Deliverable D11 (D2.3)

Harmonization of oxidative potential of PM monitoring for application in pilots

RI-URBANS

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By

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Deliverable D11 (D2.3): Harmonization of oxidative potential of PM monitoring for application in pilots

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Comments	This is a report from WP2, T2.2, on the evaluation of oxidative potential assays, analytical methods used, the association between air pollution and OP, and the relationship of OP with health outcomes. In addition, the report includes recommendations on the combination of OP assays to be applied and on the challenges associated with the previous knowledge of the chemical composition of PM before their application

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1. About this document

1.1 Background

WP2 of the RI-URBANS project will provide new inputs and exposure approaches with improved spatial resolution at the urban scale, which can be used for future health studies. In the last few years, there has been an increase in the development of exposure metrics in air pollution, such as the oxidative potential (OP), intended to better assess the link between air pollution and adverse health outcomes. However, as it occurs with key PM components for health effects, the major emission sources contributing to OP may be different from those which account for most of the particulate matter (PM) mass and, therefore, imply a different vision (and priorities) for air pollution control. Furthermore, these contributors are variable from one place to another, and firm knowledge about the air pollution characteristics is fundamental for a good establishment of OP assessment approaches.

Despite the increase of OP studies in the last years, there is still a need to establish whether OP is a better predictor of health impacts in human populations than PM mass or can be considered as complimenting mass-based metrics. There are several OP methodologies available based on lung antioxidant (or surrogate) depletion when in contact with PM. Several probes co-exist and exhibit different sensitivities towards pollutants, as the different lung antioxidant categories result in different outputs (and not always correlated) for different OP assays. Here, we deliver an integration of results on the parameters and sources of importance targeted by OP analysis and an assessment to implement these analyses in the pilot test demonstrations.

1.2. Scope of this deliverable

The RI-URBANS D11 (D2.3) revises the last advances and practices for evaluating the association between air pollution and oxidative potential. We integrate an overview of the measurement methods and the integration of OP in different epidemiological studies. The report includes recommendations for future studies of health impacts through the application of OP assays. This deliverable is made in reply to T2.2 that uses OP as an additional metric to assess potential toxicological effects of PM_{10} and $PM_{2.5}$, in relation to PM components and their source contributions using online and off-line techniques. PM_{10} and $PM_{2.5}$ source apportionment data from WP1 is being used to identify the predominant sources contributing to OP with inputs from available data and pilot studies providing both OP and source contribution data (T4.4). This evaluation is done for Athens, Paris, Zurich and Barcelona.

This is a public document that will be distributed to all RI-URBANS partners for their use and submitted to the European Commission as a RI-URBANS deliverable D11 (D2.3). This document can be downloaded at https://riurbans.eu/work-package-2/#deliverables-wp2.

2. Introduction

In recent years, several studies have demonstrated associations between exposure to ambient air pollution and adverse human health outcomes (Burnett et al., 2018; Hart et al., 2015; Laden et al., 2006; Lelieveld et al., 2020; Lepeule et al., 2012, WHO et al., 2017, 2021). Particulate matter (PM) comprises complex chemical mixtures resulting from natural and anthropogenic sources and atmospheric processes. Epidemiological studies have suggested that the largest effects of air pollution can be linked to long-term exposure to PM.

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Health effects attributable to PM are complex and diverse and overall, $PM_{2.5}$ is now considered to be the largest environmental contributor to adverse health effects globally (WHO, 2017). PM may act through different mechanisms such as oxidative stress and inflammation, genomic alterations, impaired nervous system function, epigenetic alterations, among others. Thus, it is not possible to cover all these effects with single monitoring air quality parameters.

PM can induce oxidative stress at the cellular level (Li et al., 2008). By definition, oxidative stress is the additional production of reactive oxygen species (ROS) relative to antioxidant defence (Shankar and Mehendale, 2014). ROS are species having an unpaired electron which can react with other molecules close to their place of production. They comprise radical and non-radical oxygen species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^\cdot$). Generally, ROS production in the cells is regulated mainly by enzymes. This regulatory process is called the antioxidant system (Alkoussa et al., 2020). The antioxidant species can play a valuable role at low doses by interacting in physiological processes and a negative role by causing damage through their ability to interact with several cellular components (Sies, 2018). As a result, many biological processes can allow free radical species to evolve in the body, which can cause harmful effects and premature ageing in cells. Thus, oxidative stress occurs when ROS overwhelm the cellular antioxidant defence system, either by an overproduction of ROS or a decrease in the cellular antioxidant capacity, once this redox balance is no longer maintained, such as after exposure to PM or stress (Cassee et al., 2013; Gao et al., 2020; Kelly and Fussell, 2017). The capacity of PM to invoke oxidative reactions or to generate ROS in a biological media is quantified by its oxidative potential (OP) (Bates et al., 2015; Cho et al., 2005; Sauvain et al., 2008; Uzu et al., 2011). Consequently, OP of PM is increasingly being studied as a relevant metric that measures health impacts instead of PM concentration. Indeed, much of the ambient particle mass does not contribute to the toxicity of PM (Park et al., 2018). In addition, OP is a relatively simple measure of PM redox activity but reflects a complex interplay of particle size, composition, and chemistry, which induce oxidative stress by free-radical generation.

Epidemiological studies have shown that PM air pollution contributes to cardiorespiratory morbidity and mortality and is recognized as an important contributor to the global disease burden (Lim et al., 2012). Moreover, numerous recent studies have confirmed that increased exposure to OP in PM (short or long-term in urban and suburban environments) can lead to increased cardiopulmonary or neurological diseases and mortality in cases of cancer, asthma, or stroke (Bates et al., 2015; Dabass et al., 2018; Delfino et al., 2005; Donaldson et al., 2001). While the biological and in-cellular mechanisms underlying these associations have not yet been fully understood, current evidence suggests that oxidative stress plays a substantial role in PM-induced health effects, including systemic/respiratory inflammation and DNA damage (Weichenthal et al., 2016).

In recent years, there has been an increased interest in measuring and developing OP studies, applying different in vivo or in vitro assays, together with aerosol characterisation, to estimate the main sources related to PM OP. A wide range of acellular chemical methods now allows to evaluate the OP of atmospheric particles, as typically, acellular assays allow faster measurement and are less labour compared to cell cultures or in vivo methods (Bates et al., 2019). These include several methods, such as the dithiothreitol assay (DTT), ascorbic acid assay (AA), 2,7-dichlorofluorescein assay (DCFH), electron paramagnetic resonance (EPR) spectroscopy, glutathione assay (GSH), Ferric-Xylenol Orange hydroperoxide assay (FOX), 9,10-bis (phenylethynyl) anthracene-nitroxide (BPEAnit) ROS assay (BPEAnit-Me), among others. These acellular assays display different sensitivities to specific particle components that may contribute to increased aerosol OP. In addition, applying these commonly used assays simultaneously allows different mechanisms of ROS generation to be assessed, allowing a complete idea of the oxidative mechanisms triggered by PM exposure.

