A Semi-automated Instrument for Cellular Oxidative Potential Evaluation (SCOPE) of Water-soluble Extracts of Ambient Particulate Matter

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Abstract. Several automated instruments exist to measure the acellular oxidative potential (OP) of ambient particulate matter (PM). However, cellular OP of the ambient PM is still measured manually, which severely limits the comparison between two

10 types of assays. Cellular assays could provide a more comprehensive assessment of the PM-induced oxidative stress, as they incorporate more biological processes involved in the PM-catalyzed reactive oxygen species (ROS) generation. Considering this need, we developed a first of its kind semi-automated instrument for measuring the cellular OP based on a macrophage ROS assay using rat alveolar macrophages. The instrument named SCOPE - Semi-automated instrument for Cellular Oxidative Potential Evaluation, uses dichlorofluorescein diacetate (DCFH-DA) as a probe to detect the OP of PM samples extracted in

15 water. SCOPE is capable of analyzing a batch of six samples (including one negative and one positive control) in five hours and is equipped to operate continuously for 24-hours with minimal manual intervention after every batch of analysis, i.e., after every five hours. SCOPE has a high analytical precision as assessed from both positive controls and ambient PM samples (CoV <17%). The results obtained from the instrument were in good agreement with manual measurements using tert-Butyl hydroperoxide (t-BOOH) as the positive control (slope = 0.83 for automated vs. manual, R^2 = 0.99) and ambient samples

20 (slope = 0.83, R^2 = 0.71). We further demonstrated the ability of SCOPE to analyze a large number of both ambient and laboratory samples, and developed a dataset on the intrinsic cellular OP of several compounds, such as metals, quinones, PAHs and inorganic salts, commonly known to be present in ambient PM. This dataset is potentially useful in future studies to apportion the contribution of key chemical species in the overall cellular OP of ambient PM.

1 Introduction

25 Epidemiological models have traditionally relied on mass of the particulate matter (PM) as a metric to associate the health effects such as wheeze (Doiron et al., 2017; Karakatsani et al., 2012), asthma (Holm et al., 2018; Wu et al., 2019; Zmirou et al., 2002), myocardial infarction and coronary heart disease (Yang et al., 2019), ischemic heart disease and dysrhythmias (Pope et al., 2004) and heart rate variability (Breitner et al., 2019; Pieters et al., 2012; Riojas-Rodriguez et al., 2006) with the inhalation of ambient and indoor PM. However, mass is not a wholesome metric as it does not capture the diverse range of

30 particle physicochemical characteristics. Apparently, the assumption that an increase in PM mass alone leads to a proportionate

increase in the mortality would yield erroneous estimates if we do not account for the complexity of PM chemical composition and the resulting intrinsic toxicities. There are also mounting evidence that toxic effects of different chemical components are not simply additive, but there exists both synergistic and antagonistic interactions (Wang et al., 2020; Yu et al., 2018). Therefore, we need a metric of the PM along with mass that can provide some relevant information to assess its toxicity.

35   Oxidative stress has emerged as one of such metrics, which has been identified as a crucial step in the progression of many human diseases.

Oxidative stress is caused by an imbalance between reactive oxygen species (ROS) generation and their subsequent scavenging by lung antioxidants (Kryston et al., 2011; Li et al., 2008; Møller et al., 2010; Rao et al., 2018; Reuter et al., 2010). Thus, measuring the ability of PM to induce ROS generation in the respiratory system, also called the oxidative potential (OP), could

40   be considered as one of the markers of its toxicity and accordingly several acellular assays have been developed in the recent past to measure the OP of PM. These include the dithiothreitol (DTT) assay (Charrier and Anastasio 2012; Fang et al., 2015), ascorbic acid (AA) assay (Künzli et al., 2006; Visentin et al., 2016), glutathione assay (Künzli et al., 2006; Mudway et al., 2005), and hydroxyl radical ($\bullet$OH) measurement (Vidrio et al., 2009; Xiong et al., 2017). Along the similar lines, several cellular assays have also been developed, which involve molecular probes that can detect ROS through their transformation

45   from non-fluorescent to fluorescent forms (Dikalov and Harrison 2014; Kuznetsov et al., 2011; Landreman et al., 2008; Wan et al., 1993). However, measurement of OP of PM using both cellular and acellular assays is often a labor-intensive and time-consuming process and therefore manually analyzing a large number of ambient samples for spatiotemporal resolution of OP is a cumbersome process.

In the last few years, a number of automated instruments have been developed based on acellular assays which could provide

50   rapid and high-throughput analyses of the PM chemical OP (Berg et al., 2020; Fang et al., 2015; Gao et al., 2017; Venkatachari and Hopke 2008). There have also been a number of online instruments which can be deployed in the field making it possible to collect real-time OP or ROS data (Brown et al., 2019; Huang et al., 2016; Puthussery et al., 2018; Sameenoi et al., 2012; Wragg et al., 2016; Zhou et al., 2018). Although, acellular assays have many advantages over cellular assays such as ease of application, low maintenance and no risk of microbial contamination, they are unable to capture the complex biochemical

55   reactions occurring in a biological system as a response to PM exposure. This could be one of the reasons for their inconsistent correlations with various biological responses such as DNA damage and expression of inflammatory cytokines in previous studies (Crobeddu et al., 2017; Janssen et al., 2015; Øvrevik 2019; Steenhof et al., 2011). Cellular assays have an edge in this regard as these assays directly expose biological cells to chemical constituents of the particles, thus capturing some, if not all, of the biochemical processes related to the oxidative burst. However, to the best of our knowledge, no automated instrument

60   has ever been developed to provide a rapid high-throughput analysis of the cellular OP induced by the ambient PM. In comparison to chemical assays, cellular assays are even more time and labor-intensive. Due to this strenuous nature of the cellular protocols, there have been very limited comparison between the chemical and cellular OP measurements. There is a

need for the development of an automated instrument for the cell-based measurement of OP, which could not only analyze a large number of samples in shorter period with minimal manual intervention, but could also open up the possibilities for
65   developing a field-deployable real-time instrument measuring cellular OP. Having such an automated instrument would be able to provide a direct comparison of cellular and acellular assays, thus screening the important chemical OP endpoints. Such advances will also help in integrating the OP data in toxicological and/or epidemiological studies by yielding a relatively large dataset on these measurements.

In this paper, we describe the development of a semi-automated instrument for cellular oxidative potential evaluation (SCOPE).
70   SCOPE is the first of its kind instrument to measure the cellular OP induced by the water-soluble ambient PM extracts in murine alveolar cell line NR8383, using an automated protocol. The instrument is capable of analyzing a batch of six samples (including one negative and one positive control) in five hours. SCOPE is equipped to operate continuously for 24-hours with minimal manual intervention after every batch of analysis, i.e., after every five hours. We also calculated the detection limit of this instrument and evaluated its performance by measuring precision and accuracy using both positive controls and ambient
75   samples. Finally, we demonstrated the ability of SCOPE to analyze a large number of both ambient and laboratory samples, and developed a dataset on the intrinsic cellular OP of several compounds, such as metals, quinones, polycyclic aromatic hydrocarbons (PAHs) and inorganic salts, commonly known to be present in the ambient PM.

