-510]. In fact, other studies have shown that metal content removal with Chelex resulted in significant lowering of the PM related toxicity in rats and cell cultures [27], as well as a significant decrease in antioxidant depletion in an RTLF model [277]. Although there is compelling evidence suggesting that the metal content is a principal driver of PM<sub>10</sub> oxidative potential, further components such as quinones [511] and endotoxins [512] cannot be excluded. This project did not aim to characterize the individual components of PM<sub>10</sub> because previous studies have explored this question [114] and also it was not technically possible due to near complete use of the PM filter surface area in the synRTLF exposures. Overall, the antioxidant depletion calculations aimed to represent an integrated measure of the toxicological response of multiple individual characteristics of the varied and complex PM mixture [79, 281, 445].

Interestingly the results showed that location (roadside or background) had an air pollutant specific effect on oxidative potential. In the case of PM<sub>10</sub> the results support the contention that higher antioxidant consumption would be expected at roadside compared to background. This finding is in alignment with laboratory studies demonstrating the redox contrast of PM<sub>10</sub> collected from locations equivalent to the ones used in this project [113, 114, 513] and this is also in agreement with epidemiological studies showing that the traffic component of PM<sub>10</sub> substantially drives the health impact of outdoor pollution [514]. Similarly, other epidemiological studies have consistently supported that individuals living in proximity to high trafficked roadways (characterized by high PM mass concentrations) exhibited increased respiratory problems compared to individuals living in less polluted locations [196, 198, 515-517]. However, in these studies the challenge of disentangling the independent effects of each air pollutant re-emerges and socioeconomic confounders related to exposure should also be taken into account.

A more surprising antioxidant depletion hierarchy was observed for the gaseous pollutants. Specifically, a comparison of their antioxidant depletion capacity under identical concentrations and conditions demonstrated that O<sub>3</sub> had a significantly higher oxidative capacity than NO<sub>2</sub> across all antioxidants (Chapter 5); however applying their real life concentration demonstrated that environmental occurrence influences this presumed hierarchy. At roadside (in London), NO<sub>2</sub> concentrations were about 7 times higher than the equivalent O<sub>3</sub> values, thus NO<sub>2</sub> surmounted O<sub>3</sub>'s oxidative potential for ascorbate and urate. At the same location, reduced glutathione reactivity did not differ significantly, thus reflecting the lower reduced glutathione reactivity noted in the laboratory exposures ( $\text{AH}_2(\text{O}_3) \geq \text{UA}(\text{O}_3) > \text{GSH}(\text{O}_3) \geq \text{AH}_2(\text{NO}_2) >> \text{UA}(\text{NO}_2) > \text{GSH}(\text{NO}_2)$ ). The total antioxidant depletion effect however pointed towards NO<sub>2</sub> presenting a significantly higher oxidative potential than O<sub>3</sub> at Marylebone road, typically in toxicological studies O<sub>3</sub> is presented as more reactive gas than NO<sub>2</sub> [148, 382, 384, 385] however without consideration of their real life concentrations.

A different gaseous hierarchy was observed at the background location. At North Kensington, O<sub>3</sub> levels had a higher trend than NO<sub>2</sub>, indicatively the median of the former was ~1.5 times larger, as a result O<sub>3</sub> resulted in significantly higher ascorbate, urate and reduced glutathione oxidation than NO<sub>2</sub> at the same site. This hierarchical contrast observed between the roadside and background locations following the concentration application, highlights the importance of considering the environmental levels of any air pollutant prior to projecting their potential biological impact.

A major target of the protocol development was to compare the aforementioned pollutants while utilising the same exposure conditions (e.g. temperature, synRTLF, time points) as well as the same antioxidant depletion methods. Exposures of synRTLF to O<sub>3</sub> and NO<sub>2</sub> (same concentrations, and air flow) were achieved via a mixed interfacial exposure system, that is, the gases were streamed above synRTLF while the latter was placed on an orbital shaker to facilitate mixing. While concurrently collected roadside and background PM<sub>10</sub> disc cuts were placed inside eppendorfs containing synRTLF and mixed at the same speed as the mixing platform used for the gaseous exposures. Thus, there was an evident difference believed to model the *in vivo* exposures, that is, synRTLF was in direct contact with the PM<sub>10</sub> samples while the gaseous diffusivity was dependent on their solubility and antioxidant reactivity [51, 52, 287, 404, 415]. A further reason that a gaseous impingement was not favoured is that it could potentially cause an overestimation of the antioxidant reactivity towards the pollutants due to

increased reaction rates compared with the *in vivo* situation in which gaseous solubility is also important. A further potential confounder was the extreme evaporation observed in pilots testing the ‘bubbling’ method, even at shorter exposures (2h).

It is appreciated however that the extent of the PM<sub>10</sub> deposition will be dependent on factors such as ventilation rate [291, 518], breathing pattern (e.g. oral versus nasal inhalation) and respiratory health [290, 292]. These complex physiological parameters were beyond the scope of the current *in vitro* model; however, future studies could replicate this project by placing synRTLTF in a robotic respiratory tract simulator. In addition, PM<sub>10</sub> is thought to be deposited at the upper airways [288], in contrast, the gaseous pollutants examined are mostly absorbed at the lower airways [56, 57, 519], these differences in air pollutant deposition patterns have also been confirmed in animal and human studies utilizing radio labeled markers [366, 520-522]. To complicate matters further, urate is a more prominent antioxidant in the upper respiratory tract [73] but PM<sub>10</sub> does not react with it *in vitro* [234, 280, 288, 454], therefore in terms of this specific oxidative marker, the gaseous pollutants examined presented a greater adverse effect. However, it should be acknowledged that whilst this project focused on the interaction of the aforementioned pollutants with the small molecular weight antioxidants further antioxidant defences are present in the RTLTF and could potentially function to prevent air pollution driven injuries.

