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Atmospheric Chemistry and Physics Discussions

Interactive comment on "Gaseous VOCs rapidly modify particulate matter and its biological effects – Part 1: Simple VOCs and model PM" *by* S. Ebersviller et al.

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Referee's Comment: The manuscript describes in vitro experiments with a pulmonary cell line (A549) exposed to different kinds of gaseous and particulate model components relevant for ambient conditions. The experiment is very well designed and performed. Especially the systematic approach of investigating cellular response due to gas-phase and particulate phase components separately as well as under combined exposure conditions with gas-phase and particle-phase selective samples is innovative

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and interesting. Furthermore, the fact that the authors utilize air-liquid interface cell cultures (instead of the traditionally used submerged cell cultures) to examine the toxicity of airborne toxins under physiologically realistic conditions is essential for the merit of this study. This study provides well-stratiïňĄed data for studying the interplay between gas phase and particulate phase components in terms of cellular in vitro toxicity. Based on this unique data set it presents direct and convincing evidence for the “effect modiïňĄcation” phenomenon, the enhanced toxicity of PM in the presence of toxic gaseous components. The ïňĄndings of the authors are innovative and relevant for a deeper understanding of the processes governing the toxicity of ambient particles.

Author’s Reply: Dr. Schmid, thank you for your excellent comments on this manuscript. It is very good to have a pulmonary aerosol investigator helping to clarify our communication of this trans-disciplinary subject.

Referee’s Comment: MINOR ISSUES 1) I concur with referee #1 that the authors should discuss the experimental details of the two systems for gas-phase-only (GIVES) and particles-only exposures (EAVES) in more detail. The issue of having negligible particle-related dose in the GIVES and negligible gas-phase dose in the EAVES is the basis for the study. Hence, it requires more rigorous discussion especially with respect to the following aspects: - Please explain, why the EAVES can be expected to have much less gas-phase deposition than the GIVES, since gas-phase components will be present in both systems and the exposure times are not that much different (4h versus 0.5-1h). - What are the particle deposition efiňĄciencies in both systems? Typically this will depend on particle size. The geometric number mean diameter of the MOA particles is about 200nm (Fig. 2). BUT mass or possibly even better surface area is likely to be toxicologically more relevant dose metric than particle number. For the relatively broad size distribution presented in Fig 2 the geometric mean surface area and mass diameters will probably lie between 500 and 1000 nm. At this size range not only diffusion, but also sedimentation may play a role in particle deposition. Was this considered? - This information should then be used to estimate the cell-delivered

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doses for both gaseous and particle phase components as one of the most important parameters for assessing substance related toxicity.

Author's Reply: As stated in our response to Referee one, it is clear that we should have provided more information in the reviewed manuscript about our unusual biological exposure systems. We are more familiar with publishing in journals with very limited word counts and tend to avoid repeating anything that can be accessed in published work. Thus, in the submitted manuscript, we did cite peer-reviewed publications in which the design details and test findings for the EAVES sampler have been described, discussed, and supported; in the revised manuscript we have added additional citations on its testing. We also cited three published papers in which the GIVES sampler had been previously described and used; in the revised manuscript we have added additional citations. The GIVES is a commercial device and we had provided the manufacturer and model number. We also gave a six-line brief description and a one-line explanation of why PM does not cause an exposure in the device.

In response to both Referees, in the revised manuscript in section 2.6.1 we have expanded the GIVES discussion (Section 2.6.1) by reporting calculations and including a citation to settling times of mass-mode-diameter PM in the chamber (2.86x10ˆ(-3) cm/s for 1 μm particles, of which there were about 82 particles per cmˆ3 in our chamber tests) to estimate, applying various simplifying assumptions, how long the exposure would have to be in the GIVES to give a deposition similar to that in the EAVES PM sampler. Depending upon the assumptions, this time varies from 150 days to 12 hours, and the latter assumes that all particles that entered the GIVES be deposited uniformly across the footprint of the exposure chamber, which of course does not happen. Thus, cells in the GIVES do have some exposure to PM, but the extent is so limited that it cannot be detected by biomarker changes, making this sampler 'virtually' a gas-only sampler.