2 Materials and Methods

2.1 Chemicals

80   Copper (II) sulphate pentahydrate [Cu (II)] (≥98%), Luperox® TBH70X, tert-Butyl hydro peroxide (t-BOOH) solution (70 wt. % in water), iron (II) sulphate heptahydrate [Fe(II)] (≥99%), manganese (II) chloride tetrahydrate [Mn(II)] (≥98%), zinc (II) nitrate hexahydrate [Zn(II)] (≥98%), iron (III) chloride hexahydrate [Fe(III)] (≥97%), lead (II) acetate trihydrate [Pb(II)] (≥98%), aluminum (III) nitrate nonahydrate [Al(III)] (≥98%), chromium(III) nitrate nonahydrate [Cr(III)] (≥97%), cadmium (II) nitrate tetrahydrate [Cd(II)] (≥98%), vanadium (III) chloride V(III) (97%), nickel (II) chloride hexahydrate [Ni(II)]
85   (99.9%), 9,10-phenanthraquinone (PQN) (99%), 1,2-naphthaquinone (1,2-NQN) (97%), 1,4-naphthaquinone (1,4-NQN) (97%), 5-hydroxyl-1,4-naphthaquinone (5-H-1,4-NQN) (97%), pyrene (Pyr) (98%), naphthalene (Naph) (99%), anthracene (Anth) (97%), phenanthrene (Phen) (98%), benzo[a]pyrene (B[a]P) (≥96%), fluorene (Flu) (98%), benz[a]anthracene (B[a]A) (99%), sodium chloride (NaCl) (≥99%), ammonium nitrate (NH₄NO₃) (≥99%), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), zymosan A from saccharomyces cerevisiae, Ham's F-12K growth media, and fetal bovine serum (FBS) were purchased from
90   Sigma Aldrich Co. (St.Louis, MO). Ammonium chloride (NH₄Cl) and calcium chloride (CaCl₂) were purchased from VWR Life Sciences. Ammonium sulphate (NH₄SO₄) and potassium chloride (KCl) were purchased from Fisher Scientific. Salt

glucose media (SGM) at two different concentrations (1X and 10X), which was prepared according to the composition discussed in Klein et al., (2002), was provided by the Cell Media Facility at UIUC.

2.2 Stock Solution Preparation

95 The stock solutions of quinones (PQN, 1,2-NQN, 1,4-NQN) were prepared in DMSO and stored in a freezer at -20 ℃ for one week. The stock solution of 5-H-1,4-NQN was prepared on the same day of the experiment as it was found to be relatively unstable (e.g., change in color over a period of more than 24 hours) compared to other quinones. The stock solutions of PAHs (anthracene, pyrene, naphthalene, fluorene, phenanthrene, Ba[A]P, and Ba[A]A) were prepared in methanol and stored in a freezer at -20 ℃ for one week. On the day of the experiment, the stock solutions of both quinones and PAHs were diluted

100 using Milli-Q deionized water (DI, resistivity = 18.2 MΩ/cm) to appropriate concentrations. All the metals, inorganic salt and t-BOOH solutions were freshly prepared using DI on the day of the experiments and immediately used.

2.3 Cells

Alveolar macrophages form the front-line of defense in pulmonary region of respiratory system against attack by the foreign particles. These cells play a major role in preliminary responses such as phagocytosis, secreting pro-inflammatory cytokines

105 and killing pathogens. We used a murine alveolar cell line, NR8383, which resembles greatly to the primary macrophages in terms of their expression of cytokines and other biological responses, thus serving as a good model for the PM toxicity studies (Lane et al., 1998; Lin et al., 2000). The cells were maintained on glass culture plates in Ham's F12-K medium containing 5% FBS and incubated at 37 ℃ with 5 % CO_2 concentration. The cells were cultured by transferring floating cells from culture plates to fresh plates every four weeks. The cells generally divide and double in concentration within 48 hours (Helmke et al.,

110 1987), after which the floating cells are removed for further growth of the attached cells by adding fresh media. Since Ham's F-12K media could itself contribute to the fluorescence, it was replaced by 1XSGM after counting the initial cell density and subsequent centrifugation, such that final concentration of the cells in SGM is 2000 cells/μL.

2.4 Probe Preparation

A 45 mM stock solution of DCFH-DA was prepared and aliquoted into different vials (30 μL per vial). These vials were stored

115 in a freezer (at -20 ℃) and used within a month. To prepare the final probe solution, a portion of the content of one vial (i.e., 25 μL of 45 mM DCFH-DA) was diluted 100 times just before the experiment, using 10X SGM.

2.5 System Setup

We adapted the method of macrophage ROS assay from Landreman et al., (2008) which is the most widely used protocol for measuring the cellular OP of ambient PM. In this assay, DCFH-DA is used as an ROS probe. The reaction mechanism of

120 DCFH-DA with ROS is well established (Rosenkranz et al., 1992; Wan et al., 1993). Briefly, DCFH-DA is a cell permeable compound which undergoes deacetylation by intracellular esterase to form DCFH. DCFH is oxidized by a variety of ROS to

form a fluorescent product called DCF. The intensity of fluorescence provides a direct measure of the ROS generation. In our protocol, all the components of the assay, i.e., PM extract (or negative control/positive control; 138 μL), molecular probe (DCFH-DA; 39 μL), and cells suspension (177 μL at a final concentration of 1000 cells per μL in the reaction vial (RV)) are added together and incubated for 2 hours. Next, a small aliquot is withdrawn and transferred to a spectrofluorometer after dilution to measure the fluorescence.

