Notes to Editor:

1. We have highlighted in red all of the changes made for the final submission throughout the main text. These include the changes suggested by the Reviewers.
2. Below we provide a point-by-point response to the Editor and the reviewers.

Anonymous Referee #2

Received and published: 14 June 2016

This paper assesses the toxicity of isoprene SOA through exposure of human lung cells to SOA formed in a chamber. The SOA is deposited directly onto cells and inflammatory biomarkers are monitored. Additional tests with resuspended filter-collected SOA confirms the response is due to particles and not gases formed or originally injected into the chamber (NOx, O3, VOCs). Toxicity is inferred from comparison of the biomarker responses to a seed aerosol (approx. 170 ug/m3 of MgSO4 and H2SO4) to the seed aerosol plus SOA (approx. 170 ug/m3 of acid seed + 30 to 40 ug/m3 isoprene SOA). By essentially noting an increase in the ratio of these biomarkers (SOA+seed/seed) the authors conclude isoprene SOA is toxic to humans. Combined with an earlier paper (Kramer et al., 2016), the authors are asserting that isoprene SOA is toxic. The results of this paper should be of great interest to the air quality community considering the large implications of what is being proposed; biogenic SOA is toxic, and possibly as toxic as diesel emissions (Kramer et al, 2016). Unfortunately, the results are not highly convincing and I fear that these types of publications generally mislead the community since they leave the impression that biogenic SOA is a health hazard, when really, in this case for example, all they show is that cells responded to very high concentrations of a form of SOA produced in these laboratory experiments. For this reason I do not believe this paper should be published without some major discussion up front qualifying the results.

Our intention was not for the reader to assume our conclusion was that isoprene-derived SOA is toxic to humans, but rather it induces inflammatory gene expression in the exposed human lung cells and warrants further study. Although the Kramer et al. (2016) study shows that ROS potential (using the DTT assay) of isoprene-derived SOA is similar to some previously assessed diesel particles, this does not necessarily mean these isoprene-derived SOA components are more toxic to humans. To really make this judgment, *in vitro* and *in vivo* studies are needed. We therefore initiated work to expand on the Kramer et al. (2016) study by examining the potential adverse biological effects within human lung cells resulting from isoprene-derived SOA exposure, with a specific focus on inflammatory-related genes examined in past studies (Doyle et al., 2004; Doyle et al., 2007; Hawley et al., 2014b; Lichtveld et al., 2012). Further *in vitro* studies will allow for more exploration of mechanisms of inflammation, whereas chemical-based assays (like DTT) mimic redox reaction potential within organisms that may lead to oxidative stress and eventually inflammation.

To clarify our intention of our study, we have added the following text to the biological implications section (page 19, lines 418-423):

"In vitro studies such as this one using a direct deposition model cannot fully elucidate mechanisms of lung inflammation and potential pathogenesis but serve as a necessary part of hazard characterization, particularly for a complex air mixture that has not been fully studied (Hayashi, 2005; Paur et al., 2011). Therefore, further *in vitro* studies exploring the health implication of the elevation of *IL-8* and *COX-2* due

specifically to isoprene-SOA exposure are necessary and may in turn justify further extension to in vivo work."

Regarding the high concentrations of isoprene-SOA in the chamber, the high aerosol seed concentration was needed to produce atmospherically relevant compositions of isoprene-SOA. In particular, we selected the conditions of our experiments to mimic compositions of southeastern U.S. aerosol, as shown in Figure 2 and in several recent publications (Rattanvaraha et al., 2016, ACP; Budisulistiorini et al., 2015, ACP). Additionally, a high concentration of particles was needed in the chamber to dose a reasonable amount of particles onto the cells using the direct deposition device.