## 5.5 Conclusion

It is clear from research studies that poor ambient air quality has adverse effects on human health. However, in most of these studies, NO<sub>2</sub> concentration at the community level was correlated with PM and O<sub>3</sub>, making it difficult, if not impossible to disentangle whether the associations represent a toxic effect of NO<sub>2</sub>, *per se*, or whether they reflect a surrogate effect.

These experimental findings constitute the first to compare the effects of O<sub>3</sub>, NO<sub>2</sub> and PM<sub>10</sub> in a lung lining fluid model under the same experimental conditions. The gaseous pollutants examined resulted in depletion of the water soluble antioxidants present in the human RTLTF model in a time and concentration dependent manner (for PM<sub>10</sub> source related characteristics were more important than initial concentration). This observation supports the premise that the RTLTF antioxidants are acting as a first line of defence against inhaled air pollution, while the results supported differential antioxidant consumption with ascorbate being overall the primary target. Following the application

of the air pollutant's real life concentrations to the experimentally calculated antioxidant depletion rates it was revealed that (on the days considered) PM was responsible for higher ascorbate and glutathione consumption across all tested days and sites when compared with NO<sub>2</sub> and O<sub>3</sub>. However, for urate depletion, PM was not responsible and NO<sub>2</sub> could even be more important than ozone, given its higher concentration at roadsides. Location played a more important role for the relative toxicity hierarchy of the gaseous pollutants. At roadside, NO<sub>2</sub> levels greatly surpassed the O<sub>3</sub> levels and this resulted in a higher oxidative potential calculation of the former, despite O<sub>3</sub> exhibiting a higher antioxidant depletion capacity in the controlled laboratory experiments. At background, O<sub>3</sub> levels presented a higher trend than the NO<sub>2</sub> levels and therefore the antioxidant depletion hierarchy was in accordance with the laboratory experiments, that is, antioxidants were more reactive when exposed to O<sub>3</sub>. Overall this study draws attention to the importance of utilising realistic concentrations when exploring the toxicological impact of different air pollutants and hopes to pave the way for more experimental studies comparing their effects under the same controlled conditions.

## 6 Concluding Discussion

### 6.1 Study Summary

There are more *in vitro*, *in vivo* and epidemiological studies than ever before suggesting that air pollution results in ill health, though the NO<sub>2</sub> specific effects are still being debated as it is challenging to disentangle its independent effects [4]. This scientific enigma prompted this study to investigate the relative toxicity of NO<sub>2</sub> in the respiratory tract; further air pollutants examined included O<sub>3</sub> and PM<sub>10</sub> which constitute two major ambient pollutants in the developed world. An RTL model was used because *in vivo* this aqueous material has a strategically important location, being the first physical interface encountered by any inspired agent [80]. It has a range of protective biochemical species and of these defences, I focused on the water soluble antioxidants as they function as sacrificial substrates *in vitro* and *in vivo* when exposed to air pollutants and are thought to be the principal targets [51, 415].

In fact similar studies have been conducted previously for these gaseous pollutants and have demonstrated their high reactivity towards the antioxidant molecules [268, 269]. The unique point of this study was that identical conditions were utilised in order to allow for direct comparisons. In addition creative methodological development led to the design of an equivalent exposure protocol for PM<sub>10</sub>. This process involved the calculation of antioxidant depletion rates under the same controlled conditions (RTL, temperature, time points, quantification methods and calculations) and the incorporation of the pollutants daily real life concentrations in order to achieve direct comparisons.

The results showed that on a unitary basis O<sub>3</sub> resulted in significantly higher antioxidant depletion than NO<sub>2</sub> across all antioxidants. Also, differences existed in the antioxidant consumption rates within each exposure condition, that is, there was an antioxidant depletion hierarchy. Ascorbate exhibited the highest reactivity and reduced glutathione the lowest, while in the case of the NO<sub>2</sub> exposures there was a more distinct hierarchy with urate exhibiting significantly lower oxidation than ascorbate and these results were in accordance with previous controlled studies (Chapter 5). Consideration of the pollutants daily mean concentration revealed that interestingly NO<sub>2</sub> exhibited overall a higher antioxidant depletion capacity at roadside than O<sub>3</sub> and the opposite was true at the background site. These findings reflected a) the importance of location as a determinant of air shed composition b) the very high NO<sub>2</sub> levels in the UK.

The oxidative potential of the aforementioned gases was compared with that of PM<sub>10</sub>. The latter resulted in significantly higher depletion of ascorbate and reduced glutathione at both sites, though a major difference was that the particles did not cause any measurable depletion of urate, in accordance with previous studies [234, 277, 454]. For this reason the total antioxidant depletion effects of each air pollutant were calculated by adding their antioxidant consumption effects, and these results revealed that the higher effects of PM<sub>10</sub> persisted in this scenario. A further important note related to the PM<sub>10</sub> samples is that while their antioxidant depletion capacity presented some variation that was day specific, the roadside pair always presented higher oxidative effects than the background pair and for both locations these results were not correlated with the initial weight of the filter. Thus, the roadside filters carried the greatest toxicity potential, in accordance with previous studies comparing PM samples from roadside locations and those situated away from major roads [113, 114, 449-451]. The oxidative potential of the samples was composition dependent, and the metal content can be hypothesized to be a principal driver of these effects [277, 491].

