

Response to Reviewers Comments

On dithiothreitol (DTT) as a measure of oxidative potential for ambient particles: evidence for the importance of soluble transition metals

Atmospheric Chemistry and Physics

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Note: Reviewer comments are first, in bold, followed by our responses in plain text.

Anonymous Reviewer #1

1. It should be pointed out that substances present in the test mixture act as electron transfer agents, or catalysts of the reaction which are recycled, i.e, reduced by DTT or superoxide and reoxidized by oxygen species in a cyclic manner (see Li et al 2009a reference).

We do mention that the reaction is catalytic in redox-active species, on pg 11326 starting on line 10: "Oxidation of DTT by metals is a catalytic process, as it is for quinones (Kumagai et al., 2002). For example, in our experiments 1.0 μM of Cu(II) oxidizes 13.2 μM of DTT in 15 min. Cu(II) likely accepts an electron from DTT, forming Cu(I), which rapidly donates an electron to dissolved oxygen to form superoxide and re-form Cu(II)."

Further, some metals can participate in other reactions such as the Haber Weiss and Fenton reactions with superoxide and hydrogen peroxide to complicate the overall stoichiometry. In other words, the consumption of DTT can be due to direct reaction of the analyte components with the electron transfer agent or its reaction with a reduced oxygen species such as superoxide, hydrogen peroxide or hydroxyl radical.

We were also concerned that the formed reactive oxygen species (ROS) would react directly with DTT, causing an overestimate of the oxidative potential of the redox-active species. Kumagai et al. (2002) investigated this possibility. They observed both superoxide and hydrogen peroxide (HOOH) production from mixtures of DTT and phenanthrenequinone (PQN) and used superoxide dismutase and catalase to test if these ROS directly oxidized DTT. The addition of superoxide dismutase with PQN did not change the result, while catalase reduced DTT loss from PQN. While this indicates that HOOH can react directly with DTT, the article did not quantitatively estimate the effect of HOOH, so it was unclear how large this effect may be. If HOOH is very reactive with DTT it will, as the reviewer points out, "complicate the overall stoichiometry" of the assay.

To investigate this issue, we had measured concentrations of HOOH in the reaction mixture of DTT and quinones and found that the HOOH was relatively high (up to 30 μM) by the end of the reaction time. We also found 3-13 μM HOOH in the DTT blank (without metals or quinones added) and wondered if this explained background DTT loss. We tested the ability of HOOH to degrade DTT by adding 5 to 35 μM of HOOH to the DTT assay (without any other chemical species added). We found that the direct reaction of HOOH with DTT accounts for up to 20% of DTT loss in the blank and in PQN samples. The direct reaction of HOOH with DTT, therefore, appears to be small but potentially

significant. We have not measured HOOH production from DTT with metals so we cannot estimate the fraction of DTT activity from HOOH in these solutions.

As far as we know there has been no investigation into the reaction of hydroxyl radical ($\cdot\text{OH}$) with DTT. It is likely that less than 3% of HOOH is converted to $\cdot\text{OH}$ within the 15 minute reaction time (Shen and Anastasio, 2012), corresponding to maximal $\cdot\text{OH}$ concentrations between 0.1 and 1.0 μM (after 15 minutes). We know that iron produces little HOOH (Shen and Anastasio, 2012), likely converting any residual HOOH to $\cdot\text{OH}$ (Charrier and Anastasio, 2011). We found that iron had little DTT reactivity, which indicates that $\cdot\text{OH}$ reactions with DTT may not be important.

We have added a short discussion of this topic at the end of section 3.1 (and in supplemental section S5) along with a figure showing the rate of DTT loss from HOOH (supplemental Figure S6).

2. Although the authors have clearly shown that metals participate in the reactions, their role in a particular sample must necessarily be dependent on the sample itself.

We do state that the DTT response will depend on the specific chemical composition of each PM sample: page 11330, starting at line 7. For a discussion of this and other topics related to ambient metal speciation, please see our response to comment 1 of Dr. V. Verma's comments below.

Preliminary DTT rate data from simple metal mixtures (supplement figure S7) shows a slight enhancement in response from metal mixtures (<20%). Additional experiments are needed to confirm this response over a wider range of concentrations and with more complex mixtures. However, this initial result provides some confidence that the response from mixtures is essentially additive, as we assume.