As stated in the text on page 15, lines 333-335, *"Based on deposition efficiency characterized by de Bruijne et al. (2009), the estimated dose was 0.29 μg cm^{-2} of total particle mass with 23% attributable to organic material formed from isoprene photooxidation (0.067 μg cm^{-2} of SOA)."*

We put this dose into the context of an exposure through the addition of the following text in the discussion on page 16, lines 360-369:

"There are many ways to classify in vitro particle dosimetry based on the various properties of particles (Paur et al., 2011). For this direct deposition study, we chose to classify dose as SOA mass deposition per surface area of the exposed cells to mimic lung deposition. Gangwal et al. (2011) used a multiple-path particle dosimetry (MPPD) model to estimate that the lung deposition of ultrafine particles ranges from 0.006 to 0.02 μg cm^{-2} for a 24-hr exposure to a particle concentration of 0.1 mg m^{-3}. Based on this estimate, a dose of 0.067 μg cm^{-2} of isoprene SOA in our study can be considered a prolonged exposure over the course of a week. In fact, most other in vitro studies, require dosing cells at a high concentration sometimes close to a lifetime exposure to obtain a cellular response. Despite this limitation, in vitro exposures serve as a necessary screening tool for toxicity (Paur et al., 2011)."

So, how toxic is isoprene SOA formed under these conditions, is it a health concern? As noted above, a reasonable conclusion from this work is simply for these concentrations, which are much higher than ambient, human lung cells responded, period. If these results could be directly compared to other forms of SOA, than some discussion of relative toxicity could be presented and a context provided. Lack of context is a major flaw and makes the paper results nearly impossible to interpret (see more on this below).

The present study is an initial step in long planned analysis of the biological impacts of isoprene SOA exposure on lung cells. More comprehensive studies encompassing expanded gene expression analysis and dose-response relationships will further inform the evaluation of the potential for toxicity. We do not intend to make conclusive statements about isoprene-SOA being a health concern when it is still a new topic of study. In addition to the newly added text in the biological implication as stated in a previous comment, we have added this point in the conclusion sections on page 19 on line 427-428 as follows:

"The present study is an initial step in a long planned analysis of the biological impacts of isoprene SOA exposure on lung cells"

Additionally, we have revised the final line in the abstract to more accurately reflect our intentions of the paper as follows:

"The present study is an attempt to examine the early biological responses of isoprene SOA exposure in human lung cells."

Furthermore, these authors recently published a related manuscript (Lin et al., ES&T letter, 2016), except in Lin et al SOA is formed from reactive uptake of MAE and IEPOX and more genes are measured. In a sense, the materials presented here could have been easily folded into Lin et al to provide more context and would have made a much stronger publication (for both papers). How does one put the findings reported in this work in the context of those reported in Lin et al? Why is this paper not cited in this work?

Based on Lin et al. (2016), the purpose of this paper was to investigate the effects of photochemically generated isoprene-SOA using our more complex outdoor photochemical chamber. Furthermore, unlike Lin et al. (2016), this paper focuses on utilizing a direct deposition method to better mimic inhalation exposure as stated in the introduction on page 4, lines 87-88, and on page 5, lines 98-99 as follows:

"The objective of this study is to generate atmospherically relevant isoprene-derived SOA and examine its toxicity through in vitro exposures using a direct deposition device"

"Additionally, for a more atmospherically relevant exposure, isoprene-SOA was photochemically generated in an outdoor chamber to mimic its formation in the atmosphere."

The paper was not cited because at the time of submission, Lin et al. (2016) had not been published. This work is now cited in the text as a potential explanation of the elevations of the two genes studied. This has been added in the biological implications section on page 18, lines 405-413.

"Following the discovery of the potential importance of isoprene-SOA in generating ROS, Lin et al. (2016) showed that isoprene-SOA formed from the reactive uptake of epoxides alters levels of oxidative stress-associated genes, including COX-2 in human lung cells. Oxidative stress caused by ROS plays a major role in lung inflammation and the induction of oxidative stress can lead to IL-8 expression (Tao et al., 2003; Yan et al., 2015). Pathway analysis showed that gene expression of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway was induced in cells exposed to isoprene-SOA (Lin et al., 2016) which has been reported to alter the expression of IL-8 through mRNA stabilization (Zhang et al., 2005)."

Lin et al. (2016) has also now been cited in the introduction on page 4, lines 85-86:

"…and recently isoprene-SOA formed from the reactive uptake of epoxides has been shown to induce the expression of oxidative stress genes (Lin et al., 2016)."

and on page 5, lines 102-104

"An in vitro study that followed supported the potential for isoprene-SOA to affect the levels of oxidative stress genes (Lin et al., 2016)."