This report reviews the current state of knowledge on the association between health endpoints and OP of ambient PM in different environments, along with the sensitivities of different OP assays to PM chemical composition,

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emission sources, and physical properties. We also briefly discuss particle-bound ROS measurements and epidemiological associations between OP and health effects. Nevertheless, our report mainly focuses on OP measurement techniques due to the growing research linking these assays to adverse health outcomes. Additionally, there is a limited number of studies evaluating the selection of the OP assay to be developed and the sensibility of OP tests to the emission sources and chemical composition of PM, so future work is needed before conclusions about the relevance and choice of the OP test to be performed and recommended.

3. Overview of OP measurement methods

3.1 Oxidative Potential and Particle-bond ROS

Oxidative potential relies on the idea that exposure to PM can induce oxidative stress. Thus, OP is used as a health-based indicator to characterise exposure reflecting the oxidative capacity of PM in the lung, the main route of PM entrance, over time (Massimi et al., 2020). By definition, the OP measures the ability of PM to deplete lung antioxidant molecules in synthetic airway fluids (Crobeddu et al., 2017; Goldsmith et al., 1997; Landreman et al., 2008; Moufarrej et al., 2020). Therefore, it has been increasingly used as an exposure metric to link atmospheric particles and specific health endpoints.

There are many OP assays, and protocols can vary according to the probe within each assay. Therefore, applying different OP protocols, laboratory conditions and PM concentrations will conclude in a different result for a given OP assay, making results across studies difficult to compare. Also, these differences include the selection of solvents for the PM extraction (buffer; ultrapure water; methanol), the type of extraction (vortex, ultrasound…), filter types for PM sampling, incubation times and temperature and the use of metal chelators to purify some reactants. Recently, some studies have adopted simulated lining/lung fluids (SLF) to mimic physiological conditions as much as possible (Charrier and Anastasio, 2012; Godri et al., 2011). Diverse types of SLF co-exist with complex compositions (Gamble's solution with a surfactant, artificial lysosomal fluid (ALF)) or more simple compositions with low molecular weight antioxidants such as ascorbate (Asc), glutathione (GSH), uric acid (UA), and citrate (Cit) buffered at pH 7.4 with an ionic strength that mimics lung lining fluid (Gonzalez et al., 2017). Evaluating these discrepancies between the OP methods is fundamental to optimising the assays and better understanding the association between PM and health outcomes (Bates et al., 2019). To date, there is currently no standardized methodology for measuring oxidative potential. Several methods coexist, and each brings a different vision of the OP of aerosols.

In the next sections, we describe the different and most common measurement techniques applied for OP analysis.

3.2 Offline measurements

Acellular OP assays are commonly applied as they are less time-consuming and less labour-intensive than in vitro assays. These assays simulate the PM contact into the lung using chemical probes and measure the OP of PM either through direct ROS measurements (e.g., electron spin resonance (ESR) spectrometry by the generation of OH radical in the presence of H_2O_2 or OH assay measuring •OH production) or indirectly (e.g., the depletion of a given antioxidant, such as oxidation of dithiothreitol (DTT), ascorbic acid (AA), reduced glutathione (GSH)). Each assay represents a valid assessment of a fraction of the PM toxicity, which could help to elucidate complex mechanisms of PM ROS generation.

The Ascorbic acid assay (OP^{AA}) measures the ability of PM to deplete a lung antioxidant (Ascorbic acid), which is proportional to the ROS generation rate. This test was initially developed to measure trace metal oxidation. Due to its simplicity of measuring the OP of PM by a single antioxidant, it has been widely used in the last few years. OP^{AA} is quantified by incubating concentrations of AA with PM samples in a UV/VIS-spectrophotometer. The optical

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density at 265 nm is unique to AA, and since ascorbate depletes exponentially, there is a linear relationship between the log-transformed optical density and time. Therefore, the percentage of AA depleted after an incubation period and the rate of AA depletion are metrics used to represent the PM oxidative potential. (Fang et al., 2016; Pietrogrande et al., 2019).

The Dithiothreitol assay (OP^{DTT}) measures the ability of PM to deplete a cellular reductant (dithiothreitol), which is proportional to the ROS generation rate. It was designed to simulate the in vivo generation of superoxide radicals, wherein DTT was used as a surrogate of the cellular reducing agents (NADH or NADPH). The DTT assay was initially proposed to measure quinone-catalysed oxidation. It's probably the most widely used OP assay for being the oldest and quite easy to implement. The assay involves the incubation of PM samples with DTT, and the remaining DTT over time is estimated by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction of DTT and DTNB forms 2-nitro-5-thiobenzoic acid (TNB), which can be quantified using a UV/VIS spectrophotometer. When DTT is in excess, the consumption rate of DTT is proportional to the concentration of the redox-active species and thus is used as a measure of PM OP (OP^{DTT}) (Calas et al., 2018; Fang et al., 2016; Simonetti et al., 2018).

The glutathione assay (OP^{GSH}) relies on the depletion of the GSH, a lung antioxidant by PM. It has been less frequently used than other assays (Crobeddu et al., 2017; Gao et al., 2020; Shahpoury et al., 2019; Weichenthal et al., 2019, 2016), possibly due to the complexity of methods for separation and analysis of GSH and its oxidation product glutathione disulfide (GSSG) (Shahpoury et al., 2022). However, in a recent study, a new method has been developed, including thiol antioxidants and their oxidation products, including cysteine (CSH) and cystine (CSSC) which were not considered in the past (Shahpoury et al., 2019).

The ROS present within and on PM can be also assessed using various fluorescence techniques adapted from intra-cellular ROS measurement techniques. These particle-bound ROS methods suspend particles in a reagent and measure the spectra of specific oxidation by-products. Among them, the oldest and the most widely used is the OP^{DCFH} method, based on the reaction of the ROS in PM with DCFH, a non-fluorescent reagent, with horseradish peroxidase. This reaction produces a fluorescent compound, 2,7-dichlorofluorescein (DCF). Because not all oxidants can react with DCFH and also because it is sensitive to some reactive nitrogen species, the fluorescence intensity generated from the reaction did not necessarily capture the total oxidative capacity and is less used in the last years (Massimi et al., 2020; Perrone et al., 2016). Although it is of interest when OP is assessed in parallel to in-vitro toxicological tests since the same probe can be used with lung epithelial cells. Similar to the OP^{DCFH} assay, other methods were developed to measure the continuous generation of ROS over time, using electron spin resonance (ESR) (Bates et al., 2019). In particular, ESR measures PM-induced hydroxyl radical (•OH) formation by detecting the electron paramagnetic resonance signals, in the presence of hydrogen peroxide, via Fenton-type reactions and their trapping by the nitrone DMPO (Calas et al., 2018; Hellack et al., 2014; Shi, 2003). The OP^{ESR} measures the particle-bound ROS formed during the dissociation of PM in ultrapure water, and in general, antioxidants are not included since they could act as radical scavengers and negatively affect the EPR signal (Shahpoury et al., 2022). Similarly, the OP^{OH} assay measures the hydroxyl radicals (•OH) induced by PM measured after the reaction with terephthalic Acid (TPA) by measuring the adduct formed, namely hydroxyterephthalic acid (hTPA). Fluorescence emission of hTPA is obtained after the addition of solutions of TPA in phosphate buffer and ascorbate (Asc) (Dominutti et al., 2023). The •OH production catalysed by PM is only significant in the presence of a reducing agent that helps to recycle PM components (chiefly transition metals in the case of Asc) to their reduced form, allowing a small amount of PM to generate a measurable amount of ROS.