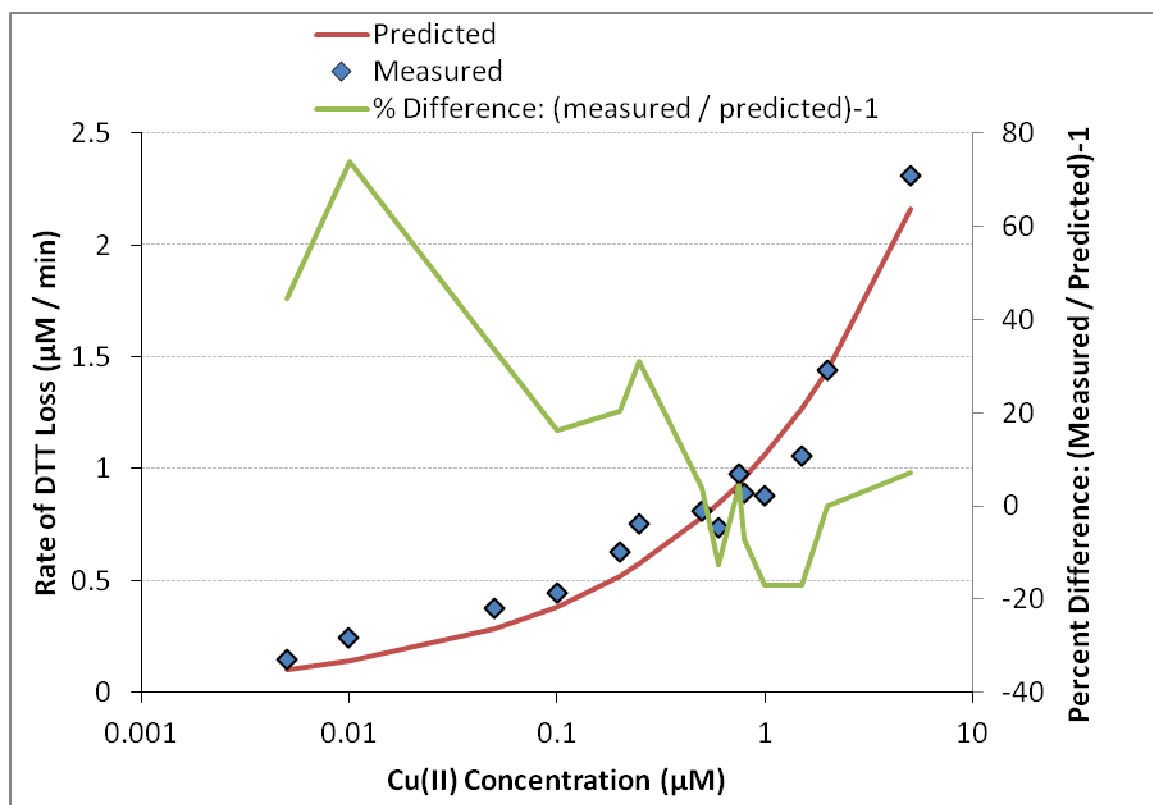
Anonymous Reviewer #2

1. Figure 5, which shows the relative contributions of PM components to DTT consumption in a hypothetical PM sample, is I think a little misleading given the large variability in the concentrations of these species. The authors do, in fairness, explicitly address this issue in the manuscript, but is there perhaps a better way to represent this graphically? Perhaps a bar chart for the relative contribution for each component at the median concentration with error bars showing the range of ambient concentrations measured?

We agree with the reviewer and have added a panel to Figure 5 showing the range of DTT rates associated with each chemical species to better illustrate the potential variability in DTT response.

2. For the calculation of the contribution of copper to the hypothetical PM (and perhaps also for the SJV samples), the copper concentration is in the range where the regression equation seems to be systematically lower than the measurements (Figure 4). This suggests that the copper contribution to the hypothetical particle sample may be higher and that the metals may account for more than 80% of DTT loss in the SJV samples according to this analysis (although see the comments below).

This is an interesting point. As shown in the figure below, the predicted values for the lowest three copper concentrations are 20 to 80% lower than the measured values. All other data are within +/- 20% of their predicted values. While this suggests that the DTT response will be larger than predicted for very low concentrations of copper, the magnitude of change is relatively small (0.04 to 0.10 μM / min) compared to the total DTT response of our "typical" PM estimate at 1.44 μM / min. For atmospherically relevant concentrations of copper, typically between 0.3 and 2 μM , the measured values are within 20% of their predictions.