With regard to the EAVES sampler, we have added a brief summary of its operational principles and a summary of the previous test findings. This was done in Section 2.6.2

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in which we added a brief paragraph describing the physical and flow operations of the EAVES sampler and its environment. This as followed by an 18-line summary of the published peer-reviewed findings for the operational characteristics. We also added statements that clarified that cells in the EAVES do have some exposure to the sample gas, but it has been repeatedly demonstrated in testingâĂŤincluding this study's results—that such exposure is so limited that the cells show no detectable response to gases for the 1-h exposure time and for the unique flow conditions of the sampler. These outcomes make the EAVES sampler 'virtually' a PM-only exposure device. Further, in the largely revised Section 4, we have in both the TOLALD and ACRO discussions identified comparisons of the experimental graphical results in this manuscript where this operational feature of the EAVES is clearly evident.

PM deposition efficiencies for the EAVES device were given in the referenced method paper (de Bruijne, et al. 2009). Recognizing that this device is a full electrostatic precipitator its deposition efficiency is very high and mildly size dependent. Also, not all the aerosol in the sample is deposited on the cell wells, but SMPS tests of most test and sample aerosols showed that 90% of all particles between 19 and 882 nm, representing 98% of the total mass were deposited in the device. Test results for the cell wells with fluorescent-labeled polystyrene latex spheres of size 198 nm and 513 nm at air concentrations of 1.71 and 2.36 mg/m3 gave 1.96 $\pm$ 0.50 and 4.11 $\pm$ 0.84 μg / h in each cell well. These correspond to collection efficiencies of 35.1 $\pm$ 9.3 and 47.0 $\pm$ 9.8 %.

We agree completely with the Referee's statement that determining the cell-delivered dose is vitally important to accurately evaluating dose-response characteristics of exposures. The behaviors of gases and PM in the GIVES is a complex fluid-dynamic problem that will not, we feel, provide a definitive answer to the question of the dose of each toxicant that reaches the interior of the cell and may, therefore, distract the reader from the main purpose of the manuscript – the observation of the 'effects modification' phenomenon.

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In addition, we share the Referee's interest in examining the effects of surface area and mass dosing on cellular response, but do not feel that an extensive additional analysis of these parameters is appropriate to this demonstration of principle work. We hope to examine those factors in future studies.

Our primary purpose for including the size distribution plot shown in Fig. 2 was to assure the reader that the PM the cells were exposed to was unchanged in the VOC mixtures (relative to the MOA exposure in clean air). As stated in the MS, this removes the question of size-dependent PM effects from our assessment of 'effects modification.'

Referee's Comment: 2) While the interpretation of the p-Tolualdehyde data with and without MOA particles seems straight forward, the corresponding Acrolein data are much more puzzling as recognized by the authors. The IL-8 response (Fig. 6a) suggests that Acrolein is not signiïňĄcantly taken up by the MOA particles – as suggested by gas-particle portioning theory. On the other hand, Fig. 6b seems to indicate that the MOA particles, which are not toxic per se (see Clean air + MOA control), are substantially more toxic in the presence of Acrolein, which seems to suggest a signiïňĄcant partitioning of Acrolein to MOA. This seems contradictory. Furthermore, when looking at the LDH data (Fig 7) the gas-phase response is signiïňĄcantly reduced in the presence of MOA (Fig 7a) and the Acro+MOA response is larger than the Clean Air + MOA response. Both effects indicate signiïňĄcant uptake of Acrolein by MOA, which – again - is inconsistent with theoretical predictions of Acrolein partitioning. Hence, only the results from Fig 6a seem to agree with partitioning theory. The authors argue (P. 5084 LL29), that these inconsistencies are likely related to the higher sensitivity of LDH to small concentration changes relative to IL-8. Hence, the small amount of PM-adsorbed Acrolein has no effect on IL-8 reduction due to gas phase Acrolein (Fig. 6a), but a notable effect on LDH (Fig. 7a). But this explanation is not sufiňĄcient, since – to my understanding – LDH release is a much more severe insult to the cells than IL-8 release. Hence, LDH should not respond to small amounts of a toxic substance. Thus