The Ferrous-Orange Xylenol (FOX) assay is based on the measurement of the oxidation rate of Fe^{2+} to Fe^{3+} in the presence of a specific ligand (orange xylenol) and sorbitol (Laulagnet et al., 2015). When oxidants are introduced into the reactive mixture, the produced Fe^{3+} combines with orange xylenol, increasing the absorbance at 580 nm (Dominutti et al., 2023). In addition, the presence of sorbitol in the FOX mixture acts as a chemical amplifier through

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the iterative conversions of OH into H_2O_2 via radical reactions (Gay and Gebicki, 2000). The FOX assay is a recognised method for determining oxidants (hydroperoxides) in fats and oil material (Bou et al., 2008).

All these offline OP assays generally involve filters from air samplers. It's of importance to note that both the air sampling and the preparation of the samples before performing OP assays will influence the results. Its assumed that filters must be kept cold after collection like for any common chemistry analysis: at least at 4°C before a rapid analysis or at -20°C if the OP analysis is delayed. This request is to prevent from filter sampling of PM that may lose reactive species before analysis (Sameenoi et al., 2012, Bates et al., 2019). Then the filters may be prepared prior analysis on different ways. It has been shown that the choice of solvent extraction will affect the OP results as well as the time and the type of extraction. As solvents, water, methanol, phosphate buffer and lining fluids (Gamble solution and Artificial lysomal fluid) are the most used. Methanol is more efficient than water (Yang et al., 2014, Verma et al., 2012) but reflects poorly lung physiological conditions. Water and simulated lining fluids (SLF) extraction depicts OP results in the same order of magnitude but filters extracted in SLF show generally a decrease in OP due to ligand complexations in the lung fluid with an unpredictable coefficient (Calas et al., 2018, Cigánková et al., 2021)). Water extraction may overestimate the real pulmonary bioaccessibility of PM compounds in the lungs but research is still ongoing to assess whether SLF extraction is better use prior OP measurements. Finally it has also be shown that the type of extraction may lead to different OP results whether a simple incubation or an extraction of different time and different instrument (ultrasounds, vortex or gentle agitation) may lead to discrepancies (Yang et al., 2014; Calas et al., 2018).

The final OP of PM after measurement can be either normalised by the volume of sampled air to assess human exposure (OP_v) or normalised by the particulate mass concentration (OP_m) or by % of antioxidant loss over the reaction to represent the intrinsic property of particles linked to PM composition and sources.

3.3 Online measurements

The application of acellular offline OP methods has the advantage of providing a robust, high-throughput screening and low-cost approach to the PM OP by different simultaneous OP assays for the same PM sample. However, these techniques also have some limitations regarding the collection of fast OP and underestimation of ROS concentrations due to their short lifetime and achieving long-term monitoring time series. In the last years, a number of automated prototypes have been developed to enable real-time OP analysis from online measurements (Campbell et al., 2019; Gao et al., 2017; Huang et al., 2016; Venkatachari and Hopke, 2008; Wang et al., 2011; Wragg et al., 2016). These instruments collect the total aerosol (or only PM) into a water collection device, e.g., particle-into-liquid sampler (PILS), liquid spot sampler (LSS), or mist chamber (MC) to an analytical system (Puthussery et al., 2018). The offline OP assays and chemical probes frequently used are applied and adapted for online approaches. To our knowledge, no commercial device is available to date.

Venkatachari and collaborators developed one of the first online devices based on the reaction of DCFH with HRP to form the fluorescent product DCF that is subsequently detected by fluorescence spectroscopy (Venkatachari and Hopke, 2008). Similarly, Wang and colleagues developed an effective automated particle-bound ROS sampling analysis system. Their approach employs the DCFH fluorescence method of collecting $PM_{2.5}$ into an aqueous slurry containing the DCFH solution by using a sharp-cut cyclone and a particle-into-liquid-sampler (PILS) (Wang et al., 2011). To obtain the total ROS (gas + particles), King and Weber included a mist chamber scrubber, alternating with gas-phase ROS by using a filter, and determining particle-phase ROS by the difference between total and gas ROS (King and Weber, 2013).

Over the years, some advances were added to the previous prototypes based on DCFH, mainly related to the oxidation products of organic compounds. Fuller et al., (2014) were able to prevent the ozonolysis of oleic acid by improving the extraction of PM, and later, the same group demonstrated the instrument's sensitivity to a range of

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organic peroxides and SOA generated from the ozonolysis of both α-pinene and limonene (Wragg et al., 2016). More recently, a new online and offline device improved the separation of DCFH and peroxidase working solutions, and they were able to avoid ultrasonic filter extraction for offline analysis (Zhou et al., 2018).

Other chemical probes were also applied in the development of online devices. Relying on their previous devices, the group of Kalberer also accommodated a new ascorbic acid-based chemical assay to quantify PM OP (Campbell et al., 2019). The OP of a given sample is measured based on the quantification of the oxidised form of ascorbic acid (AA), the dehydroascorbic acid (DHA). Under acidic conditions, DHA reacts with o-phenylenediamine (OPDA) to form the product 3-(1,2-dihydroxyethyl)-fluoro[3,4-b]quinoxaline-1-one (DFQ), a highly fluorescent compound which can be detected by fluorescence spectroscopy (Campbell et al., 2019).

The OP^{DTT} assay was also adopted for OP online purposes by different research groups and improved over the years (Eiguren-Fernandez et al., 2017; Fang et al., 2015; Gao et al., 2017; Koehler et al., 2014; Puthussery et al., 2018). Koelher et al., introduced a device based on the DTT assay that couples a particle-into-liquid-sampler with microfluidic-electrochemical detection (Koehler et al., 2014). A semi-automated OP^{DTT} device was developed by Fang et al., (2015) and adapted to both water-soluble and insoluble particle components by Gao et al., (2017), and turned into an online device by Puthussery et al., (2018) by coupling an automated analytical system for DTT activity determination by collecting $PM_{2.5}$ suspension in water (Fang et al., 2015; Gao et al., 2017; Puthussery et al., 2018). Another online OP^{DTT} monitoring system was developed by gathering a liquid spot sampler (LSS), a three-stage laminar-flow water condensation device for particle collection, and a classical detection of DTT activity via light absorbance spectroscopy (Eiguren-Fernandez et al., 2017).