3. While summing the individual contributions to DTT loss in the PM samples is certainly a useful way of looking at the mixtures to a first approximation, it is clearly possible that the chemistry is more complex than this. Have the authors done any measurements with mixtures of metals and/or quinones and metals to see if the effect of each component is truly additive? Clearly there may be many other components present in the ambient PM samples that increase or inhibit the reaction rate of the metals and quinones.

We have tested several binary mixtures of metals and quinones, and the results indicate that the DTT response is essentially additive in these simple cases. We have added a figure of these results (Fig. S7) and a short discussion of this topic to the manuscript (section 3.3).

4. Is there anything that can be learned from looking at the redox potentials for the components studied? Is there any relationship between this property and the reactivity of the components in the assay?

This is an interesting question. However, as shown in the table below, reduction potentials of individual chemical species do not predict their relative DTT activity.

Compound	Reduction Potential (V)*	Rate of DTT loss (μM DTT / min) from 0.1 μM of Compound**
Fe(III)/Fe(II)	0.77	0.0052
Cu(II)/Cu(I)	0.16	0.38
BQN	0.09	0.013
PQN	-0.12	1.4
1,4NQN	-0.15	0.053
DTT	-0.32	n/a

* Quinones from (Cenas et al., 1994) and metals from (Weast and Astle, 1981). More positive values reflect a greater affinity for taking electrons and being reduced. **Based on regression equations from Table 1 of the manuscript.

Why is there no relationship between reduction potential and DTT activity for these species? One possibility is that the reduction potentials of the species are altered by the presence of ligands. While the redox potentials above are for the species without ligands. Cenas et al. (1994) found that complexation with glutathione can alter the reduction potentials of quinones. Similarly, we have found that ROS production from iron and copper can be altered by the presence of ligands (which may be due to changes in reduction potential or in kinetic rate constants) (Charrier and Anastasio, 2011). DTT complexes with metals, as we mention in the manuscript, and this complex could certainly change the reactivity of the metals.

Another possible reason for the lack of a relationship between DTT activity and reduction potential is that the activity of a compound in the DTT assay might depend upon kinetic rate constants rather than thermodynamic reduction potentials. Because the reaction mechanisms seem more complex than redox behavior we felt it was outside the scope of this manuscript to include a discussion of this issue.

5. The inhibition of quinone reactivity by EDTA is a very interesting result. Is it possible that trace metals not removed by chelex treatment are involved in the reaction of quinones with DTT?

Based on the new DTT mixtures data (Fig. S7) the DTT response from mixtures of metals and quinones is essentially additive. Mixtures of Fe(II) and PQN give the same response as the sum of the individual species, while mixtures of Cu(II) and PQN give a response that is 14% higher than the sum of individual species at a concentration of 0.5 μ M Cu(II) (much higher than trace Cu(II) should be). Thus it is unlikely that residual metals explain the large suppression of DTT response from quinones in solutions containing EDTA.

Comments from V. Verma

1. The authors used individual pure metal solutions. I do not think these stock solutions exactly represent the metals in ambient PM matrix which might have been in various oxidation states together and complexed with other species (e.g. organic compounds). Thus, the actual chemistry of metals' action and their contribution in DTT activity of ambient PM might be substantially different than what is estimated in their experiments.

Certainly our experiments do not reflect the full complexity of metal speciation in ambient PM; this is a nearly impossible task since their speciation in PM is largely unknown. However, we show that DTT loss from six independent PM_{2.5} samples collected at two locations conform closely to our predictions of metal activity. Based on our current data, it appears that our prediction explains the ambient data very well. This agreement suggests that the DTT solution (e.g., 0.1 M phosphate, 100 μ M DTT, and pH 7.4) might drive metal speciation away from their original (PM) state towards a more similar "DTT assay" speciation. Of course more data is needed on this topic, as we state in the manuscript, but we feel we have provided sufficient evidence to show that metals are likely significant in the DTT assay, especially for ambient PM_{2.5}, which has higher concentrations of soluble metals. Note that we did test different oxidation states for iron and vanadium and included these results in our estimates of reactivity in ambient PM extracts; see, for example, Table 1 and Fig. 4.

2. As deduced from the above point, the estimation of DTT activity in a hypothetical PM (Figure 5) based on the limited lab results using only metals and quinones/PAHs, might include huge uncertainties. It appears from the previous work on DTT assay that there are not just quinones which are active in this assay, but an array of organic compounds having a wide range of volatility profiles as shown in Biswas et al., 2009 and Verma et al., 2010. In fact these studies highlight a point that the association between organic compounds and DTT is not purely based on co-variability with the metals as attributed in this study, but there is mechanistic evidence about the active role of these compounds in DTT activity.