The following are some major issues.

What type of SOA is being formed? It is not clear chemically, exactly what type of isoprene SOA is being produced in these experiments. Put another way, how does this isoprene compare to what one would be exposed to the ambient environment (maybe specify specific types of locations). It is not clear how

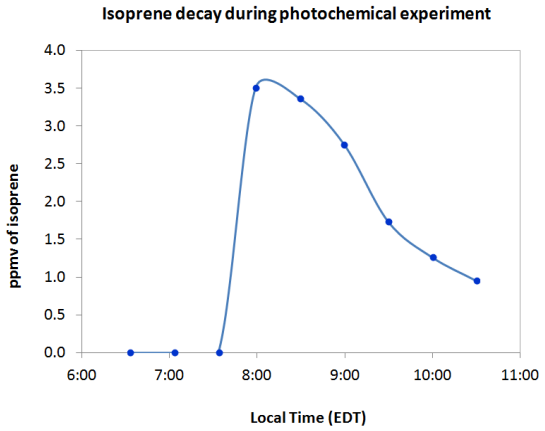
just the presence of certain isoprene tracers observed in both the chamber and at YRK confirm the SOA is identical to ambient (at least identical to what was measured at YRK).

As shown in Figure 2, the vast majority of the isoprene-derived SOA tracers measured and quantified using GC/EI-MS and UPLC/ESI-HR-QTOFMS are derived from the low-NO channel, where IEPOX reactive uptake onto acidic sulfate aerosol dominates. Our purpose of using the Yorkville, GA (YRK) sample as an example was to demonstrate that this was the case as YRK is a low-NO region. Recent SOA tracer measurements from the Southern Oxidant and Aerosol Study (SOAS) campaign made by our group at Look Rock, TN, Centerville, AL, and Birmingham, AL, also show that the IEPOX-derived SOA constituents dominate the isoprene SOA mass in summer, even in urban areas like Birmingham, AL (Budisulistiorini et al., 2015b; Rattanavaraha et al., 2016). In addition, we have shown that even in downtown Atlanta, GA, that IEPOX-derived SOA dominates the isoprene SOA mass (Budisulistiorini et al., 2013; Budisulistiorini et al., 2016). We have added this point concerning IEPOX-derived SOA tracers dominating the isoprene SOA mass in ambient $PM_{2.5}$ on page 13-14, lines 298-316, as follows:

"The chemical composition of aerosol, collected onto filters concurrently with cell exposure and characterized by GC/EI-MS and UPLC/ESI-HR-QTOFMS, are shown in Fig. 2. No isoprene-SOA tracers were observed in the filters collected from dark control experiments. The dominant particle-phase products of the isoprene-SOA collected from photochemical experiments are derived from the low-NO channel, where IEPOX reactive uptake onto acidic sulfate aerosol dominates, including 2-methyltetrols, C_5-alkene triols, isomeric 3-MeTHF-3,4-diols, IEPOX-derived dimers, and IEPOX-derived organosulfates. The sum of the IEPOX-derived SOA constituents quantified by the available standards accounted for ~80% of the observed SOA mass. The MAE-derived SOA constituents 2-methylglyceric acid and the organosulfate derivative of MAE, derived from the high-NO channel, accounted for 1.4% of the observed SOA mass, confirming that particle-phase products generated were predominantly formed from the reactive uptake of IEPOX onto acidic sulfate aerosols. As demonstrated in Figure 2, all the same particle-phase products are measured in the $PM_{2.5}$ sample collected in Yorkville, GA (a typical low-NO region), demonstrating that the composition of the chamber-generated SOA is atmospherically relevant. Recent SOA tracer measurements from the Southern Oxidant and Aerosol Study(SOAS) campaign at Look Rock, TN, Centerville, AL, and Birmingham, AL, also support the atmospheric relevance of IEPOX-derived SOA constituents that dominate the isoprene SOA mass in summer in the southeastern U.S. (Budisulistiorini et al., 2015a; Rattanavaraha et al., 2016)."