However, it should be mentioned that complex lifestyle factors that are beyond the scope of this thesis are expected to strongly influence the impact of each of the aforementioned pollutants. Indicatively, background sites are more representative of residential areas thus for an average person spending more hours a day in a background site, O<sub>3</sub> would potentially carry a greater oxidative role than NO<sub>2</sub>, though cycling to work via roadside paths could increase the NO<sub>2</sub> exposure dose and potentially surpass the total O<sub>3</sub> effects. Such comparisons are, however, further complicated by the indoor microenvironment, and parameters such as ventilation type and building occupancy.

## **6.2 Controlled Studies can Assist Evidence Based Health Policies**

Public health policies involve group decision making and complex input, in the 21<sup>st</sup> century the aim is to base such decisions on knowledge and evidence though it is of course naïve to believe that this is the sole determinant, as political factors can compromise this approach [523, 524]. The European Commission has expressed a clear support of science based decision making, indicatively, Janez Potočnik, the EU Commissioner for Environment has stated that the '*EU air policy must be based on the latest science*' [525]. However, scientific evidence offers limited influence on public policy when among other reasons there is a lack of consensus about the findings due to their complexity [526].

In the field of air pollution, epidemiological studies are of imperative importance but carry understandably several complexities such as a difficulty in disentangling the effects of different air pollutants as they are often generated from the same emission sources, and an intricacy in establishing the extent of the human exposure. Thus, controlled laboratory studies can facilitate such comparisons by employing identical methodological protocols for different air pollutants. However, as mentioned in the gaseous comparison section (Chapter 5) controlled studies have arbitrarily employed higher O<sub>3</sub> concentrations under the assumption that it is more toxic than NO<sub>2</sub> or have used different experimental conditions. Therefore, the current study aimed to facilitate the comparison of the major air pollutants by employing identical conditions where possible and equivalent conditions when it was not possible, it is hoped that further studies will employ a similar strategy. A subjective evaluation of the findings by the regulators is certainly still required, but to an extent facilitated.

### **6.3 Interpretation of Epidemiological Studies**

Epidemiological studies have clearly shown that exposure to air pollution is associated with ill health. Though, as discussed in the introductory chapter, disentangling the independent effects of each air pollutant is a challenging but important process as it can lead to more efficient public health policies. Undoubtedly epidemiological studies represent a far more complex and logistically difficult approach than the *in vitro* model used in this project, still however the current results can possibly assist in addressing the plausibility of pathways that might lead to health effects, as well as assessing the relative importance of these air pollutants in causing respiratory morbidity.

The results of this study support epidemiological studies showing antioxidant genes interacting with air pollution, and antioxidant genetic polymorphisms being related to air pollution susceptibility [527-529]. Indeed, the fact that all three air pollutants exhibited antioxidant depletion capacity in the RTLTF model supports the contention that the RTLTF antioxidant moieties have an intrinsic reactivity towards air pollutants and can act as a protective screen against their oxidative effects by acting as sacrificial substrates, thereby conserving sensitive biomolecules [79].

The simulation of the air pollutant's interaction with the antioxidants of the RTLTF has highlighted the temporal and spatial variability in antioxidant depletion, a finding that

may partly explain some of the inconsistencies observed in epidemiological studies. Specifically, roadside derived PM<sub>10</sub> samples exhibited significantly higher antioxidant depletion capacity than their background derived pairs. Location was very important for the gaseous pollutants as well. Application of their real life concentrations revealed that NO<sub>2</sub> was more important at roadside and conversely O<sub>3</sub> exhibited higher oxidative effects at background. This heterogeneity was further complicated by the fact that all air pollutants examined presented temporal effects, that is, their oxidative potential was day dependent (an even shorter time frame could exist), though no clear seasonal or other pattern emerged. Therefore, had we utilized the overall average concentration of the gaseous pollutants, as it is often done in epidemiological studies, their oxidative contrast would have been reduced and their site dependent hierarchy would not have been observed. Similarly, the fact that PM<sub>10</sub> oxidative activity was site dependent and importantly was not correlated with filter weight, demonstrated that utilising only the ambient concentration of PM in epidemiological studies is probably not an ideal predictor of PM's biological activity. The findings of this work potentially suggest that the development of personal air pollution monitoring systems (so that devices may be worn by people for a long period of time without discomfort) in conjunction with the use of GPS technology could extensively advance the epidemiological field. The use of such equipment would enable an accurate measurement of an individual's air pollution exposure at different locations and hence could lead to a more successful disentanglement of the pollutant's biological effects.