The schematic diagram of SCOPE based on this protocol is shown in Fig. 1. The instrument consists of four major units: cells reservoir and samples holder, fluid transfer unit, incubation-cum-reaction unit, and measurement unit. The cells reservoir and sample holder consist of a set of seven vials (15 mL each) – one containing NR8383 cells suspended in 1XSGM, one amber vial containing DCFH-DA solution, five vials containing samples (i.e., four PM samples and one positive control). All the vials of this unit were placed in an Eppendorf Thermo-Mixer (Eppendorf North America, Hauppauge, NY, USA), which is maintained at 37 °C while continuously shaking at a frequency of 600 RPM. The fluid transfer unit consists of three Kloehn programmable syringe pumps (IMI precision, Littleton, CO, USA) (Pump #1, 2, and 3; see Fig. 1) and a 14-port multi-position valve (VICI® Valco Instrument Co. Inc., Houston, TX, USA) connected to Pump #2. The incubation-cum-reaction unit consists of 17 RVs [amber vials, 2 mL each; 1 for negative control (DI) in triplicate, 1 for positive control (t-BOOH) in duplicates and 4 for PM samples in triplicates] held in another Eppendorf Thermo-Mixer which is maintained at 37 °C and continuously shaking at a frequency of 800 RPM to keep the contents of all the vials well-mixed and suspended. 14 of these RVs are connected to Pump # 2 through the multi-position valve. Each RV connected to the multi-position valve is accessed by changing the valve position [using a valve actuator (VICI®)] to its respective number. Since multi-position valve has only 14 ports, rest 3 RVs are directly connected to Pump #3. Both of these pumps (i.e., #2 and 3) transfer the content from various reservoirs (e.g., cells, DI and DCFH-DA) to RVs, and also transfer a small aliquot from these RVs (50 μL from each RV) to the measurement vial (MV) after 2 hours of reaction. Finally, the measurement unit consists of a Fluoromax-4 spectrofluorometer (Horiba Scientific, Edison, NJ, USA) equipped with a Flowcell (Horiba Scientific, HPLC Flowcell- 25 μL volume) to measure the fluorescence generated from the reaction of DCFH and cellular ROS. Pump #1, which is connected to the MV and the spectrofluorometer, first dilutes the aliquot withdrawn from the RV and then transfers this diluted mixture from MV to the spectrofluorometer for fluorescence measurement.

2.6 OP Measurement Protocol

The protocol for measuring cellular OP involves two stages –the first stage consists of manual preparation of the cells, DCFH-DA probe, and PM extracts, while the second stage involves incubating the cells with PM and DCFH and measuring the fluorescence in an automated manner. After preparing the cells, DCFH-DA and different PM extracts (i.e., completion of first stage), all the vials are manually transferred to the cell reservoir and sample holder. The second stage (automation stage) further consists of two phases – reaction phase and measurement phase. The complete algorithm of 2nd stage is shown in Fig. 2. In the reaction phase, various reactants (i.e., cells, DCFH-DA and PM extract or positive control of DI) are transferred from their respective reservoirs to the RVs using pump # 2 and 3. This is done in a sequence of steps: in the first step, 138 μL of DI is

155   withdrawn using Pump #2 and transferred via the multi-position valve to three RVs marked for negative control (i.e., triplicate analysis of the negative control). In the second step, 177 μL cell solution is withdrawn from the cell reservoir using Pump # 2 and transferred sequentially to all 14 RVs via multi-position valve. Simultaneous to this step, 177 μL cell solution is withdrawn from the cell reservoir using Pump # 3 and transferred to three RVs connected to that pump. In the third step, 39 μL DCFH-DA is transferred using pump #2 and pump #3 to the respective RVs connected to them (i.e., 14 RVs connected to pump #2

160   through the multi-position valve and 3 RVs directly connected to pump #3) following the same sequential order as for addition of the cell solution. Finally, 138 μL of positive control (t-BOOH) and PM extracts are transferred to the respective RVs using Pump #2 and # 3, i.e., t-BOOH and 3 PM extracts are transferred using Pump # 2 via multi-position valve, while one PM extract using Pump # 3 directly connected to 3 RVs. After all the RVs are loaded with the reactants, SCOPE performs a single round of self-cleaning, in which all the valves and tubing of the instrument are rinsed with DI using the fluid handling unit

165   (i.e., all three Kloehn pumps).

After 2 hours of cells' exposure to PM, the measurement phase starts in which the fluorescence of DCF formed in each RV is measured in a sequential manner. Each measurement involves three steps- 1) withdrawing an aliquot of 50 μL from the RV (using Pump #2 for 14 RVs connected to it, and Pump #3 for the remaining 3 RVs) and transferring it to the MV; 2) diluting the aliquot 100 times by adding DI using Pump #1 to the MV, and finally 3) pushing the diluted aliquot through Flowcell of

170   the spectrofluorometer using the same syringe Pump #1. The withdrawal of the aliquot from different RVs follows the same order as for their preparation, such that the cells in each vial undergo exposure to the PM extract or DI or t-BOOH for exactly 2 hours. The spectrofluorometer is preset at an excitation/emission wavelength of 488 nm/530 nm. Between successive fluorescence measurements of different RVs, the Flowcell, MV and the tubing connected to the multi-position valve are thoroughly rinsed with at least 10 mL of DI. After all RVs are measured for fluorescence, the instrument performs a final

175   round of thorough self-cleaning, wherein each valve and tubing are cleaned (three times) with 70% ethanol followed by DI. All the RVs and MV after this cleaning step are disposed and replaced manually with clean empty vials. SCOPE takes about five hours for complete analysis of one batch of six samples (i.e., 4 PM extracts, one negative and one positive control). For the next batch of analysis, cells, DCFH-DA and samples are manually replaced with freshly prepared vials. In our experiments for this manuscript, one batch was run per day, although it is possible to run up to three batches (a total of twelve PM samples)

180   per day.

2.7 Ambient PM Sample Collection and Preparation

Ambient $PM_{2.5}$ samples were used in this study for assessing the precision and accuracy of SCOPE. These ambient samples were collected as a part of the Midwest Sampling Campaign and the sampling procedure and collection protocol are described elsewhere (Yu et al., 2019). Briefly, $PM_{2.5}$ samples were collected on quartz filters (Pall Tissuquartz TM, 8"x10") using a

185   high-volume sampler (flow rate of 1.13 m³/min; $PM_{2.5}$ inlets, Tisch Environmental; Cleves, OH) from five different sites in the midwestern USA: a road-side site in Champaign (within the UIUC campus), a rural site in Bondville (IL), and three urban sites in Chicago, IL (university campus of Illinois Institute of Technology), Indianapolis, IN (Indiana University-Purdue

University campus), and St. Louis, MO [a part of National Core Pollutants (NCore) Network of USEPA]. All the samples used in this study were collected between May 2018 and May 2019. A total of 50 samples from all the five sites (10 from

190 Indianapolis, 9 from Chicago, 10 from St. Louis, 7 from Bondville and 14 from Champaign) were used for conducting the performance evaluation, i.e., assessing precision and accuracy of the instrument. Further details on these samples (i.e., dates of collection, exact mass loadings etc.) are provided in Table S1 of the supplemental information (SI).

2.8 Filters Extraction

A single circular section of 1" diameter was punched from the high-volume filter, immersed in DI and sonicated for 60 minutes

195 in an ultrasonic water bath (Cole-Palmer, Vernon-Hills, IL, USA). The volume of DI was determined based on the $PM_{2.5}$ mass loading on each punched section, such that the final concentration of the extract for exposure in the RV is 30 µg/mL. After sonication, the extracts were passed through a 0.45 µm pore size polytetrafluoroethylene (PTFE) filter to remove any insoluble particles and/or filter fibers. The water-soluble $PM_{2.5}$ extracts were then used to measure the OP of the PM.