More specifically, it seems that isoprene OA presented in this paper is formed with NO injected into the chamber, with no additional HO2 source. Was isoprene decay measured over time? Under what NOx conditions are most isoprene reacted, and what does the RO2 react with? Self-reaction, with NO, or with HO2? From Figure 1, about half of the SOA is formed where there is NOx. Even after NO is zero, given the large amount of isoprene injected (several ppm), the RO2 + RO2 could be prevalent. It's not clear how "low-NOx" products (RO2+HO2) can be formed in these experiments, and that IEPOX-derived SOA can account for 80% of the SOA formed here. Is an HO2 source added to the chamber? Presumably the SOA in Yorkville is formed under low NOx conditions. More discussed regarding the chamber reactions are needed to justify relevancy to ambient data.

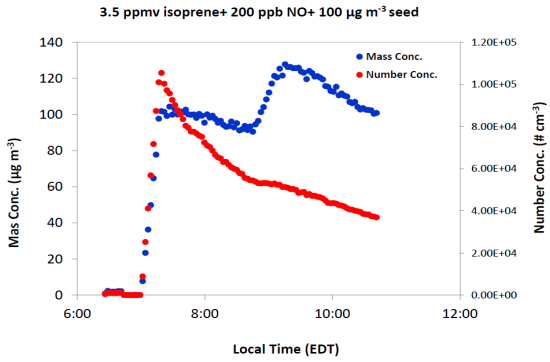
Isoprene decay was measured over time to identify its presence in the chamber and whether it reacted. The following is an example of the measured isoprene decay in our photochemical experiments as measured by the GC/FID.



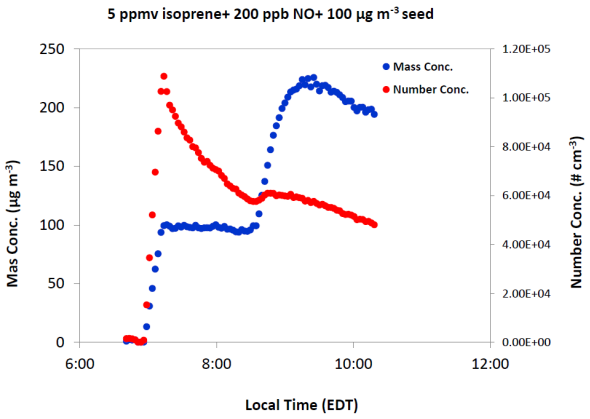
Our goal of using the high isoprene/NO ratio was to create an SOA composition similar to the southeastern U.S. Our chemical results shown in Figure 2 clearly support this, and since we were focusing our cellular exposures to isoprene SOA, we believe we achieved our goal.

To clarify the reviewer's questions, HO_2 in the chamber was rapidly formed during the OH-initiated oxidation (i.e., photooxidation) of isoprene. Although we did not add additional sources of OH to the chamber, the photolysis of nitrous acid (HONO) formed at chamber walls provides an intrinsic source of OH radical formation. In addition, the photochemical chamber experiments were conducted at high relative humidity (~70%). The photolysis ozone in the presence of water vapor also provides a source of OH radical.

We believe the majority of RO_2 in our chamber experiment reacts with HO_2, as demonstrated by our particle phase chemical characterization data. However, we agree with the reviewer that the RO_2+RO_2 self reactions could be prevalent in a chamber experiment when the initial isoprene/NO ratio is too high and produces aerosol via nucleation. Based on our aerosol size distribution data, the SOA formation in our chamber experiment (with initial isoprene of 3.5 ppmv) was mainly via condensation without new particle formation.



When we increased the initial isoprene/NO ratio (increasing the initial isoprene to 5 ppmv), we did observe new particle formation.



Thus, we are confident that our chamber-generated isoprene SOA for cell exposure has an atmospherically relevant chemical composition.

Compare the SOA in these experiments to that presented in their previous paper in Atmos Env (Kramer et al., 2016) where these authors assert that isoprene SOA is as toxic as diesel, based on the DTT assay. It seems the experimental conditions are similar to the manuscript here. However, apparently 2-methylglyceric acid is formed in these experiments (Figure 2 of this manuscript), but not in Kramer et al (Figure 2)? Why? Please provide detailed and specific comparisons on the chemical form of the isoprene formed in these two studies.