The importance of selecting pertinent biological markers was highlighted in this project and this observation is very relevant to epidemiological studies as well. Specifically, I was interested in the major water soluble antioxidants in the RTLF and hence utilized realistic concentrations of urate, ascorbate and reduced glutathione. However, PM incubation in synRTLF did not result in any measurable urate depletion in my model. If urate had been the only antioxidant in my system or the only one quantified I would have reached a very different conclusion about PM's oxidative activity. This finding is particularly striking as PM resulted in a significantly higher total antioxidant consumption effect than the gaseous pollutants, despite not depleting one of the three moieties. Similarly, the antioxidant depletion capacity contrast between O<sub>3</sub> and NO<sub>2</sub> was found to be about 2.6 times smaller when ascorbate was used for comparison instead of urate or reduced glutathione, as ascorbate presented the primary protective

antioxidant in the synRTLTF. Thus, these results suggest that a) a lack of significant biological effects in certain epidemiological studies, should not be necessarily interpreted as a complete lack of toxicity in the same concentration range b) subtle methodological differences can lead to different study results and hence very different conclusions.

The RTLTF model utilized for this work was based on the premise that the pathophysiological actions of the air pollutants observed in human and animal studies are mediated through free radical mechanisms. While there are interpretative restrictions in this study it can be hypothesized (based on the days that were analysed) that PM would induce a greater oxidative stress response *in vivo* in the RTLTF than the gaseous pollutants at environmentally relevant concentrations. In addition, the results of this study suggest that in countries like the UK which have higher levels of NO<sub>2</sub> than O<sub>3</sub>, the potential health effects of the former may have been underestimated. Certainly NO<sub>2</sub> was less reactive than O<sub>3</sub> in the *in vitro* model, though the great concentration difference at roadside overcompensated for its lower oxidative potential. These findings support the view that NO<sub>2</sub> is likely not to be a mere marker of air pollution but a toxic pollutant in its own right. Unfortunately, to this day specific rates of antioxidant depletion cannot be translated to definite presentation of pathophysiological symptoms, as this is thought to depend on the individual's redox state and antioxidant replenishment capacity. Nevertheless, these results would potentially suggest that the public and especially individuals susceptible to oxidative deterioration would be likely to benefit by further reduction of the NO<sub>2</sub> levels.

Overall, these results suggest that all air pollutants examined can cause significant antioxidant depletion in the RTLTF under realistic conditions, PM exhibited overall the greatest antioxidant depletion capacity, though the likely health impact of NO<sub>2</sub> exposure at roadside locations should not be ignored.

#### **6.4 Study Limitations**

The current project presented some innovative aspects but also potential limitations that are worth exploring. Firstly, the RTLTF model that was utilized presented an oversimplification of the human RTLTF, indicatively, lipids, proteins and immune system cells were absent. Previous O<sub>3</sub> and NO<sub>2</sub> *in vitro* studies have, however, demonstrated that as the RTLTF model complexity increases the antioxidant depletion

rates decrease, but interestingly the rates remain within the same order of magnitude and the reactivity hierarchy is consistent irrespective of the model used [268, 269]. The fact that even single antioxidant solutions exhibit depletion rates that are within the same order of magnitude as bronchoalveolar lavage samples can be attributed to the high reactivity of the antioxidants towards the gaseous pollutants. There are no studies comparing the antioxidant reactivity of different RTL models exposed to PM under the same conditions, though a similar pattern can be expected. In the current study the same synRTL was used for all experimental series in order to facilitate comparisons, and while the model is very simplified, physiological temperature and pH were chosen, in contrast to typical chemical kinetic studies.

A further potential limitation of this study is the exposure length; 2 hours were chosen following pilot work indicating that evaporation was a confounding factor at longer exposures. The rate applications were based on the 2 hour exposures which presented a consistent loss with time, though in real life more complex kinetics could be expected in prolonged exposures, especially if there was limited antioxidant replenishment. In addition, the *in vitro* set up used in this project was not sophisticated enough to take into account the impact of different breathing patterns such as oral versus nasal inhalation, and changes in tidal volume. These parameters have a similar impact on the O<sub>3</sub> and NO<sub>2</sub> absorption, and for these gaseous pollutants an identical set up was used, thus the results are comparable. Arguably, the lack of a breathing pattern simulation could have influenced more the PM<sub>10</sub> findings, as this would influence the amount of PM deposited in the RTL. However, the results showed that there was no correlation between the initial weight of the PM filter and the resulting antioxidant depletion, thus the quantified results were influenced more strongly by the composition of different PM samples than by the dose *per se*. Lastly, it would have been interesting to have included in the analysis particles of smaller size, though such particles were not available. The higher toxicity of smaller particles is more evident in *in vivo* studies, as they can penetrate deeper in the lungs, in acellular models the order of significance of PM<sub>10</sub> and PM<sub>2.5</sub> varies depending on the experimental protocol, for example in similar studies measuring oxidative potential via dithiothreitol, electron spin resonance, and ascorbate, PM<sub>10</sub> resulted in higher oxidative effects [452].

## 6.5 Future Studies

This study was the first to compare NO<sub>2</sub>'s and O<sub>3</sub>'s effects in an RTLTF model under identical conditions, also PM<sub>10</sub>'s effects were considered under equivalent conditions. Future studies, could extend these findings by employing more complex *in vitro* models such as cell cultures of the respiratory tract, as those models would offer a greater range of measurable markers. In addition, collaboration with engineers could lead to the development of a more realistic *in vitro* exposure set up that takes into account breathing parameters mentioned in the previous section, for example PM could be drawn across the RTLTF in a physiologically relevant way.