Some online instruments also appear with combined OP assays on parallel lines assessing both OP^{DTT} and OP^{AA} (Uzu et al., 2022) or a multi OP assays into a semi-automated instrument (Yu et al., 2020)

The Particle Into Nitroxide Quencher (PINQ) instrument relies on the 9,10-bis (phenylethynyl) anthracene-nitroxide (BPEAnit) ROS assay (Brown et al., 2019). The purpose-built aerosol collection device continuously samples PM, regardless of size or chemistry, directly into a liquid sample, which is later transferred into a specially designed flow-through fluorimeter for ROS quantification (Brown et al., 2019). The device presents a high time resolution (1 min) with low detection limits and high stability of the BPEAnit probe.

Beyond chemical probes, several properties of ROS and combustion aerosols make the quantification for online sampling difficult. PM-bound ROS react readily with the atmosphere (Fuller et al., 2014). Therefore, methodologies involving either long periods of collection or delays between collection and measurement risk to underestimate the concentration of PM-bound ROS in PM samples, up to 60% (Zhou et al., 2018). Finally, it has to be noted that despite considerable effort to develop instruments to assess OP online, their achievement remains challenging. Due to the large limit of detection of such devices, their use in low-medium polluted environments is still limited and such research prototypes need some care that is poorly compatible with their routine deployment.

4. The link between PM characteristics and OP

Oxidative potential can be envisaged as a comprehensive metric of PM properties (size, chemical composition, surface area, sources ...) linked to the particles' toxicity through oxidative stress (Calas et al., 2018). Therefore, OP could help to depict those particle characteristics responsible for some observed health effects. In the next sections, we present the main PM characteristics being evaluated by the literature and the evidence showing the link between PM and OP.

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4.1 PM Chemistry

There is an increasing number of studies evaluating the relationship between the chemical composition of PM and OP. Specific OP responses rely on the chemical composition associated with the emission sources of the PM measured. Recent studies concern volume-normalised OP since it reflects the exposure to redox-active PM after inhalation, representing the relationship between the intrinsic PM OP and the total PM concentration.

PM is a complex mixture that encompasses many different compounds. PM includes some trace metals and organic compounds that are known to be able to contribute to free radical reactions, resulting in redox-active species contributing to the OP response. Several transition metals were found in PM composition, such as Al, As, Ba, Ca, Cd, Co, Cr, Cs, Cu, Fe, K, Mn, Mo, Ni, Pb, Sb, Sn, V and Zn. If the soluble forms of those metals are known to participate in redox reactions and to mainly affect OP (Bates et al., 2019), insoluble components also contribute (Baumann et al., 2022; Calas et al., 2017). These metals predominately originate from anthropogenic sources, including vehicular mechanical engine wear, tail-pipe emissions, brake wear, coal-fired power plants, residual oil combustion processes, and metal refineries (Pant and Harrison, 2013; Uzu et al., 2011). Among the different metals, copper (Cu), iron (Fe) and manganese (Mn) normally had the largest contribution to the OP of PM collected from the urban sites (Bates et al., 2015; Charrier et al., 2015; Fang et al., 2016; Fujitani et al., 2017; Shirmohammadi et al., 2017). Transition metals have been positively correlated with •OH and H_2O_2 production measured by the OP^{ESR} assay. Specifically, iron (Fe(II) and Fe(III)) and soluble copper are critical to •OH generation and can have synergistic effects with each other to produce •OH in SLF (Bates et al., 2019 and references therein). Metals also have an effect on other OP assays, as already discussed in several studies performing OP^{AA}, OP^{DTT} and OP^{GSH} (Calas et al., 2018). OP^{DTT} responds to metals, with significantly impacts of those samples enriched with soluble copper and manganese (Charrier and Anastasio, 2012). However, this assay is not affected by iron like other OP assays suggesting that OP^{DTT} may not fully capture Fenton chemistry or synergistic effects on ROS, specifically regarding •OH generation (Xiong et al., 2017). In the case of OP^{AA}, several studies have demonstrated positive correlations between this assay and some transition metals, such as Fe, Cu, Cr, Mn, Co, and Ni, including total and soluble fractions (Calas et al., 2018; Janssen et al., 2015; Pant et al., 2015; Visentin et al., 2016; Yang et al., 2014).

In recent years, few studies have also shown the sensitivity of different OP assays towards organic components of PM. Among them, organic carbon (OC), quinones, polycyclic aromatic hydrocarbon compounds (PAHs) and humic-like substances (HULIS) are among the most studied organic species (Calas et al., 2017; Kramer et al., 2016; Lin and Yu, 2011; Perrone et al., 2016; Shirmohammadi et al., 2017; Verma et al., 2015).

In addition to OP reactivity towards PM compounds, the dependence of OP to ageing and atmospheric processing has to be considered. This may lead to enhancing or suppressing OP signal in a non-linear way (Daellenbach et al., 2020; Liu and Chan, 2022; Shahpoury et al., 2021; Wong et al., 2019). Finally, an emerging evidence concerns the surface-mediated dependence of oxidative potential of components of fine particulate matter. This surface-mediated dependence may concern the PM substance properties themselves as seen in insoluble metallic particles and bioaerosols (Samake et al., 2017; Shahpoury et al., 2021; Uzu et al., 2011), or the lung lining fluid interface and uptake pathways due to the effect of ligand and complexation (Baumann et al., 2022; Calas et al., 2017) that may also affect OP in a non-linear way.

4.2 Emission sources

Over the last 10 years, very large improvements in source apportionment methods were developed to determine and quantify the main sources and processes influencing the PM measured at a given location. In addition, some other studies have focused on linking OP to specific emission sources (Bates et al., 2019 and references therein) or establishing predictive regression models based on the concentrations of PM constituents (Weber et al., 2018; Weichenthal et al., 2016). Many studies have recently developed source apportionment techniques, such as principal component analysis (PCA), positive matrix factorisation (PMF), random forest analysis and multilayer

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perceptron neural models (Borlaza et al., 2021; Daellenbach et al., 2020; Grange et al., 2022; in 't Veld et al., 2022; Shen et al., 2022; Srivastava et al., 2018; Weber et al., 2021, 2019). Particularly, the PMF model has had several developments and benefited from a series of standardised procedures and quality assurance controls (Belis et al., 2019).