The SOA analyzed in Kramer et al. (2016) was the same SOA generated for our cell exposures. 2-MG was present in all SOA but was not labeled in Kramer due to its small quantity. However, its peak was identified in our paper to show its presence as an SOA tracer even though it is minimal due to the dominance of IEPOX derived SOA.

Are experiments done under dry or humid conditions?

The isoprene SOA were generated under humid conditions to ensure cell viability for exposure. Relative humidity in the chamber was at least 70% during isoprene injection for photochemical experiments as stated on page 8, line 172, and added on page 6, lines 135-137:

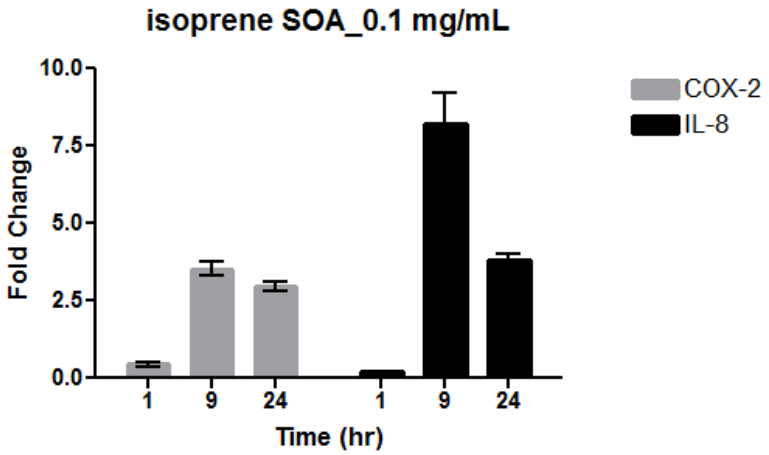
"This chamber experiment was replicated on three separate sunny days with temperatures ranging from 24.9°C to 26.8°C with a relative humidity of approximately 70% in the chamber"

Issues with Cell Details: The passage numbers used in these experiments seem very high. Please comment on the passage numbers and how determined.

Records of passage number were kept each time cells were passaged and only passages 52-60 were used for our exposures. Passage numbers in literature can be much higher such as in Wu et al. (2011) which used BEAS-2B cells having passage numbers 70-80.

From the results, it doesn't look like the time point is maximized for COX-2. Why was the specific time point used in these experiments they chosen? Is it representative of exposure? Is it to maximize gene expression, etc?

For the purpose of this study, nine hour post-collection time point was chosen to be consistent with Lichtveld et al. (2012) who used the same exposure device on this outdoor chamber facility. There will be changes to the levels of mRNA expressed at any given point of collection and gene expression does have a time profile. Because of the nature of these chamber exposures, one post-collection time point was chosen and the isoprene-SOA exposures were compared to the dark control exposure for that specific time. However, a time course analysis was conducted using resuspension techniques to show that *COX-2* and *IL-8* are maximized at 9 hours as shown in the graph below.

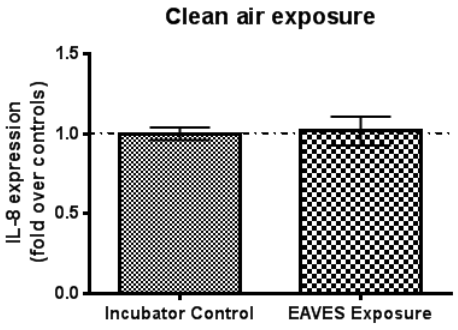


For the filter resuspension exposure, the cells are seeded 2 days prior to exposure and there's no mention of media change. If nutrients are not replenished are the cells highly stressed?

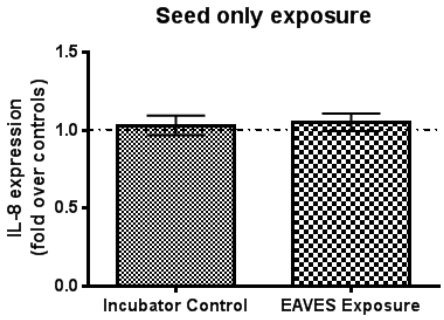
The BEAS-2B cell protocol involves a change of media every 3-4 days for cell culture. All cells for the exposures and the corresponding controls were handled in the same way.