Moreover, the gaseous concentrations used were much lower than the typical *in vitro* study and in fact there is no other study in this area utilizing lower concentrations. Nevertheless, exposures below 50ppb are relevant, especially for O<sub>3</sub>, and while a lower concentration range was not possible to investigate in this study for accuracy reasons mentioned in the methods section, future studies could test lower concentrations and confirm that the linear extrapolations were indeed accurate. Furthermore, this study was the first to analyse simultaneously collected PM<sub>10</sub> filters in a large number of days, however, this analysis was limited to two locations. Future studies, could analyse the oxidative potential of daily PM samples derived from several contrasting locations (such studies exist, presented in Chapter 4) but importantly they should ensure that collections are conducted concomitantly. Also, collecting multiple samples each day (e.g. every 8h) could help increase the resolution of the analyses.

Lastly, this study developed a direct exposure protocol for PM filters that excluded the need of extraction and resuspension methods that can potentially alter the physiochemical properties of PM [530]. Though, this method can be further improved as PM was aged and volatile components are likely to have been lost, and also this is possibly the reason why exposure of PM disc cuts to the gases did not alter its oxidative potential. Thus, future studies could use a stream of gas to directly expose RTLTF to the airshed of different locations under controlled conditions. This set up would provide real life PM exposures, however, further air pollutants would be present and would complicate the identification of independent effects. Theoretically though, a detailed concomitant measurement of other air pollutants and environmental conditions and the presence of enough measurements that contain variation could assist a statistical disentanglement of the pollutant's oxidative effects under realistic conditions.

## 6.6 Overall Conclusion

The airways are covered with a thin aqueous material, the RTLf and this protective screen is well invested with water soluble antioxidants which display a high intrinsic reactivity towards  $\text{NO}_2$ ,  $\text{O}_3$  and PM. This study aimed to shed light on the independent oxidative activity of  $\text{NO}_2$  by comparing its effects with those of  $\text{O}_3$  under identical experimental conditions and with  $\text{PM}_{10}$  under equivalent conditions. The initial studies quantified the relative rates of consumption of the antioxidant species by each of the aforementioned pollutants. These studies revealed the following hierarchies:  $\text{AA} \gg \text{UA} > \text{GSH}$  ( $\text{NO}_2$ ),  $\text{AA} \geq \text{UA} > \text{GSH}$  ( $\text{O}_3$ ) and  $\text{AA} > \text{GSH}$  ( $\text{PM}_{10}$ ). Thus, overall, ascorbate was the primary target and reduced glutathione the least reactive, a finding that is in accordance with previous studies. Direct comparison of the gaseous results revealed that  $\text{O}_3$  had a higher oxidative effect than  $\text{NO}_2$ , however following application of their real life concentrations  $\text{NO}_2$ 's effects were more significant at roadside and the opposite was true at background, while PM resulted in significantly higher antioxidant depletion at both sites. Therefore, these results further highlight the protective role of antioxidants in the RTLf against air pollutants. In addition, they support the possibility that  $\text{NO}_2$  could independently result in adverse respiratory effects and that PM carries the greatest oxidative potential. Concluding, the innovative aspect of this study was the use of same controlled conditions and the consideration of the pollutant's real life concentrations, future studies in more complex models could further disentangle the independent actions of the aforementioned pollutants.

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## Appendix A Extrapolation of Ozone Rates in Literature

In order to quantitatively compare the O<sub>3</sub> antioxidant depletion rates presented in chapter 3 with previous studies in the literature it was necessary to extrapolate these rates from graphical representations for two studies that did not provide these values. In this process, the antioxidant concentration at the different time points was visually identified and noted down, and in the case that a percentage value was depicted it was converted to absolute concentrations based on the starting concentration. The ‘overall antioxidant depletion rate’ calculations were identical to the ones described in the gaseous overall rate calculations, however, when only a single O<sub>3</sub> concentration had been tested replicating that exact calculation was impossible. In such instance, the antioxidant depletion rate was divided by the single O<sub>3</sub> concentration in order to include the ‘per ppb’ value and derive a value equivalent to the overall rate. All figures used are depicted below.

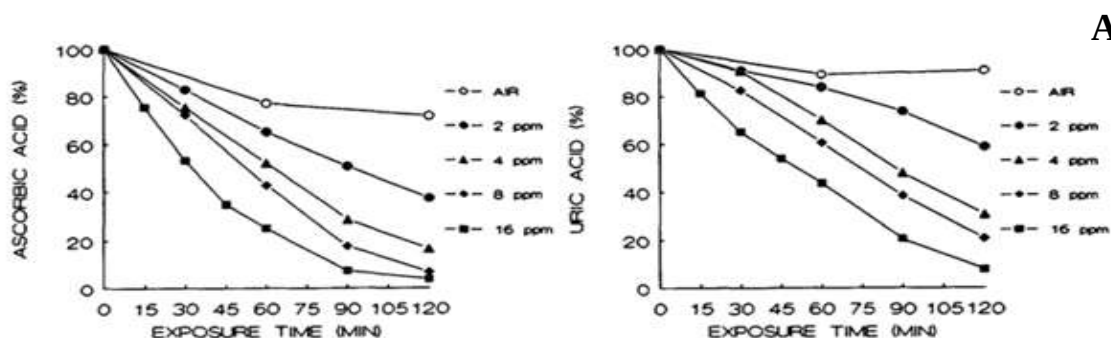


Figure A has been extracted from Van der Vliet *et al.*, 1995 (Figure 1, p.46)