3 Results and Discussions

200 ### 3.1 Instrument Calibration

The results of OP of the samples (i.e., field blank filter, positive control or PM extract) are reported as the percentage increase in fluorescence relative to the negative control (i.e., cells treated with DI), which is consistent with many previous studies (Sun et al., 2011; Thayyullathil et al., 2008; Wan et al., 2012; Wang et al., 2012). Normalizing by the negative control which is analyzed in the same batch of the samples is important, because absolute fluorescence of the cells treated with negative control

205 is not stable and vary in each experiment. Two factors could cause this variability in apparent response of the cells. First, DCFH-DA, being a photo-chemically active compound (Castro-Alférez et al., 2016; Chen et al., 2010), could itself undergo possible decay and slight photo-degradation over time. Second, the exposed cells could be under different developmental stages, which affects their metabolic activity and the subsequent generation of ROS. Both of these factors yield substantial variability [Coefficient of variation (CoV) = 35%, as obtained from the experiments conducted on 20 different days] in absolute

210 fluorescence of the cells treated with DI (see Fig. S1 in the supplementary information). However, normalizing the fluorescence of a sample with that of the negative control minimizes this variability. For example, CoV for the ratio of the fluorescence caused by the positive controls (zymosan, concentration = 100 µg/mL) versus respective negative controls was only 16 % (Fig. S1). Therefore, fluorescence of all the samples (i.e., filter blank, field blank, positive control or PM extract) was normalized with that of the negative control, analyzed in the same batch of the samples. This normalized fluorescence of the sample was

215 then blank corrected by subtracting corresponding fluorescence of the blank, which was DI for the positive control and field blank filter extract for the PM extract.

Fig. 3 shows the response curve for various concentrations of t-BOOH (3.51 – 87.83 mg/mL), which was used to calibrate the instrument. The calibration equation shown in Fig. 3 was used to convert the blank-corrected OP (% increase in fluorescence)

to the equivalent units of mg/mL t-BOOH (see Sect S1 in the supplementary information for calculations). At concentrations
220   higher than 87.83 mg/mL t-BOOH, the curve becomes non-linear (see Fig. S2 in the supplemental information), but here we
show only linear portion of the curve for the convenience of calculating the calibration equation.

3.2 Limit of Detection (LOD)

The LOD of SCOPE is defined as three times the standard deviation of multiple blanks. For this study, the LOD was calculated
from the field blank filters (FB, N=10) analyzed in different batches. As discussed earlier, the OP response from these blanks
225   was expressed as percentage increase in fluorescence with respect to corresponding negative control (analyzed in the same
batch as FB). The LOD obtained from the average and standard deviation of this data is converted to equivalent units of t-
BOOH (mg/mL) using the calibration equation shown in Fig. 4. The LOD obtained by this method is 1.26 mg/mL t-BOOH.
Note, an ideal expression of LOD should have been in terms of the threshold PM mass required to yield a signal significantly
distinguishable from the blanks, however, expressing LOD in those units is complicated given the variability associated with
230   PM, such as different chemical composition of the PM samples collected on different days and the variability in extraction
procedures (e.g., volume of water used for PM extraction and the filter area which can be submerged in that volume).

3.3 Precision

For determining analytical precision of SCOPE, three different types of samples, i.e., positive controls (9.75 mM t-BOOH and
100 µg/mL Zymosan), Cu (II) solution (20 µM), and water-soluble PM extracts were used. For PM extracts, ten different
235   circular sections (each 1" in diameter) were punched from ten different spots on one of the high-volume filters collected in the
Midwest Sampling Campaign (Sect. 2.7) and extracted in DI (Sect. 2.8). The blank corrected % normalized OP response of
the PM extract was further normalized by the PM mass used in the RV for exposure to the cells. Table 1 shows the average,
standard deviation and CoV obtained from the measurements of various samples.

The instrument showed a CoV less than 20% for most cases suggesting high reproducibility of the results. Among the positive
240   controls, CoV for zymosan was the highest (39%), as compared to 14 %, 14 % and 16 % obtained for Cu (II), PM samples and
t-BOOH, respectively. We suspect that higher CoV for zymosan is partly due to water-insoluble nature of zymosan (Gao et
al., 2012; Venkatachalam et al., 2020), which is often used as a suspended particle in phagocytosis assays (Sung et al., 1983;
Thomas et al., 2007; Underhill, 2003). This could lead to deposition of zymosan particles inside tubing of the instrument,
leading to an underestimation in the OP measurement. In contrast, t-BOOH is highly water-soluble [700,000 mg/L
245   (OECD/SIDS, 1995)] and thus involves no such complications. t-BOOH has previously been shown to induce oxidative stress
not only in macrophages (Lopes et al., 2017; Prasad et al., 2007; Roux et al., 2019) but also in a variety of other cells such as
hepatocytes (Kučera et al., 2014), sperm cells (Fatemi et al., 2012), and lung fibroblast cells (Lopes et al., 2017). These results
suggest that t-BOOH could be a more reliable positive control than zymosan for the macrophage ROS assay, particularly for
the automated operation of our instrument.

3.4 Accuracy

The accuracy of SCOPE was evaluated by comparing the instrument's response with that obtained from the manual operation using both positive controls and ambient PM samples. We prepared different concentrations of t-BOOH from 0.04 to 9.75 mM (0.04, 0.39, 0.97, 1.95 and 9.75 mM, in the RVs used for exposure to the macrophages), and analyses were conducted both manually and using the instrument. Fig. 4 shows the comparison of manual and automated measurements of ROS induced by various concentrations of t-BOOH. The slope of the automated versus manual measurements for the positive control was ~0.83 with a very high coefficient of determination ($r^2 = 0.99$). The automated measurements were slightly but consistently lower than the manual measurements. This bias could probably be caused by the error introduced during transfer of cells using the fluid-transfer unit (i.e., some loss of cells in valves or tubing), leading to slight inconsistency of the cell density in RVs. Though, this deposition of the cells is not expected to yield cross contamination of the samples, given a rigorous cleaning procedure (as discussed in Sect. 2.6) employed during operation of the instrument.

One of the major objectives of developing SCOPE was to enable a high through-put analysis of the PM samples. To demonstrate this ability of the instrument, fifty ambient $PM_{2.5}$ samples collected from various sites in the Midwest US (Sect. 2.7) were analyzed and the results from the automated instrument were compared with manual measurements. The results are expressed in terms of the equivalent units of t-BOOH (mg of t-BOOH per mg of PM), and the comparison is shown in Fig. 5 (see Sect. S1 of the supplementary information for the calculation procedure). Overall, there was very good comparison between the manual and automated measurements, with a slope of 0.83 and a coefficient of determination (r^2) = 0.71.