Biswas 2009 examined heavy duty diesel particles, and Verma (2011) examined ambient ultrafine PM (<180 nm); redox activity in these particles may be dominated by organic species, compared to the ambient PM_{2.5} which we investigate here. We mentioned this in two places in the manuscript: (1) page 11330, starting at line 7: "...the relative contribution of any given species to DTT loss will depend on the actual concentrations of soluble metals and quinones in the PM. This is an especially important caveat since ambient particulate concentrations of soluble metals and quinones vary by one to two orders of magnitude (Figs. S4 and S5)." and (2) page 11333, starting at line 4: "...much of the current literature has examined ambient ultrafine PM or diesel exhaust, which may be enriched in organics and quinones compared to metals, causing organics to dominate the DTT response in these cases."

In order to clarify our conclusion that metals likely dominate the DTT response in ambient PM_{2.5}, and to emphasize that organics are also important contributors to the DTT response (especially for ultrafine and diesel PM) we have changed the manuscript as follows (additions in italics; deletions in strikethrough): (1) Introduction, pg 11320 line 1: "Some later studies found correlations between DTT loss and the metal content of ambient PM, but these relationships were attributed to covariance between metals and carbonaceous redox-active organic species, which were thought to be responsible for DTT oxidation (Ntziachristos et al., 2007; Hu et al., 2008). *Recent studies have found that heating diesel or ambient ultrafine particles reduces DTT response by 40 to 100 percent, but does not change their soluble metal content, indicating that semi-volatile organics dominate the DTT response for these particle types, which are likely enriched in organics (Biswas et al., 2009; Verma et al., 2011).* Very recently, Lin and Yu (2011) found DTT loss from laboratory solutions of Cu(II) and Zn(II), but not from Fe. While most of the current literature indicates that DTT assay responds strongly to certain organic species, other measures of oxidative potential from ambient PM indicate that metals are most important for ROS production."

(2) Section 3.4, pg 11331, starting at line 17: "Our results indicate that a number of transition metals can oxidize DTT and that metals ~~overall~~ account for the majority of DTT loss from typical ambient PM_{2.5} where *the concentrations of DTT-active metals are generally much higher than those of quinones (Fig. 5b).* This is a relatively new finding in the DTT literature and is in contrast to several past studies that have stated that the DTT assay is insensitive to metals."

(3) We have also added a panel to figure 5 in order to better characterize the variability in DTT response from metals and quinones based on their reported ambient PM_{2.5} concentrations.

3. As authors pointed out themselves that DTT activity measured on the stored filters does not include the contribution from semi-volatile species, I think it is a serious issue which makes it difficult to compare with the estimated summed contributions from the measured metals.

We did not state that our stored filters do not include semi-volatile species, and we do not believe this to be true. On page 11331, line 6 we state "...it is possible that semi-volatile and reactive species such as quinones were lost during storage, which would artificially inflate

the contribution of metals to DTT loss.” We do not know the stability of the organic species in our PM samples, which were stored in the dark at -20 °C. Clearly there is a need to repeat our experiments on freshly collected samples in order to assess the contributions of metals and quinones towards DTT loss.

As reported in Table 3, there is a very wide range of DTT activity (0.005 -0.170 nmol/min/ug) in different studies. Thus, it is difficult to say that the measured activity on those stored filters (0.015 – 0.075 nmol/min/ug) represents the real measurement in ambient PM.

Yes, there is a wide range of DTT activities in ambient PM, but we are encouraged that our measured DTT rates are in the middle of this range. We don't use the Table 3 data to say that our measurements are the “real” values, but rather to illustrate that we do not appear to be missing a significant fraction of their DTT activity. Of course more work needs to be done to study the role of metals in DTT activity, as we point out in this section of the text.

Author's comment for revised manuscript:

While performing additional experiments to respond to the reviewers' comments, we found that the concentrations of three of our quinone stock solutions were slightly different than originally calculated. We have now corrected the data throughout the manuscript and figures: benzoquinone concentrations were reduced by 6.9%, 1,2-NQN concentrations were reduced by 3.5% and the 1,4-NQN concentrations were increased by 20.8%. These corrections caused only very minor changes to some numbers in the tables and text, had little to no effect on the figures, and did not change our main points or conclusions.

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