For example, urate levels exposed to 2ppm O<sub>3</sub> changed as follows; from 100 to 92, 82, 78 and 60% at 0, 1800, 3600, 5400 and 7200 seconds. The starting concentration was 300μM; therefore running a regression of these concentrations against the time points resulted in  $1.57 \times 10^{-9} \text{ mol l}^{-1} \text{ s}^{-1}$ . Similarly the depletion rates for the 4ppm, 6ppm and 16ppm were  $3.03$ ,  $3.31$  and  $3.86 \times 10^{-8} \text{ mol l}^{-1} \text{ s}^{-1}$ . Therefore, the overall antioxidant depletion capacity was calculated by a regression analysis of these rates against their respective concentrations (2000, 4000, 6000 and 16000ppb), resulting in  $1.32 \times 10^{-12} \text{ mol l}^{-1} \text{ s}^{-1} \text{ ppb}^{-1}$  overall urate depletion rate.

## Appendix A (contd.)

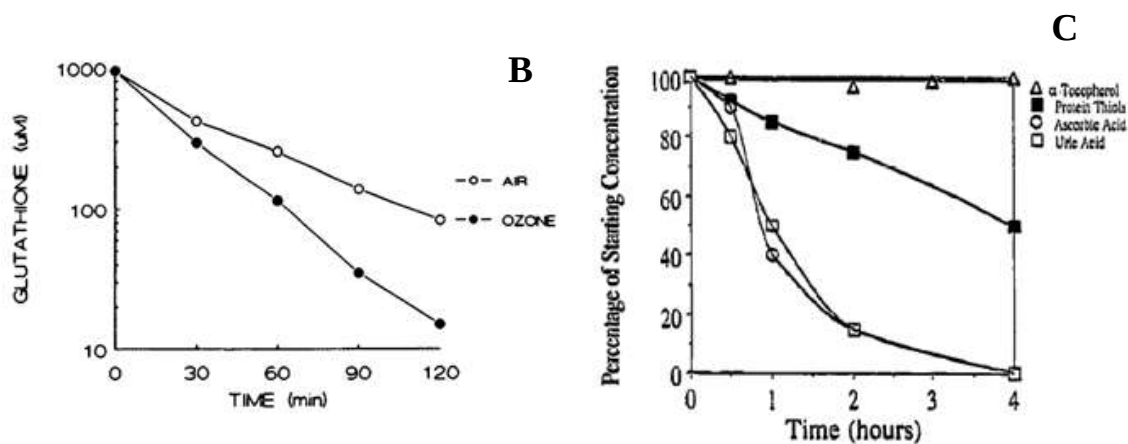


Figure B has been extracted from Van der Vliet *et al.*, 1995 (Glutathione; Figure 3, p.47) and Figure C from Cross *et al.*, 1992 (Figure 1, p. 270). These rates are based on single concentration exposures, therefore the rate calculation process is same as before but a 'per ppb' estimate was achieved by dividing the rate by the respective O<sub>3</sub> concentration. For example, glutathione in Figure B is depleted from 1000 to 600, 100, 50 and 20μM at 0, 1800, 3600, 5400 and 7200s. The antioxidant depletion rate was  $1.39 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}$ . This was then divided by the O<sub>3</sub> concentration (8000 ppb) and therefore the final value was:  $1.74 \times 10^{-11} \text{ M}^{-1} \text{ s}^{-1} \text{ ppb}^{-1}$ .

### **Appendix B Normality Test of PM Antioxidant Consumption Effects**

Normality tests (Shapiro-Wilk) were carried out in order to calculate if the antioxidant depletion rates for the various PM samples could be well modelled by a normal distribution.

|                                   |                     |           |        |
|-----------------------------------|---------------------|-----------|--------|
| <b>AH<sub>2</sub> Roadside:</b>   | W-Statistic = 0.911 | P = 0.002 | Failed |
| <b>GSH Roadside:</b>              | W-Statistic = 0.808 | P < 0.001 | Failed |
| <b>AH<sub>2</sub> Background:</b> | W-Statistic = 0.906 | P = 0.002 | Failed |
| <b>GSH Background:</b>            | W-Statistic = 0.929 | P = 0.009 | Failed |

The results of the normality test revealed that non parametric tests were appropriate to use for all the PM related comparisons. Please note that urate was not included in the PM calculations as it was not depleted.

## Appendix C An Indirect Analysis of the PM Filter Metal Content

cluster analysis grouped wind direction into 7 potential clusters while also considering wind speed.

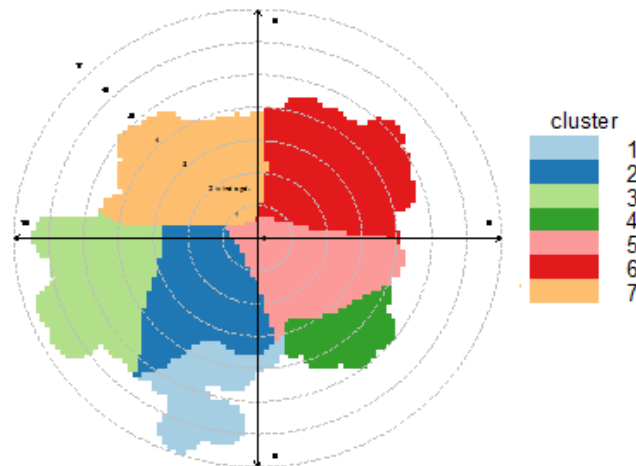


**Figure 48: Cluster Analysis of Daily Wind Data**

The example above represents the wind clusters present at the Marylebone road site, the same cluster categories but with different values were used at the North Kensington site. Different clusters are presented by a different colour, the wind speed and direction can also be identified on the diagram. Cluster 1 (south; potentially sea salt source), cluster 2 (south west; roadside sector), cluster 3 (south west; uncertain), cluster 4 (south east; possibly resuspended PM), cluster 6 (north east; long range PM), cluster 7 (north west, background sector).