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there should be no response in Fig. 7b. Furthermore, this “supposedly small” amount of Acrolein taken up by MOA induced the most severe IL-8 induction out of all of the cases investigated here (Fig 4a and 6a). Thus I cannot see a possible scenario reconciling all of these ïňĄndings. I would suggest that the authors admit to this inherent inconsistency of the data and include the following issues in the discussion:

Author’s Reply: It appears that there has been a miscommunication. In the reviewed manuscript, we did not intend to imply that cytotoxicity occurs at lower exposure thresholds than our marker of inflammation. On the contrary, we have observed significant inflammatory responses at exposure concentrations that were unable to elicit a significant response for cellular damage (as stated LL 1 of page 5084). But see more response to this in discussion below. In response we have made several changes. We do not believe that there is “inherent inconsistency” in our data. We do suffer mild ambiguity from using an accepted but older method to access responses to LDH as the Referee pointed out in another comment below. We have just become aware of this more absolute approach and are adopting in future work. The revised manuscript now includes a discussion of this shortfall in LDH assessment in section 4.1. In light of the questions or understandings expressed by both Referees, we have re-organized and re-focused Section 4 Discussion to provide a more easily followed step-by-step analysis of the gas and PM effects. Furthermore, we have inserted at the fourth step in the analysis (the evidence for the transfer of toxicity from gas-phase to particle-phase) in section 4.1, a clarification of the relationship between the two biological endpoints: IL-8 requires living cells to express; LDH is only expressed when cells die. While both markers can initially increase, as cells die off so does the expression of IL-8. We now explicitly walk the reader through the application of this understanding and show how it affects the interpretation of the biological response data. In the more confusing case of ACRO, we present and discuss alternative conclusions that can be made from the data, but ultimately settle on accepting the decrease in LDH with ACRO with PM relative to just ACRO gas-only exposure as a strong indicator that much ACRO had moved to the PM-phase. These then present a much clearer understanding of both TOLALD

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and ACRO results. We moved questions of failure of partitioning theory to account for a number of recent atmospheric observations, including ours here, into a second section, 4.2, of our Discussion. We now present some evidence that "good old standard partitioning theory" is likely not adequate for explaining our results, which would not be so obvious without the very important biological response data.

Referee's Comment: - Could these discrepancies be related to the different exposure times for GIVES and EAVES and hence the differences in cell-toxin contact times (9h incubation time plus 4h and 0.5-1h exposure time, respectively)?

Author's Reply: Please refer to our response to a similar comment made below.

Referee's Comment: - Could the fact that exposure F was the only case with RH > 90% (in the chamber), while all other exposures were performed with RH < 75% could have something to do with these "strange" results? In fact the authors hint on potential relevance of RH on P. 5085 LL2, but they fail to relate this statement to this peculiarity in RH conditions.

Author's reply: We have now revisited the text and attempted to clarify this point. Please refer to our extensive response to previous comment from which we now explain our results in much clearer terms and clarify the conditions, so that we reject the notion that our results are "strange" and therefore they are not in need of hypothesize alternative causes.

Referee's Comment: 3) Unlike Referee #1 I did not feel that the concept of the experiment design was described in an unnecessarily repetitive way. Sometimes repeating an important conceptual issue helps understanding the data better. However, I was missing some essential experimental parameters such as: - Chamber setup: length of sampling tubes, air ïňĆow rates and estimated losses in the tubing. Is the chamber convectively mixed to guarantee homogeneous distribution of pollutants? - At what conditions was the SMPS operated: Sheath, aerosol ïňĆow rate, cut-off diameter of inlet impactor? The width of the size distribution indicates that possibly a substan-

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tial part of the aerosol mass distribution is beyond the SMPS diameter limit of about 900nm. Was any other sizing instrument such as an aerodynamic particle sizer used to investigate the supermicron size range?

Author's reply: Thank you for your support of our 'hammering on' the conceptual issues and its relationship to our experimental design.

Sampling lines are all ca. 20 feet long (about 6 meters) from end to end.