Did the cells exhibit inflammatory response to the acid seed? Ie, what was the fold increase in the biomarkers for the dark seed experiments to the cells exposed to a completely clean chamber? This might give some sense as to the importance of the fold increase in SOA relative to dark (just seed) aerosol.

The effect of the acidic seed was tested in our resuspension exposures and determined to have minimal effects (no significant differences) on the BEAS-2B cells when compared to media only. The following graphs shows the fold changes of *IL-8* expression of cells exposed to a clean air chamber compared to cells left in an incubator and cells exposed to the acid seed only compared to cells left in an incubator. There were no significant differences in *IL-8* expression based on the EAVES operation and the seed only exposure. Based on this, we determined that the dark only exposures served as the best control to ensure that no other effects such as the particle concentration, equipment handling, and cell handling had effect on the cells.



Unpaired t test with Welch's correction	
P value	0.8729
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed



Unpaired t test with Welch's correction	
P value	0.8184
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed

Issues with context: The authors state that a dose of 0.067 ug/cm2 to their simulated lung surface is sufficient to induce a response. What is the relevance of this number? Ie, can it be compared to ambient concentrations in any manner, or to say a minimum dose for responses of differing aerosol components in which similar health endpoints were measured? The lung surface area is very large. To have this kind of dose spread throughout the lung would require exposure to an enormous mass of isoprene SOA. The number 0.067 ug/cm2 has little meaning without some context (see more on lack of comparison to other work below).

In vitro studies require dosing cells at a high concentration sometimes as high as an exposure experienced over a lifetime (Paur et al., 2011). When compared to other similar *in vitro* studies our dose is much lower. We have compared the dose used in our study to those of diesel studies as stated on page 17, lines 377-388 as follows:

"In a similar study using the EAVES, normal human bronchial epithelial (NHBE) cells exposed to 1.10 µg cm^{-2} diesel particulate matter showed less than a 2-fold change over controls in both IL-8 and COX-2 mRNA expression (Hawley et al., 2014b). In another study, A549 human lung epithelial cells were exposed by direct deposition for 1 hour to photochemically-aged diesel exhaust particulates at a dose of 2.65 µg cm^{-2} from a 1980 Mercedes or a 2006 Volkswagen (Lichtveld et al., 2012). Exposure to aged Mercedes particulates induced a 4-fold change in IL-8 and ~2-fold change in COX-2 mRNA expression, while exposure to aged Volkswagen particulates induced a change of ~1.5-fold in IL-8 and 2-fold in COX-2 mRNA expression (Lichtveld et al., 2012). Although the differences in cell types preclude direct comparisons, the finding of significant increases in COX-2 and IL-8 expression at doses much lower than reported for comparable increases in inflammatory gene expression levels induced by photochemically-aged diesel particulates is notable."

In addition, to further put the dose in an exposure context, new text has been added on page 16, lines 360-369 as follows:

"There are many ways to classify in vitro particle dosimetry based on the various properties of particles (Paur et al., 2011). For this direct deposition study, we chose to classify dose as SOA mass deposition per surface area of the exposed cells to mimic lung deposition. Gangwal et al. (2011) used a multiple-path particle dosimetry (MPPD) model to estimate that the lung deposition of ultrafine particles ranges from

0.006 to 0.02 µg cm^{-2} for a 24-hr exposure to a particle concentration of 0.1 mg m^{-3}. Based on this estimate, a dose of 0.067 µg cm^{-2} of isoprene SOA in our study can be considered a prolonged exposure over the course of a week. In fact, most other in vitro studies require dosing cells at a high concentration sometimes close to a lifetime exposure to obtain a cellular response. Despite this limitation, in vitro exposures serve as a necessary screening tool for toxicity (Paur et al., 2011)."