Due to the seasonality of emissions sources (e.g. biomass burning in winter), some studies report strong differences between seasons related to the main emission sources driven OP, whereas others do not (Bates et al., 2015; Borlaza et al., 2021; Calas et al., 2019; Cesari et al., 2019; Dominutti et al., 2023; Fang et al., 2016; Paraskevopoulou et al., 2019; Perrone et al., 2016; Pietrogrande et al., 2018; Zhou et al., 2019). In general, those studies performed in regions with strong seasonal changes (i.e., mountainous areas) presented higher dissimilar source contributions than those located in temperate areas (i.e., coastal areas). Additionally, several studies have already shown that different sources of PM have different sensitivities to OP tests (Bates et al., 2015; Cesari et al., 2019; Daellenbach et al., 2020; Dominutti et al., 2023; Fang et al., 2016; Paraskevopoulou et al., 2019; Verma et al., 2014; Weber et al., 2018; Zhou et al., 2019). In particular, sources with high concentrations of transition metals, such as road traffic, road dust and industrial sources, seem to have a higher intrinsic potential than other PM sources for a given OP. Nevertheless, there is still a limited number of studies integrating long-term PM samples and only sometimes consider seasonal variability. Consequently, they may not encompass a comprehensive variability of emission sources for a given site, omitting some important features on the source-related OP. This limitation is even higher in terms of spatial distribution. Most of the studies were developed in northern regions, where the discrepancies related to emission sources are less significant.

Finally, attaining a general model linking OP with site-specific PM constituents or emission sources is challenging because OP depends not only on the concentrations of redox-active species but also on factors that influence the diversity of redox reactions and the formation of ROS in the human body (Shahpoury et al., 2022). Thus, the selection of the OP assays to obtain a wide range of OP determinants will depend on previous knowledge about the PM characteristics for a given location. Combining two or three OP assays with complementary sensitivities will provide more exhaustive information about the PM characteristics underlying the OP effects on human health.

4.3 Physical properties of PM

Even though it is clear that PM chemical composition plays a role in OP, the effects that PM will have on the environment and respiratory system also depend on the PM size (Kelly and Fussell, 2017). In ambient air, this size effect is a direct consequence of the fact that emissions sources display some specific range of size distributions. For instance, combustion sources are mainly emitted in the fine mode (PM_1) whereas non-exhaust emissions from brake wear are more emitted in the coarse mode ($PM_{2.5\text{-}10}$) (Harrison et al., 2020). This leads to more or less reactive fractions of PM when assessing OP from different site typologies, making OP, PM size-fraction dependent.

That is why it has been reported that mass-normalised OP (OP_m) and volume-normalised OP (OP_V) are dependent on the PM size fraction. But Hu and co-workers have found that OP of quasi-ultrafine particles ($PM_{0.25}$) showed the strongest OP_m when compared to accumulation mode ($PM_{2.5–0.25}$) and coarse mode ($PM_{10–2.5}$) particles in Los Angeles, USA (Hu et al.,2008). Similar findings were also reported in other cities (including Beirut in Lebanon, Shanghai in China, Bern in Switzerland) and also in metal-rich industrial emissions (Daher et al., 2014; Grange et al., 2022; Lyu et al., 2018; Uzu et al., 2011). Contrarily, in't Veld and co-workers did not find a significant dependence of OP_m upon particle size (PM_{10}, $PM_{2.5}$ and PM_1) in Barcelona, Spain (in 't Veld et al., 2022). On the other hand, some previous studies showed that OP_m related to particle size is commonly smaller than one of OP_v for the same PM sample. For example, volume-normalised OP (OP_v) showed the strongest contribution at 0.5–2.5 µm in Shanghai (Lyu et al., 2018), Atlanta, USA (Fang et al., 2017b), and Italian cities (Massimi et al., 2020; Simonetti et al., 2018). This pattern was not observed in Barcelona, where the OP_v was significantly higher for PM_{10} than for $PM_{2.5}$ and PM_1, which were both in the same range (in 't Veld et al., 2022). Volume-normalized OP, more relevant to exposure than

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OP_m, illustrates consistent peaks near 2 µm across studies (Bates et al. 2019 and references therein). For example, Daher and colleagues found that OP_m presented higher values at 0.25–2.5 µm, while the OP_v was stronger at 0.25–10 µm in Beirut (Daher et al., 2014).

The contribution of PM size on the OP also varies with different acellular assays. Some studies compared the OP_v of the same PM sample using different OP assays (i.e., DTT, DCFH, AA, GSH). For example, in a study in London, OP_v^{AA} disclosed no significant differences related to particle size, but OP_v^{GSH} showed a slight increase with increasing particle size (Godri et al., 2011). In an Italian study, authors found that the OP_v^{DTT} presented higher levels for the smallest particles while the OP_v^{AA} at the largest and the OP_v^{DCFH} at the middle particles size range (Massimi et al., 2020; Simonetti et al., 2018). These discrepancies can be partly attributed to the chemical sensitivities of each OP assay, which are also related to specific particle sizes and maybe showing different mechanisms or routes for oxidative stress. For instance, OP^{DDT} shows a higher sensitivity to organic compounds, which tend to be more present in the fine mode (Rogula-Kozłowska, 2016). The results in Barcelona have shown that higher OP levels for PM_{10} were driven by anthropogenic sources related to combustion (OP^{DTT}) and industrial and road dust (OP^{AA}) sources with high contribution of metals and organics (Cu, Fe, OC, EC, Sb and Sn) (in 't Veld et al., 2022). Finally, the surface area could be a driving factor for in vivo oxidative stress (Kelly and Fussell, 2012). Surface area and size are two closely related characteristics of PM, since an increase in surface area to volume ratio is observed for smaller particles (He and Zhang, 2022). Nevertheless, the induction of ROS by the interactions between PM surface area and biological systems cannot be captured by the OP acellular assays, and more in vivo developments are needed to better understand its effect (He and Zhang, 2022).

Globally, the PM chemical composition, which varies by emission source and atmospheric photochemical processes, seems to drive the size distribution of OP, which varies by assay type (Bates et al. 2019). Consequently, PM size, chemical composition, and processing may result in ubiquitous and specific health effects.

5. OP in epidemiological studies

It was beyond the scope of this deliverable to provide a systematic review of all epidemiological studies that have evaluated OP as an alternative exposure metric in association with health. We therefore do not draw strong conclusions about the value of OP relative to other metrics. We have however identified a sizable number of epidemiological studies that evaluated OP, allowing us to assess the potential value of OP in future routine monitoring and subsequent health studies.

Numerous epidemiological studies have reported associations between air pollution, especially PM, and various health outcomes (mortality, morbidity, and respiratory and cardiovascular diseases). Lelieveld et al. (2020) highlighted that air pollution reduces 2 years of the life expectancy of Europeans. The latest EEA estimates that at a minimum 238,000 people died prematurely in the EU in 2020 due to exposure to PM2.5 pollution. Moreover, a recent study by Lelieveld et al. (2019) disclosed that the attributable excess mortality rate is about 8.8 million per year, which is about twice the global premature mortality estimated before at 4.5 million people a year. According to WHO, the most vulnerable to air pollution are the population with pre-existing respiratory or cardiovascular europe-2022.diseases, as well as elderly people and children.