3.5 Intrinsic OP of individual PM chemical species

To demonstrate the utility of SCOPE, we tested several compounds commonly known to be present in the ambient PM. These include 11 metallic species [Fe (II), Fe (III), Cu(II), Mn(II), Zn (II), Al (III), Pb (II), Cr (III), Cd (II), V (III) and Ni (II)], 4 quinones (PQN, 1,4-NQN, 1,2-NQN and 5-H-1,4-NQN), 7 PAHs (Phenanthrene, Anthracene, Naphthalene, Pyrene, Fluorene, B[a]P and B[a]A) and 6 inorganic salts (KCl, NaCl, NH_4Cl, NH_4NO_3, NH_4SO_4 and $CaCl_2$). The concentrations used for these compounds, i.e., 0.5 μM for metals, 0.2 μM for quinones and PAHs, 5 μM for KCl, NH_4Cl, NH_4NO_3, NH_4SO_4, $CaCl_2$ and 1 μM for NaCl, were in their typical ranges present in the ambient $PM_{2.5}$ and similar to those used in previous studies based on acellular assays (Charrier and Anastasio, 2012; Yu et al., 2018). We are not aware of any study which has systematically explored and compared the DCFH-based OP of individual PAHs vs. various metals or quinones in alveolar macrophages (murine cell line NR8383). Fig. 6 shows the OP of these chemical species. Among metals, Fe (II), Mn (II), and Cu (II) induced the highest response (12.40 -9.95 mg/mL t-BOOH). In contrast, metals such as Zn (II), Pb (II), Al (III), Cr (III), Cd (II), V(III) induced low response (<4.5 mg/mL t-BOOH). Ni (II) caused almost negligible oxidative stress (about 20 times lower than that of Fe (II)) and the response was lower than LOD of the instrument. Interestingly, Fe (III) induced comparatively much lower response (~3 times lower) than Fe (II), which matches with their relative redox activities as measured by the dithiothreitol (DTT) assay, i.e., 3 times lower intrinsic DTT activity of Fe (III) compared to Fe (II) (Charrier and Anastasio, 2012).

Among the organic compounds, quinones dominated the OP. PQN, 1,2-NQN, 1,4-NQN showed the highest response (7.51-4.92 mg/mL t-BOOH) and followed Fe (II), Mn (II) and Cu (II) in the decreasing order of response. In contrast, 5-H-1,4-NQN showed a very low response of 2.87 mg/mL t-BOOH. PQN, 1,2-NQN and 1,4-NQN are among the most abundantly found
285 quinones in ambient air (Charrier and Anastasio, 2012), known to show a high redox cycling capability transitioning to and from their semiquinone forms, as well as the ability to cause DNA damage and induce apoptosis in cells (Klotz et al., 2014; Shang et al., 2014; Shinkai et al., 2012; Yang et al., 2018). Therefore, a high intrinsic OP of these quinones indicates towards their prominent role in other cellular responses such as inflammation and cell death.

PAHs showed very low response (<4 mg/mL t-BOOH), and the differences among their responses were not significant at the
290 concentration tested in our experiments (i.e., 0.2 µM). Phenanthrene and B[a]P, showed the lowest responses (<2 mg/mL t-BOOH), and it seems highly unlikely for these PAHs to make any appreciable contribution to the oxidative stress of ambient $PM_{2.5}$, as measured by the macrophage ROS assay. This is in contrast to several studies conducted on bronchial epithelial BEAS-2B cells (Landkocz et al., 2017), acute monocytic leukemia THP-1 cells (Den Hartigh et al., 2010) and U937 cell line (Tsai et al., 2012), which have suggested that PAHs such as B[a]A, B[a]P, pyrene, anthracene and phenanthrene are the
295 important drivers of oxidative stress and cytotoxicity. However, these cells of human origin differ significantly from the murine cell lines used in our study in terms of their morphology (Krombach et al., 1997), expression of certain reactive nitrogen species and related enzymes (Jesch et al., 1997), and membrane proteins (Jaguin et al., 2013). Certain mechanisms, such as aryl hydrocarbon receptor (AhR)-mediated activity which activates the CYP450 gene, are necessary for the initial steps of bio-activation of PAHs (Rossner et al., 2020) to convert them into more redox-active products. It has also been shown that such
300 mechanistic pathways differ substantially among different cells (Libalova et al., 2018; Vondráček et al., 2017). Therefore, a direct comparison between our results and those studies showing a significant role of PAHs in the oxidative stress is probably not reasonable.

Among the inorganic salts, NH_4NO_3 and NH_4SO_4 showed the lowest responses (<1.5 mg/mL t-BOOH). Although, the average response of chloride salts such as KCl, NaCl, NH_4Cl and $CaCl_2$ (3.22 – 2.34 mg/mL t-BOOH) seems to be slightly higher than
305 other salts, there was no significant difference in the responses ($p > 0.05$; unpaired t-test) of any of these salts. Overall, at atmospherically relevant concentrations, inorganic salts seem to have very low contribution, if at all, to the oxidative stress as compared to the metals and quinones. This is consistent with previous studies based on ambient PM samples, showing either nil or inconsistent correlation of the macrophage ROS response with the concentration of inorganic ions (Hu et al., 2008; Kam et al., 2011; Verma et al., 2009; Wang et al., 2013; Xu et al., 2020).

4 Conclusion

In this paper, we have described the development of SCOPE for assessing the OP of water-soluble extracts of ambient PM in rat alveolar macrophages. The promising results of this instrument could pave the way for further development in automating other cellular assays. Moreover, since real-time instruments based on acellular OP assays have been developed in recent past,

the current research opens up the road for the development of such online instruments based on mammalian cell lines, possibly

315   coupling it to a real-time ambient PM sampling device (e.g., particle-into-liquid sampler or mist chamber). SCOPE is capable of analyzing up to 6 samples in a span of 5 hours without any manual intervention. The results of performance evaluation of the instrument demonstrate a high precision and accuracy for both positive control and the PM samples.

Overall, we have shown a first of its kind instrument capable of performing cellular OP measurements of PM. It substantially reduces the extent of manual labor associated with conducting cellular assays resulting in increased throughput of the results.

320   We demonstrated that SCOPE is capable of handling large number of ambient PM samples, thus, providing an opportunity for generating an extensive dataset on cellular OP, that can be used in epidemiological studies. We also generated a database of several chemical compounds commonly known to be present in the ambient PM. Metals such as Fe (II), Mn (II) and Cu (II) dominated the OP, which were followed by quinones such as PQN, 1,2-NQN and 1,4-NQN. PAHs and inorganic salts showed low OP as compared to the most metals and quinones. Further investigations on the interactions of these chemical species may

325   be undertaken in future studies.

Data availability. Supplementary data is provided with the manuscript.

Author contributions. SS developed the instrument, performed the experiments and prepared the manuscript. YW contributed

330   in filters collection, helped in developing the manual protocol for cell-based experiments and edited the manuscript. JVP helped in developing the instrument and edited the manuscript. VV conceived the idea, organized the manuscript and supervised the overall project.

Competing interests. The authors declare that they have no conflict of interest.