These clusters were then expressed in percentage terms (% hours per day). *This analysis was based on Dr Barratt's work* and it was provided in an Excel worksheet for each site. Filters were analysed overall on days that there was a single significant cluster (80-100%) in order to minimize the noise of the data.

| Date   | 1  | 2   | 3  | 4  | 5    | 6  | 7   |
|--------|----|-----|----|----|------|----|-----|
| 14-Nov | 0% | 38% | 0% | 0% | 29%  | 4% | 29% |
| 15-Nov | 0% | 8%  | 0% | 0% | 83%  | 0% | 8%  |
| 16-Nov | 0% | 0%  | 0% | 0% | 100% | 0% | 0%  |

**Table 15: Typical percentage values of daily wind clusters**

Typical percentage values of daily wind clusters. This table presents typical results of the wind cluster analysis presented in the excel file, in order to minimise the noise in the PM data were derived from days that a particular wind direction was particularly prominent ( $\geq 80\%$ ). Therefore, in this example the first row would not be an appropriate day to consider, while the second and third rows contain leading clusters and thus were included in the analysis.

The table above presents an example that has been extracted from the Marylebone Road site, the wind directions varied daily. It was aimed to analyse samples that originated from days that a specific cluster was prominent (the second and third row of the table are representative of such samples while the first is not). It should be noted that the clustering analysis resulted in seven theoretical clusters, however three clusters (1,3,4) did not occur at the site and dates examined (value was 0%) as the association between metal content and wind direction was not strong enough and thus these were not included in the analysis as they were not relevant. This analysis shed light on the metal content of the samples as different wind directions were highly correlated with different metals.

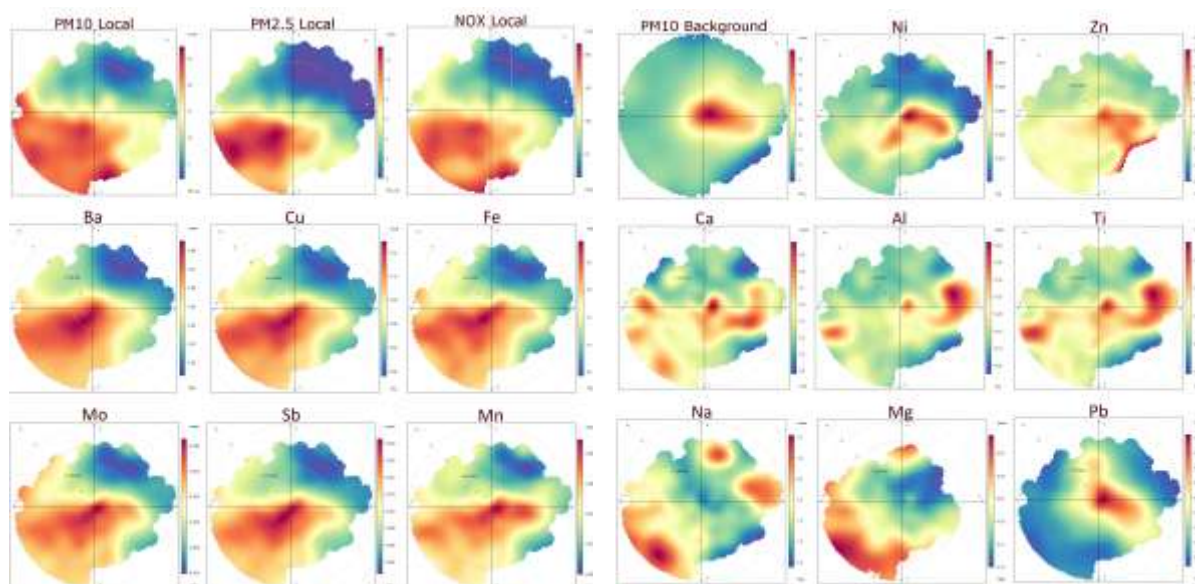


**Figure 49: PM metal content is associated with wind direction**

An analysis of the metal content of multiple PM filters from the Marylebone road and North Kensington sites revealed that the metal composition was closely associated with the wind direction and speed on a particular day. The summary of these findings is presented in this figure and different metals can be associated with different winds, while the strength of that association is also metal dependent. For example, south west winds (roadside winds) were associated with high concentrations of barium and iron. Interestingly, the overall NO<sub>x</sub> levels presented in the first row of this graph denote the very high correlation between the NO<sub>x</sub> and PM<sub>10</sub> levels (figure provided by Dr Barratt).