The final line of the paper illustrates the limitations with lack of context, it states: Taken together, this study demonstrates that atmospherically relevant compositions of isoprene-derived SOA can induce adverse effects, suggesting that anthropogenically-derived acidic sulfate aerosol may drive the generation and toxicity of SOA

This seems too strong a statement, all one may infer from this work is that if you expose lung cells to very high doses of the specific type of isoprene SOA formed in these expts (see questions how atm representative it is), they respond. But cells will respond to many things. Context through relative toxicity could have been provided by doing two identical experiments, but with differing SOA types. Say isoprene vs some aromatic species found in incomplete combustion. There is some discussion near the end of the paper attempting such a comparison, ie comparison to aged diesel exhaust (Lichtveld et al, 2012), but no definitive answer on the relative toxicity of isoprene SOA can be made because the contrast does not involve identical experiments, (ie, different cell lines were used) making it is difficult to conclude that any observed differences are due solely to the exposure of differing SOA chemical composition. I believe same, applies with Hawley et al, who used primary cells and not a cell line.

Along with the changes in text as stated above in the biological implication section, the final line of the paper has been changed for further context as follows (page 20 lines 438-440):

"The results of this study show that, because of its abundance, isoprene SOA may be a public health concern warranting further toxicological investigation through in vitro or in vivo work"

The authors agree that making direct comparisons is difficult when using different cell lines. The choice to use BEAS-2B cells over A549 was made because BEAS-2B is an immortalized non-cancerous cell line, which provides more consistent and representative results for our study design (compared to the cancerous A549 cells or to the primary cells, where responses are subject to interindividual variances). The purpose of this study was not to determine relative toxicity but to identify isoprene-SOA worthy of further *in vitro* and *in vivo* studies, as clarified in the abstract with the inclusion of the following statement on lines 46-47.

"The present study is an attempt to examine the early biological responses of isoprene SOA exposure in human lung cells"

The authors further support their observations of inflammatory response due to isoprene SOA by noting they also find that the DTT response for SOA is higher than diesel ((Kramer et al., 2016). What they fail to note is that other analysis, based on ambient data, show a DTT response to isoprene SOA, but it is vastly smaller than the DTT responses to other sources, such as those from incomplete combustion (Verma et al., ES&T, 2015). This again demonstrates the limitation of this work due to lack of context; yes there may be a response to isoprene SOA, but how important is it? These authors may note that the Verma work involved only water-soluble extracts, whereas their experiments involved methanol, and so the difference could be due to non-water soluble isoprene SOA components. But the authors note here

that the SOA constituents are “water-soluble (lines 329-330). . . and remain well mixed in the cell medium”.

We would like to point out that in Verma et al. (2015) the isoprene SOA was identified from aerosol mass spectrometry (AMS) measurements through positive matrix factorization (PMF) analysis. The isoprene SOA factor, or more precisely the 82 factor, has been demonstrated to originate from IEPOX SOA (Budisulistiorini et al., 2013). Also, in our recent study (Lin et al., 2016) we show that IEPOX SOA is a weak inducer of cellular oxidative stress gene expression in BEAS-2B cells. Thus, the conclusions from these studies are fairly consistent. However, as shown in Kramer et al. (2016) isoprene SOA has higher DTT activity compared to IEPOX SOA. The difference between Verma et al. (2015) and Kramer et al. (2016) may be due to organic peroxides not measured as part of the isoprene SOA factor (82 factor) (Riva et al., 2016). Therefore, the inflammatory response (i.e., induction of *COX-2* and *IL-8* gene expression) observed in the present study from isoprene SOA exposure could be modulated by oxidative stress. Additional work is required to validate this hypothesis.

We agree with the reviewer that the non-water soluble isoprene SOA components, such as oligomeric species (Lin et al., 2014) could have been much enriched in methanol extracts. We would like to clarify that a majority of isoprene SOA constituents are water-soluble because of their highly oxygenated character, and they appear to be much more hydrophilic compared to diesel particle extracts and remain well mixed in the cell medium during our resuspension exposure processes.

Typos: Line 307, should it be Fig 4 and following, Fig 4 should be Fig 5?

We thank the reviewer for catching this error. This has been corrected in the text.