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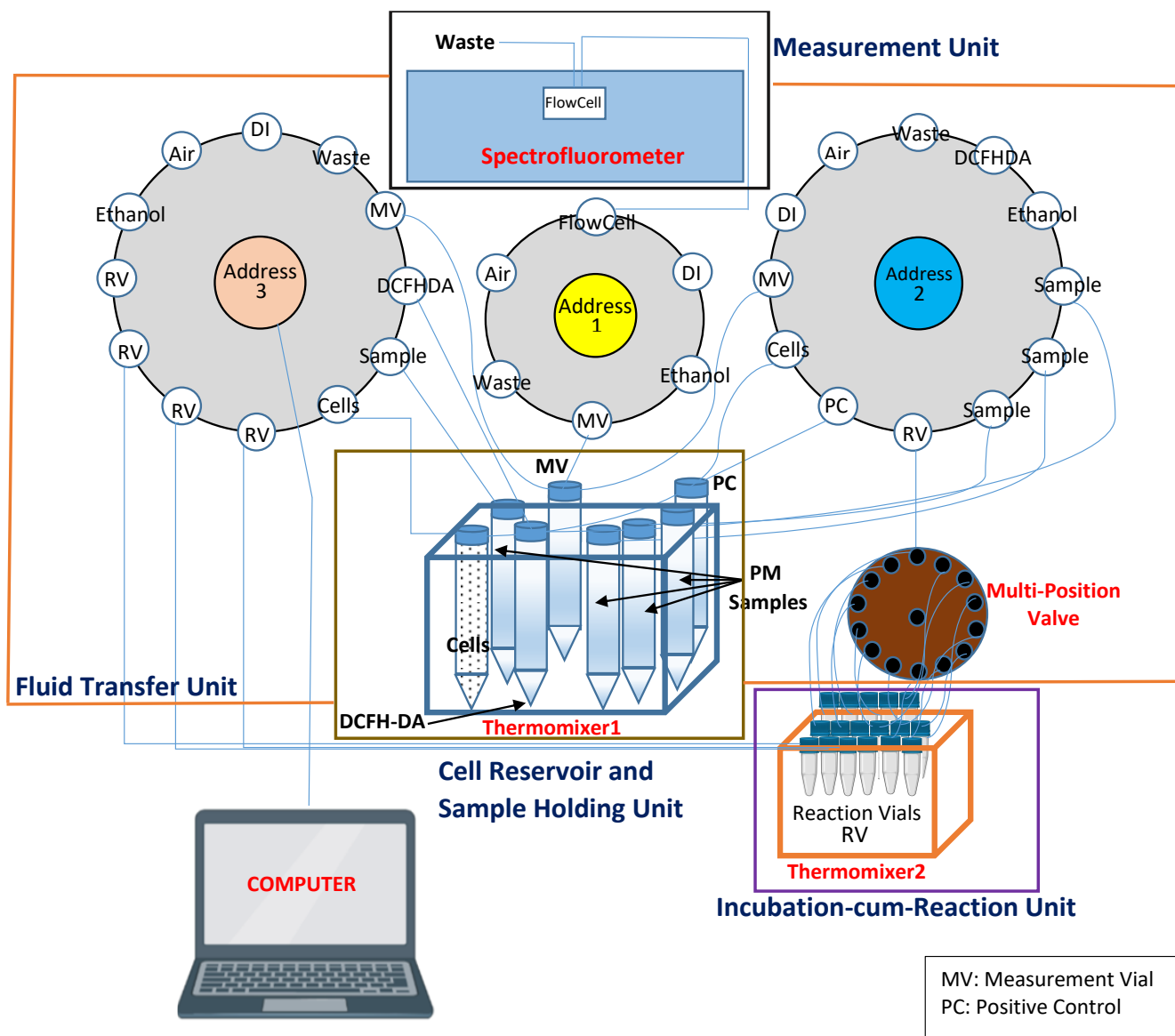
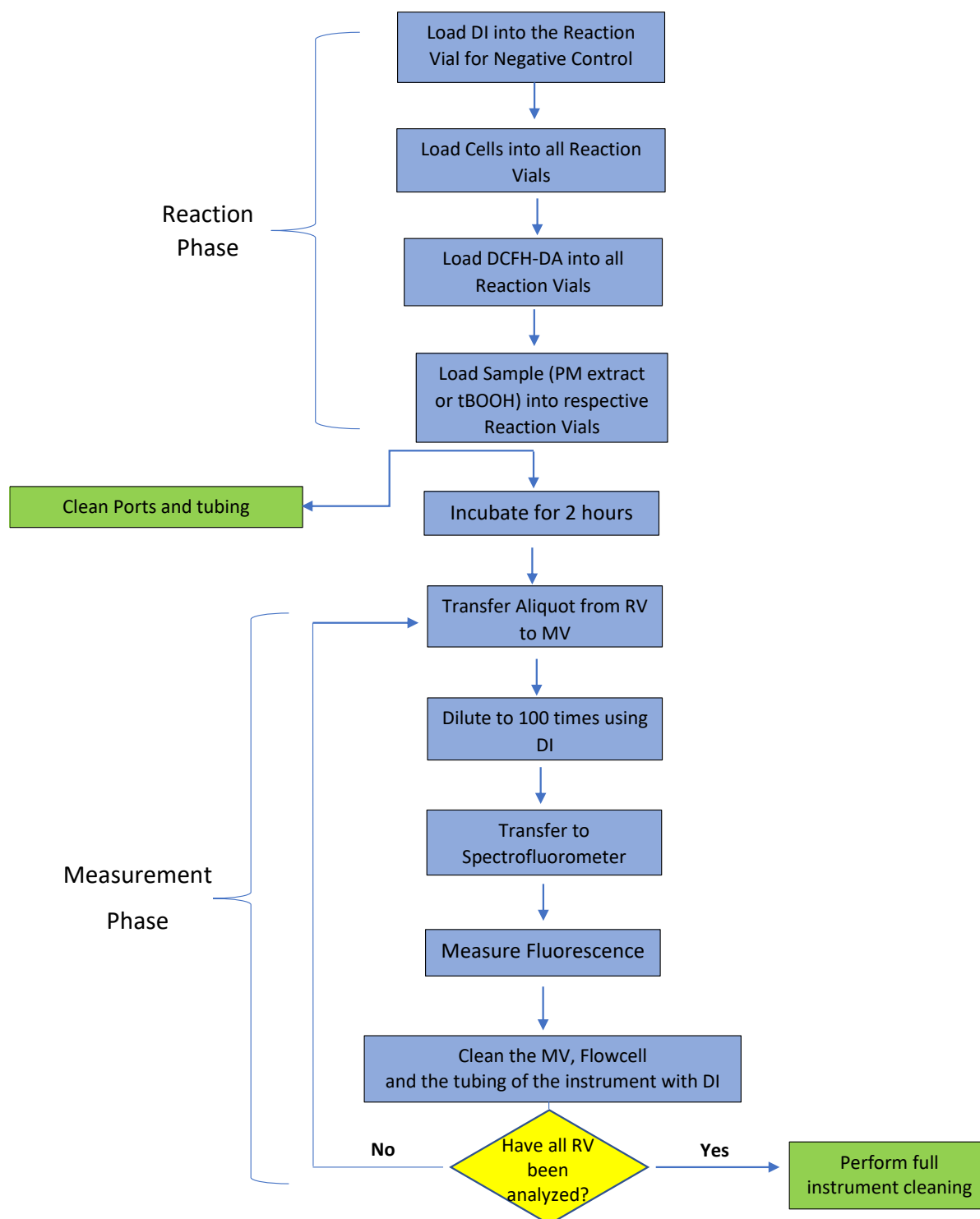


Figure 1: Automated System Setup.