In recent years, an increase in epidemiological studies has shown the associations between PM and restricted foetal growth (Maisonet et al., 2019; Li et al., 2017). A few have assessed that oxidative stress can lead to tissue damage which can potentially lead to a rupture causing leakage in the maternal circulation (Tjoa et al., 2006) and placental disease (Huang et al., 2017; Rahmalia et al., 2012) contributing to foetal growth restriction (Duhig et al., 2016; Ozsurekci and Aykac, 2016; Rashid et al., 2018). Additionally, Johnson et al. (2021) revealed that maternal exposure

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to fine and ultrafine PM produces adverse birth outcomes and impacts the child's respiratory and immune systems, as well as brain development.

In the same way, several studies suggested that long-term exposure to high levels of PM is directly linked to cardiovascular issues, with an increased mortality rate in highly exposed individuals (Chen and Hoek, 2020; Wang et al., 2016). Furthermore, epidemiological studies indicate that long-term exposure to PM increases the chance of strokes, vascular hypertension, and other cardiovascular pathologies (WHO, 2013 and 2021, Lelieveld and Münzel, 2020; Manisalidis et al., 2020). As for the respiratory system, short-term exposure to PM is closely correlated to cough, shortness of breath, asthma, and high hospitalisation rates. Long-term exposure to air pollution can also be related to chronic asthma and pulmonary insufficiency and might even promote lung cancer (Manisalidis et al., 2020). Chen and Hoek (2020) review the association between long-term exposure to $PM_{2.5}$ and mortality using cohort and case-control studies in primarily Europe and the United States. Clear evidence of association was highlighted between $PM_{2.5}$ and PM10 with mortality from all causes, cardiovascular and respiratory diseases and lung cancer. SAmong all PM constituents, metals and organic compounds are the two most important contributors to PM OP (He and Zhang, 2022). For example, Ng and collaborators found that among various $PM_{2.5}$ constituents, only organic carbon was adversely associated with lung function in asthmatics (Ng et al., 2019). Similarly, it has been found that organic carbon was the only PM component significantly associated with lung function in children with asthma (He et al., 2021).

Recently, OP of PM and their related sources have been linked to certain health outcomes. Nevertheless, clear evidence of OP as a good proxy of specific health outcomes is still needed, and there is so far no consensus toward a standardised method to measure the PM's OP. In the last years, increased evidence has associated OP^{DTT} and OP^{AA} assays with health endpoints (Bates et al., 2015; Borlaza et al., 2022; Canova et al., 2014; Fang et al., 2017b; Janssen et al., 2015; Marsal et al., 2023; Strak et al., 2017; Weichenthal et al., 2016; Yang et al., 2016). We distinguish studies of short-term exposure (using temporal contrast in exposure, typical on a daily basis) and long-term exposure (using spatial variability).

Several studies have evaluated the associations between OP assays and health endpoints. Some of them used OP observations and modelling approaches, such as land-use regression models (LUR), to evaluate the OP's spatial variability when measurements were unavailable (Bates et al., 2015; Fang et al., 2017a; Gulliver et al., 2018; Strak et al., 2017). In most studies using the **acid ascorbic probe, OP^{AA}** depicted no association with adverse health endpoints, including early-life outcomes, respiratory and cardiovascular mortality, cardiorespiratory emergencies, and lung cancer mortality (Borlaza et al., 2022; Fang et al., 2017b; Maikawa et al., 2016; Marsal et al., 2023; Weichenthal et al., 2016). But some positive associations were found for OP^{AA} and systemic inflammatory biomarkers, as well as for OP^{ESR}, in short-term exposure studies (Janssen et al., 2015; Liu et al., 2018). These results so far suggest that OP^{AA} provides limited information on the link between OP and adverse health effects, but the number of studies is still too limited to exclude its predictive capacity towards health. A study in 2022 found strong associations between outdoor fine particles, oxidising gases and respiratory hospitalisations in children and mortality when metals, sulphur and for OP (mainly for OP^{GSH} and also for specific cases for OP^{AA}) were high (Korsiak et al., 2022; Toyib et al., 2022).

On the other hand, two thiol-base probes have shown more positive associations with health. OP^{DTT} has been linked with various acute cardiac (e.g. myocardial infarction) and respiratory endpoints in several studies (Abrams et al., 2017; Bates et al., 2015; Delfino et al., 2005; Fang et al., 2017b, 2016; He et al., 2021; Janssen et al., 2015; Weichenthal et al., 2016; Yang et al., 2016). A Canadian cohort (193 000 participants) showed some significant association for OP^{GSH} and cause-specific mortality as well as lung cancer (Weichenthal et al., 2016). Recently, as a result of a long-term cohort study, Marsal and co-workers have shown a consistent association between personal prenatal OP_v^{DTT} and several early-life lung function parameters related to lung growth restriction (Marsal et al.,

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2023). Such findings are aligned with pioneering studies using the PIAMA cohort to show that respiratory health was more strongly associated with OP_v^{DTT} than with PM2.5 mass (Yang et al., 2016). Similarly, two cohort studies found some positive associations between personal OP exposure to $PM_{2.5}$ and foetal growth restrictions, specifically decreased weight and height at birth for OP^{DTT} and OP^{GSH} (Borlaza et al., 2022; Lavigne et al., 2018). Overall, a key point of most of the previously reported studies is that OP was more predictive than other components including PM mass.

Finally, one study with the emerging direct radical •OH measurement in lining fluid found some positive associations for OP^{OH} and asthma, cardiovascular disease and low-birth weight (also seen for OP^{DTT}) in Los-Angeles (Shen et al., 2022). As for OP^{ESR}, further studies involving OP^{OH} are needed to assess some statistics on such methodologies that are promising. Note that OP^{OH} should be easier to deploy because it can be assessed in a classic laboratory whereas OP^{ESR} requires some scarce specific device that may prevent routine measurements.

These studies increase evidence about the relevance of OP of $PM_{2.5}$ as a useful health-based exposure metric. They also promote the use of OP assays relying on antioxidant thiol-based probe with a wide and balanced sensitivity to PM compounds: OP^{DTT} and OP^{GSH} are to date the most promising OP assays (with probably still a small advantage for OP^{DTT} due to its wider application in more epidemiological for being the oldest assay). Our conclusions agree with a recent review of the literature (Gao, 2020).

Overall, epidemiological evidence remains limited because this field of research is relatively recent. The application of OP in health studies has been enabled very recently (less than 10 years), with the emerging of less time-consuming OP methodologies. This is why more epidemiological studies assessing OP relevance for health will be needed. But these studies have to be done with standardised OP protocols to inform about the capacity of OP to be an additional or health predictor than PM mass and how the PM characteristics and the different OP assays can link the acute and chronic health effects associated with air pollution.