**Figure 49** was generated by Dr Barratt and illustrates that wind direction and speed can often be predictive of PM<sub>10</sub> metal content and *vice versa*. Therefore, while metal composition analysis was not a primary aim of this project this theoretical model was

utilized in order to investigate potential associations between antioxidant depletion and metal components.

South west (roadside) derived winds exhibited the strongest metal associations, especially barium (Ba), copper (Cu) and iron (Fe), but also a slightly weaker association can be observed for molybdenum (Mo), antimony (Sb), and manganese (Mn). The same associations were true for north east (long range) PM but were much weaker. North west (background) derived winds presented a lower metal content, weak association can be seen with zinc (Zn), titanium (Ti), aluminium (Al), and sodium (Na).

An ordinary least squares regression analysis was utilised in order to investigate whether the ascorbate and reduced glutathione depletion rates were associated with the wind directions as it was discussed in the PM chapter.

**Appendix D Correlation Between the Calculated PM OP and Levels of NO<sub>2</sub>, O<sub>3</sub>  
and PM<sub>10</sub> on Days of Filter Collection**

|                           | NO <sub>2</sub><br>back. | PM <sub>10</sub><br>road. | PM <sub>10</sub><br>back. | O <sub>3</sub><br>road. | O <sub>3</sub><br>back. | AH <sub>2</sub> PM<br>road. | AH <sub>2</sub> PM<br>back. | GSH PM<br>road. | GSH PM<br>back. |
|---------------------------|--------------------------|---------------------------|---------------------------|-------------------------|-------------------------|-----------------------------|-----------------------------|-----------------|-----------------|
| NO <sub>2</sub><br>road.  | 0.45                     | 0.93                      | 0.40                      | -0.55                   | -0.29                   | 0.28                        | 0.12                        | 0.29            | 0.09            |
|                           | 2.17E-03                 | 2.00E-07                  | 8.02E-03                  | 1.42E-04                | 5.40E-02                | 6.26E-02                    | 4.55E-01                    | 5.67E-02        | 5.40E-01        |
|                           |                          |                           |                           |                         |                         |                             |                             |                 |                 |
| NO <sub>2</sub><br>back.  |                          | 0.44                      | 0.74                      | -0.37                   | -0.76                   | 0.14                        | 0.24                        | 0.06            | 0.22            |
|                           |                          | 2.82E-03                  | 2.00E-07                  | 1.27E-02                | 2.00E-07                | 3.55E-01                    | 1.15E-01                    | 6.92E-01        | 1.44E-01        |
|                           |                          |                           |                           |                         |                         |                             |                             |                 |                 |
| PM <sub>10</sub><br>road. |                          |                           | 0.52                      | -0.52                   | -0.22                   | 0.29                        | 0.11                        | 0.27            | 0.12            |
|                           |                          |                           | 3.75E-04                  | 3.59E-04                | 1.57E-01                | 5.62E-02                    | 4.64E-01                    | 7.80E-02        | 4.22E-01        |
|                           |                          |                           |                           |                         |                         |                             |                             |                 |                 |
| PM <sub>10</sub><br>back. |                          |                           |                           | -0.13                   | -0.34                   | 0.19                        | 0.14                        | 0.07            | 0.16            |
|                           |                          |                           |                           | 4.06E-01                | 2.64E-02                | 2.08E-01                    | 3.49E-01                    | 6.70E-01        | 3.04E-01        |
|                           |                          |                           |                           |                         |                         |                             |                             |                 |                 |
| O <sub>3</sub><br>road.   |                          |                           |                           |                         | 0.68                    | -0.29                       | -0.30                       | -0.25           | -0.28           |
|                           |                          |                           |                           |                         | 2.00E-07                | 5.84E-02                    | 4.97E-02                    | 1.06E-01        | 6.37E-02        |
|                           |                          |                           |                           |                         |                         |                             |                             |                 |                 |
| O <sub>3</sub><br>back.   |                          |                           |                           |                         |                         | -0.09                       | -0.33                       | -0.05           | -0.28           |
|                           |                          |                           |                           |                         |                         | 5.78E-01                    | 2.88E-02                    | 7.50E-01        | 6.65E-02        |
|                           |                          |                           |                           |                         |                         |                             |                             |                 |                 |
| AH <sub>2</sub><br>road.  |                          |                           |                           |                         |                         |                             | 0.77                        | 0.94            | 0.78            |
|                           |                          |                           |                           |                         |                         |                             | 9.92E-10                    | 2.62E-21        | 5.63E-10        |
|                           |                          |                           |                           |                         |                         |                             |                             |                 |                 |
| AH <sub>2</sub><br>back.  |                          |                           |                           |                         |                         |                             |                             | 0.79            | 0.98            |
|                           |                          |                           |                           |                         |                         |                             |                             | 2.92E-10        | 5.87E-31        |
|                           |                          |                           |                           |                         |                         |                             |                             |                 |                 |
| GSH<br>road.              |                          |                           |                           |                         |                         |                             |                             |                 | 0.78            |
|                           |                          |                           |                           |                         |                         |                             |                             |                 | 3.47E-10        |

Correlation between ascorbate and reduced glutathione depletion rates in a synRTLF model exposed to PM with the levels of NO<sub>2</sub>, O<sub>3</sub> and PM<sub>10</sub> on the days of filter collection. The statistical dependence of the antioxidant depletion were assessed using a spearman correlation, the rank correlation coefficients (rho) and *p* values are provided below each cell.