615 **Figure 2: Algorithm for the instrument's operational protocol.**

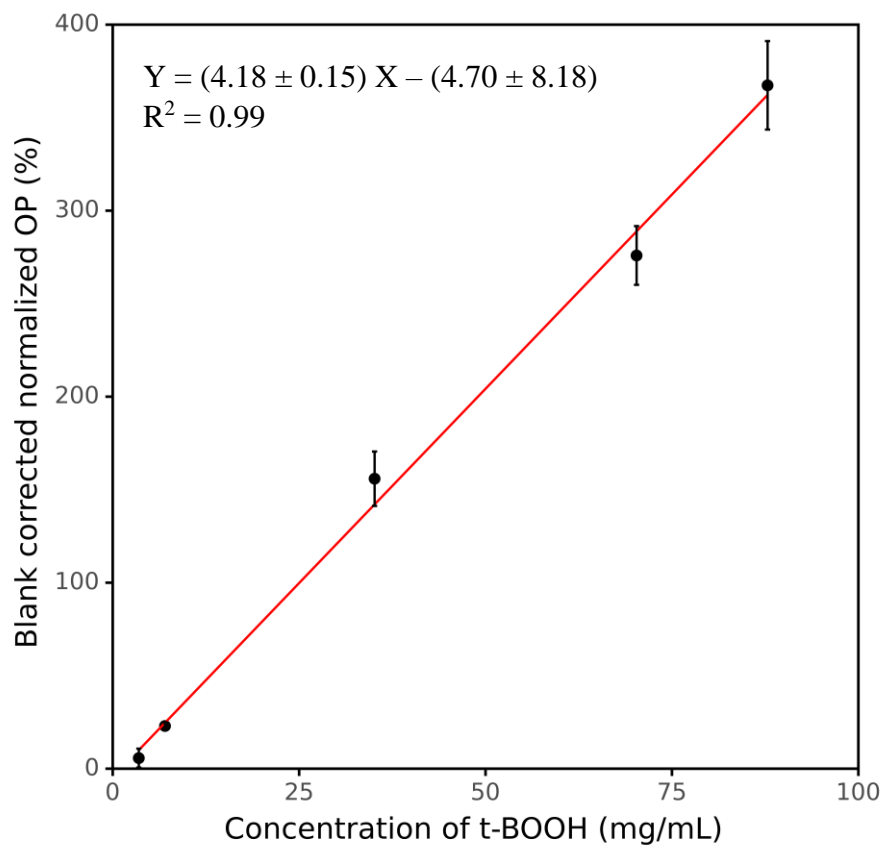



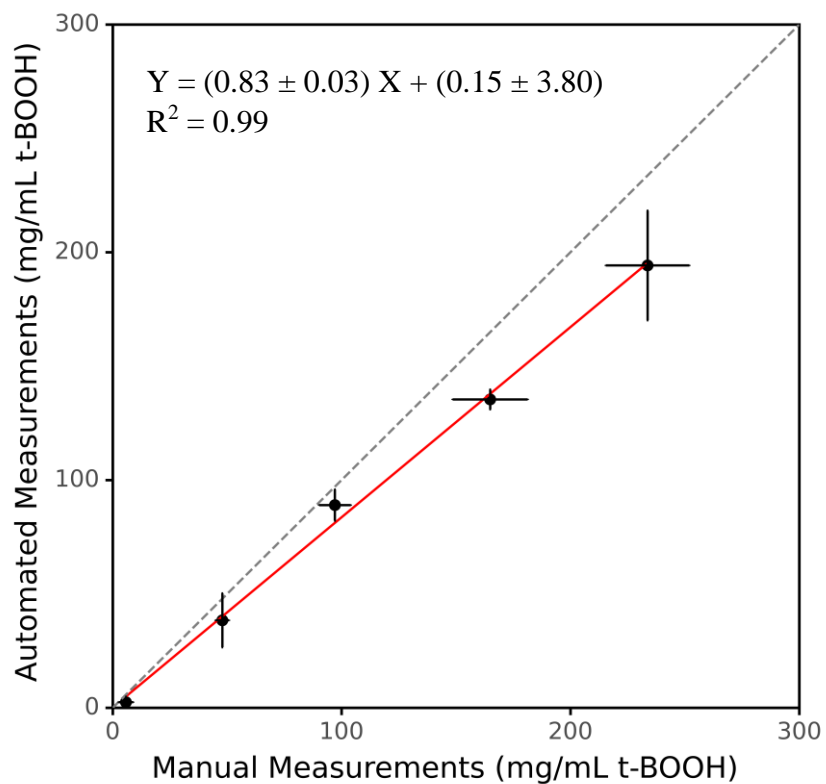
Figure 3: OP as a function of the concentration of t-BOOH, measured by our automated instrument. The values on Y- axis were obtained by dividing absolute fluorescence of the sample by absolute fluorescence of negative control and then blank correcting it (i.e., subtracting 1 from ratio and then multiplying it by 100).