6. Conclusions and recommendations

Research on OP started in 2005 and considerable effort has been put into developing protocols and then linking this new parameter to PM compounds, their emission sources and adverse health effects. Improvements in recent years in high-throughput off-line techniques and on-line OP devices, as well as in the apportionment of PM OP sources, will have a significant impact on targeting emission sources that are key to creating lung oxidative damage and reducing the health effects of air pollution.

Despite all the recent improvements in the state of knowledge on OPs, further developments are still needed to achieve standardisation of the different OP tests. This is compulsory to reach a scientific consensus for comparison purposes and to allow a wide and accurate use of this measure.

To date OP assays rely mainly on off-line measurements from air samplers. OP assays generally involve filters from air samplers incubating filtered PM extracts (water-soluble OP) or unfiltered PM suspensions (total OP including water-soluble and insoluble fractions) with chemical reagents or probes. The response is recorded over time or after incubation, according to the OP method used. It's important to highlight that preparation of the samples before running assays may affect the OP results. It's important that **filters may have been frozen (or kept at 4°C cold if the analysis is fast after collection) after sampling to avoid loss of reactive species**. The preparation of the samples before OP assays as solvent of extraction (water, methanol or lining fluid), time and type of extraction (ultrasounds, vortex or gentle agitation) may also lead to discrepancies in OP results (Yang et al., 2014; Calas et al., 2018). This will need to be standardised, but it is now too early to give explicit recommendations as these matters are the subject of ongoing research.

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As discussed in this deliverable, the OP observations and the assignment of its source-specific contributions depend on the assay used, which will consequently have implications for the conclusions obtained. The choice of a given OP methodology affects the PM source exposure ranking, which can complicate the message to deliver to local stakeholders for mitigation actions (Dominutti et al., 2023; Gao et al., 2020). **But the future choice of the best OP assay (or combination) does not depend on the link done with PM compounds and sources, but on health evidence**. To date, only five, and mainly three, OP methodologies were tested towards health endpoints. Until now, little evidence was found between OP^{AA} and diseases observed in populations, despite some associations with biomarkers of inflammation. There is a lack of studies to be conclusive for emerging direct OP assays (OP^{OH} and OP^{ESR}). This is why, in the absence of other assays with a sufficient number of studies, **thiol-based OP assays, namely OP^{DTT} and OP^{GSH}, emerge as the most promising OP assays regarding health evidence.** The evidence is sufficient to recommend additional routine monitoring on a larger temporal and spatial scale. Routine monitoring will greatly enhance the power of epidemiological studies further investigating the link between OP and health. To be more informative, should further include more traditional air pollution metrics such as $PM_{2.5}$, PM_{10} and NO_2 to evaluate the additional value of OP.

Therefore, at this stage of the state-of-the-art, a set of OP assays still recommended evaluating the links with the sources and adverse health effects. **At least two complementary assays (a thiol-based probe and another one) are useful to capture insight from a wide range of redox-active species**. To date, this is the unique way to assess the complete picture of the most oxidising PM compounds/sources and to evaluate their relation to health effects. Finally, the selection of the complementary OP tests to be applied in a given place needs to be wisely evaluated and will strongly depend on the previous knowledge about the geochemistry of sources, concentrations and chemical composition of PM evaluated. Such a strategy is also essential, since this affects the message to be delivered about the targeted sources in future mitigation policies.

Furthermore, most of the studies in the literature were developed in northern mid-latitude regions, where the levels of air pollution are less critical than those observed in low/middle-income countries. Large-scale studies are needed to better elucidate how these PM levels and regional-specific sources impact the more vulnerable population groups.

Finally, there is still a need for epidemiological evidence linking OP and adverse health outcomes to better evaluate the viability of OP as a health-relevant metric of PM exposure. Therefore, the development and testing of various OP assays with wider sensitivities to redox active species should continue as long as the link between OP-health outcomes is not clearly established to enhance its value as a strong predictor of adverse health outcomes and to provide a full view of the toxicity of PM sources.

At least, existing data from the last 15 years may be considered sufficient to support the use of OP as a health-relevant indicator of exposure that can complement PM mass concentration, but there are still significant methodological issues that need to be addressed before this measure can be deployed on a large scale and with precision.

To sum up, recommendations than can be given to date are:

- OP measurements can perform online and offline. Offline measurements from filters can be assessed on different type of filters and it's important to have blank measurements. Filters must be kept cold after sampling (at 4°C if the OP analysis is done within few days after collection or -20°c if the analysis is delayed)
- The extraction of the filters is still a research question but water and simulated lining fluid are better options than methanol to stick to physiological conditions in the lung.

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- Two complementary OP assays: a thiol-based probe and another one are recommended to provide a better picture of the potential oxidizing damages from PM compounds and to strengthen the power of epidemiological studies.

With this respect, the first international intercomparison [1] of OP assays launched by the EU RI-Urbans project in 2023 will be of utmost benefit to bring some results for a further standardisation of OP^{DTT} and will enable to update some of the previous recommendations

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[1] A simplified OP^{DTT} protocol (Annex 1) has been set up and applied by almost 20 participating groups overseas.

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Annexe 1 –

Simplified DTT RI-Urbans Protocol «Evaluation of acellular reactivity of particles by dithiothreitol (DTT) assay»

Method 1 – OP DTT assay using plate readers

Before the absorbance measurements of the samples, perform a calibration of your analytical device using a DTT calibration curve for a concentration range between 0 and 60 µM (titration with 1mM DTNB and reading of TNB formation at 412 nm) and report the results on the Excel file provided.

Reagents:

Preparation of potassium phosphate (0.1M) buffer solution at pH 7.4

Weight 13.41 g of dipotassium phosphate (K_2HPO_4, CAS [7758-11-4]) and 3.13 g of potassium dihydrogen phosphate (KH_2PO_4, CAS [7778-77-0]) and mix them in a volumetric flask of 1000 mL with ultra-pure MilliQ water. Check the pH using a pH meter reading equal to 7.4 ± 0.1

Preparation of DTT mother solution (8.3 mM)

Weight 38.6 mg of 1,4-Dithiothreitol (DTT, CAS [3483-12-3]) and add 30 ml of the potassium phosphate buffer solution (7.4 pH). Keep the solution under an ice bath or in the fridge until use.

Preparation of DTT daughter solution (0.25 mM)

This solution is obtained from 1.20 mL of the 8.3 mM DTT solution and completed to a final volume of 40 ml with potassium phosphate buffer solution (7.4 pH). Keep the solution under an ice bath or in the fridge until use.

Preparation of Dinitrothiobenzoic acid (DTNB) mother solution (10 mM)

Weight 118.8 mg of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB, CAS [69-78-3]) and add 30 ml of the potassium phosphate buffer solution (7.4 pH). Keep the solution under an ice bath or in the fridge until use

Preparation of DTNB daughter solution (1 mM)

This solution is obtained by diluting 4mL of the 10mM DTNB solution, completing a total volume of 40 mL with the potassium phosphate buffer solution (7.4 pH). Keep the solution under an ice bath or in the fridge until use.

Particulate Matter suspension solutions to be tested - samples

Solution SP1 5.0 µg/mL, solution SP2 25 µg/mL, Solution SP3 25 µg/mL, solution SP4 25 µg/mL.

Material

One transparent 96-wells plate is sufficient to process all the samples in triplicate. You can use a separate 96-wells for the calibration curve of DTT.

The samples need to be under agitation during the experiment time at 37°C.

An ice bath is required to keep the DTT and DTNB cold (at least keep the reagent solution fresh in the freezer until use)

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Procedure for plate readers automatically injected

DTT Exposure and DTNB analysis:

Set up the temperature of the plate reader at 37,4°C for the duration of the assay.

1. Draw up a grid for 96-wells plate, and locate the samples SP1 to SP4 as in the table below, leaving the first 3x4 wells for the $control_{ox}$ (inherent DTT background oxidation).

	1	2	3	4	5	6	7	8	9	10	11	12	
A	Controlox	Controlox	Controlo	SP1	SP1	SP1	SP2	SP2	SP2	SP3	SP3	SP3	**T=0**
B	Controlox	Controlox	Controlo	SP1	SP1	SP1	SP2	SP2	SP2	SP3	SP3	SP3	**T=10**
C	Controlox	Controlox	Controlo	SP1	SP1	SP1	SP2	SP2	SP2	SP3	SP3	SP3	**T=20**
D	Controlox	Controlox	Controlo	SP1	SP1	SP1	SP2	SP2	SP2	SP3	SP3	SP3	**T=30**
E	SP4	SP4	SP4										**T=0**
F	SP4	SP4	SP4										**T=10**
G	SP4	SP4	SP4										**T=20**
H	SP4	SP4	SP4										**T=30**

2. Place 20 µL of samples SP1 to SP4 into each well and 20 µl of ultrapure water in $Control_{ox}$ wells.
3. Add 220 µL of the potassium phosphate buffer solution (7.4 pH) in the sample wells SP1 to SP4 and in the control wells
4. Set up the plate reader at 37,4°C.
5. Place the plate into the reader and incubate for 10 minutes.
6. Shake the plate by the instrument for one minute.
7. Read the intrinsic absorbance of the samples/control at 412 nm.

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8. At T= 0 min, program the injector A to dispense 50µL of 0.25 mM DTT in ALL wells. Keep the solution under an ice bath or in the fridge until use.
9. At T=0 min, program injector B to dispense 50µL of 1 mM DTNB into the T=0 wells (lines A and E). Keep the solution under an ice bath or in the fridge until use.
10. Shake the plate by the reader for 30 seconds every minute for 10 minutes.
11. At T=10 minutes, dispense 50 µL of 1 mM DTNB into the T=10 wells (lines B and F) to stop the DTT consumption reaction by the samples.
12. Shake the plate by the device for 30 seconds every minute for 10 minutes.
13. At T=20 minutes, dispense 50 µL of 1 mM DTNB into the T=20 wells (lines C and G).
14. Shake the plate by the device for 30 seconds every minute for 10 minutes.
15. At T=30 minutes, dispense 50 µL of 1 mM DTNB into the T=30 wells (lines D and H).
16. Shake the plate for 60 seconds, wait 10 seconds and read the final absorbance at **412 nm**. The yellow compound (TNB) formed is stable for two hours; only one final absorbance measurement is necessary.

Procedure for plate readers without injectors

DTT Exposure and DTNB analysis:

Set up the temperature of the plate reader at 37,4°C for the duration of the assay.

1. Draw up a grid for 96-wells plate and locate the samples SP1 to SP4 as in the table above, leaving the first 3x4 wells for the control sample (inherent DTT background oxidation).
2. Place 20 µL of samples SP1 to SP4 into each well and 20 µl of ultrapure water in $Control_{ox}$ wells.
3. Add 220 µL of the potassium phosphate buffer solution (7.4 pH) in the sample wells SP1 to SP5 and in the control sample wells.
4. Introduce the plate into the reader and read the intrinsic absorbance of the solutions at 412 nm.
5. Inject 50 µL of 1mM DTNB into the T=0 min wells (lines A and E) (this is done to avoid depletion of DTT with samples at t=0 with manual injection, which is slower than injectors). Keep the DTNB solution under an ice bath or in the fridge until use.
6. Dispense 50 µL of 0.25 mM DTT in ALL wells. Keep the DTT solution under an ice bath or in the fridge until use.
7. Set up the plate reader at 37°C.
8. Introduce the plate into the plate reader and incubate for 10 mins.
9. Shake the plate by the device for 30 seconds every minute for 10 minutes.
10. At T=10 minutes, remove the plate from the instrument and inject 50 µL of 1mM DTNB into the T=10 wells (lines B and F) to stop the DTT consumption reaction by the samples.
11. Place the plate back on the reader and stir it for 30 seconds every minute for 10 minutes.
12. At T=20 minutes, remove the plate from the reader and inject 50 µL of 1mM DTNB into the T=20 wells (lines C and G).
13. Place the plate back on the reader and shake it for 30 seconds every minute for 10 minutes.
14. At T=30 minutes, remove the plate from the reader and dispense 50 µL of 1mM DTNB into the T=30 wells (lines D and H).

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15. Place the plate back into the reader and shake it for 60 seconds, wait 10 seconds and read the final absorbance at **412 nm**. The yellow compound (TNB) formed is stable for two hours; only one final absorbance measurement is necessary.

Calculate the kinetics of the DTT oxidation as:

- nmol DTT/min is obtained by substracting both the intrinsic absorption of each sample (to remove a potential matrix effect, value obtained in step 8) and the inherent DTT auto-oxidation rate of the blank (slope of Controlox sample) from the DTT consumption rate in the presence of particles (SP1-4).
- nmol DTT/min/µg is obtained by subtracting both the intrinsic absorption of each sample and inherent DTT auto-oxidation rate from the DTT consumption rate in the presence of particles and dividing it by the mass of particulate matter in the reaction.
- % DTT decay/µg is obtained by the % of DTT lost over the reaction with samples relative to the inherent DTT auto-oxidation and normalised by the reaction time and per µg of PM: first normalise every loss of DTT, AA to 100% , calculate the slope over the time and then substract the autoxidation (blank) and then devide it by the mass.

All these formulas are pre-included in the Excel spreadsheet provided.

Use it to add the results using your lab reference number, the analytical protocol and instrument used and the reference number for each sample.

Once you have reported results for the DTT simplified protocol, feel free to test the samples with your own protocols, filling the other tabs of the excel file.

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