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Figure 4: Comparison between manual and automated measurements of OP for a positive control (t-BOOH). Dotted line represents the identity line.
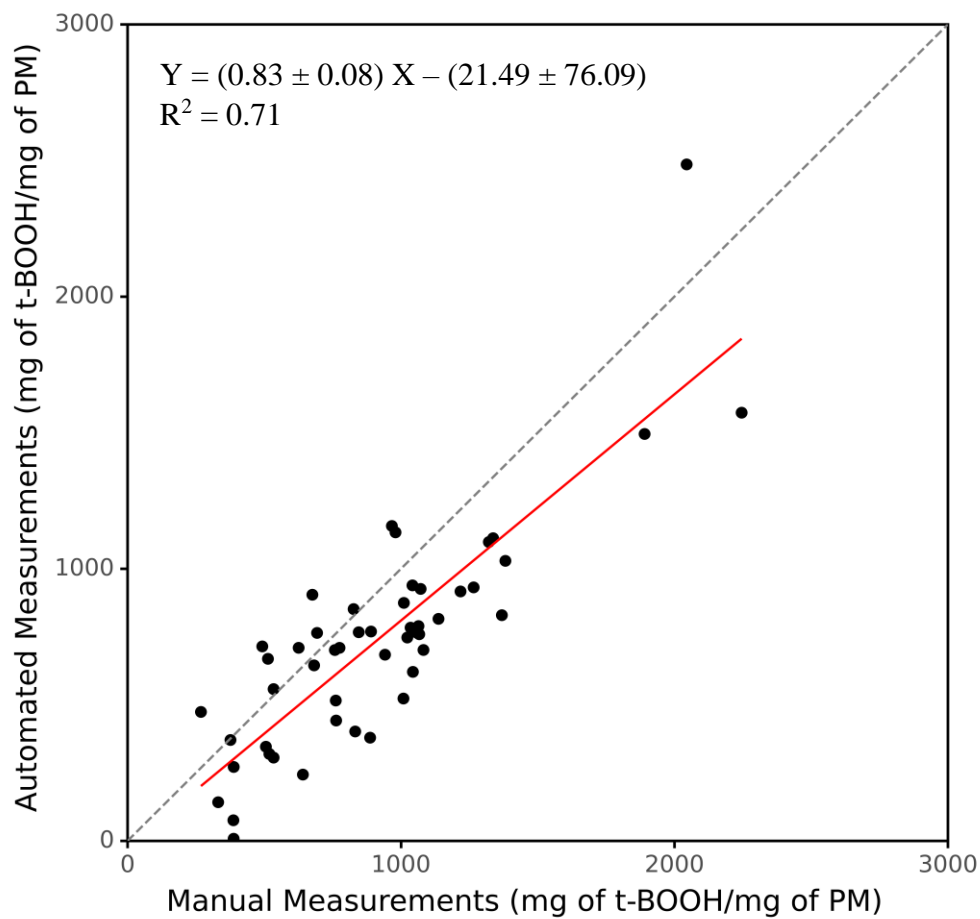
Figure 5: Comparison of the OP for manual vs. automated operation using ambient PM samples (N=50). Dotted line represents the identity line.

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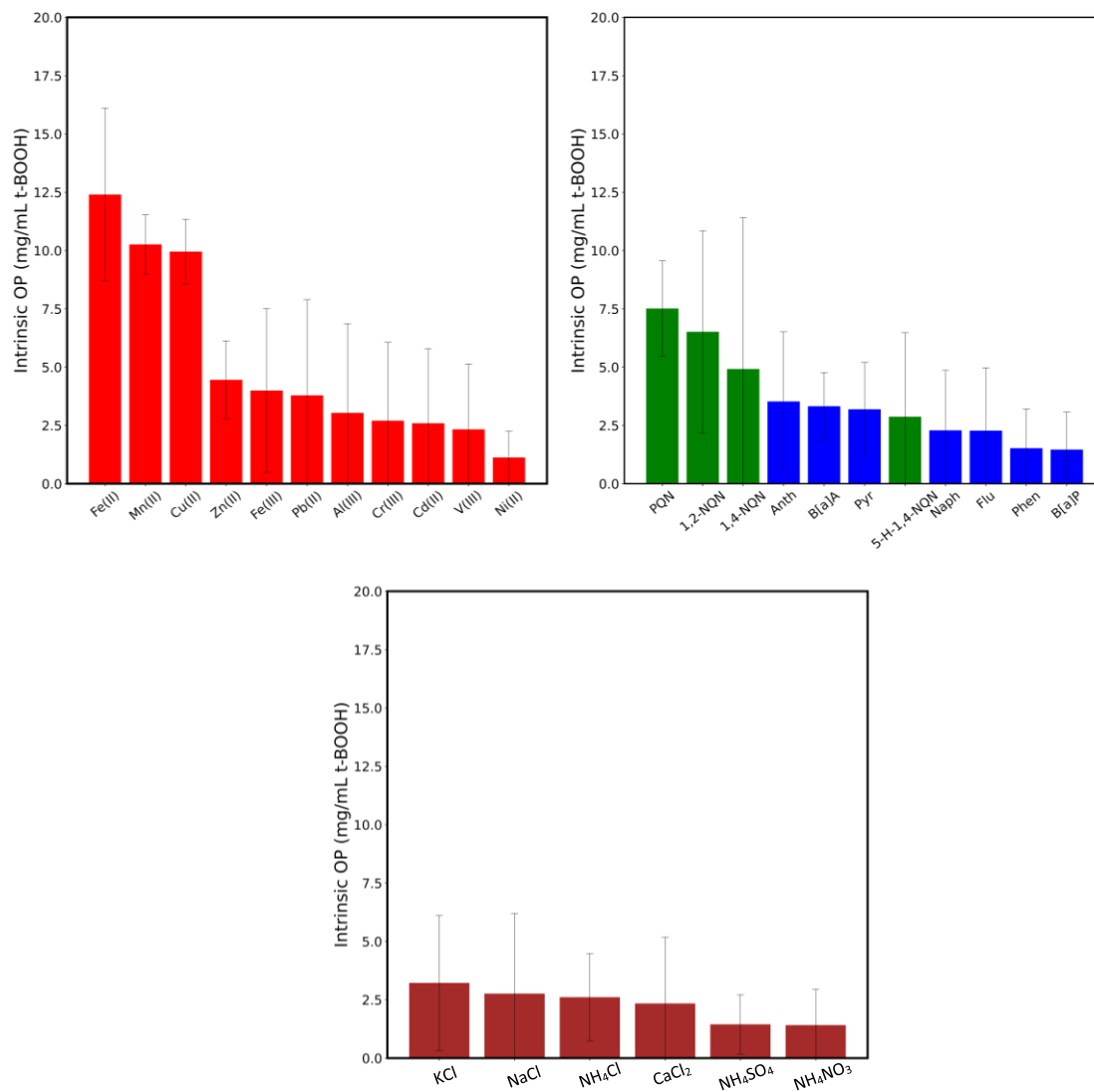
Figure 6: Intrinsic OP of 11 transition metals (panel a), 4 quinones and 7 PAHs (panel b), and 6 inorganic salts (panel c). The concentration in the RV was 0.5 µM for all the metals; 0.2 µM for all quinones and PAHs, and 5 µM for inorganic salts, except for NaCl (1 µM). Error bars represent one standard deviation from the average.

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Table 1: Limit of detection and precision of the instrument obtained through the measurements of field blanks, positive control and ambient PM samples (n = 10)

Sample	Unit	Average	Standard Deviation	LoD	CoV (%)
Field Blank	mg/mL t-BOOH	5.30	0.42	1.26	7.95
t-BOOH	% ROS response	684.71	111.13	-	16.23
Cu (II)	mg/mL t-BOOH	71.05	10.18	-	14.33
Zymosan	mg/mL t-BOOH	18.84	7.15	-	37.97
Ambient PM sample	mg of t-BOOH/mg of PM	402.01	57.93	-	14.41

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