All sampling flow rates were given in the referenced method papers. We defer to the editor as to whether or not to add this information to this manuscript explicitly.

The SMPS was operated at 0.3 Lpm sample flow rate and 3 Lpm sheath flow rate. The impactor cut off diameter is 0.071 cm. This information has been added to the Methods section of the manuscript.

We do not have routine access to an APS, but a TSI Dusttrac was used to verify that super-micron PM was not present. As we were not able to collect Dusttrac data for every experiment, and had not evaluated the Dusttrac's bias or signal drift at the time of this work, however, we treated the data as a qualitative measurement and did not include it here.

Referee's Comment: - As a related issue, the authors report both particle mass concentration and size distribution. These values should be combined to estimate the effective particle density to provide evidence that both values are consistent. In fact the authors state that this was done (P 5075 L 8-9), but they never report the results.

Author's reply: The PM mass concentration measurements were obtained from gravimetric measurements of total suspended particulate filters, while size distribution data were obtained from SMPS (an optical measurement). While we are aware that it is common practice to combine the data from these types of sources, we feel that the uncertainties inherent to each data type are incompatible and, therefore, recommends against this practice.

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Thus, while we do perform these comparisons as an internal quality check, we do not report the data as a quantitative measurement and limit our comparisons to data within each source.

Moreover, as stated above, our objective in including the size distribution plot was to demonstrate that the MOA particles did not 'grow' significantly when mixed with each VOC in the chamber (relative to MOA in clean air). This allows us to discount size-specific effects as causal to the observed modifications in response.

Referee's Comment: 4) Biological issues: - Please provide information on the cell culture conditions such as cell density, culture medium and conditions. Transwell inserts by Costar were used, but no information is provided regarding type of the wells (6-well, 12-well) and the pore size and material of the perforated membrane of the inserts. - How long were the A549 cells kept at the air-liquid interface prior to exposure? - The cells were incubated for 9h after exposure. For an exposure time of 0.5-1h and 4h in the EAVES and GIVES, respectively, this results in a total toxin-cell contact time of 9.5-10h and 13h. Does this difference in contact time have an effect on the measured response? Did you check varying the incubation time by +/-3h to see how this may affect the measured response? I would expect that for such short incubation times +/-3h this has a signiïňĄcant affect on the measured IL-8 and LDH levels.

Author's reply: This information was made available as references to method papers to avoid having an exhaustively long methods section in this manuscript. We defer to the judgment of the editor as to whether or not to include the information explicitly.

We agree that altering the incubation time by more than 30% is likely to have a marked effect on the measured response. The exposure/rest period regimes employed in this work were developed over a long time as our standard exposure protocols to facilitate comparison of results between our studies.

Moreover, the durations of exposure (as described in the manuscript introduction and response to question 2 above), are not as straightforward as they seem. The manner

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in which 'contact time' is defined can have a significant impact on how it is assessed. We could just as easily define 'contact time' as the amount of time the cells were in direct contact with each pollutant. In that case, the contact times would have been ca. 4 hours for the GIVES exposures and 9.5-10 hours for the EAVES exposures.

Referee's Comment: - LDH assay: Normalizing the effect level to the control levels without having a positive control is difiňĄcult to assess. For instance, if all cells would have been dead (apoptosis) by the time of exposure, there would be no enhancement of the LDH level due to particle exposure. Hence, it would be very helpful to also present some evidence of how viable the cells were at the time of exposure. One way of doing this without using an additional viability test (e.g. WST-1) would be to measure the LDH level of lysed control cells as an upper limit (100%) of the LDH level for complete apoptosis and then report the %-LDH level based on this 100% level. Please offer some information regarding this issue?

Author's reply: For this work (and all of our experiments), all exposure membranes were visually inspected immediately prior to exposure with a microscope to ensure both the integrity and continuity of the cellular coverage. In any instance that the viability of the cell cultures was called into question by the visual inspection, the membrane was discarded. In addition, LDH assays are performed on undiluted basolateral supernatants. In every instance where the clean air exposures or incubator controls changed color significantly more than a media blank run concurrently the entire experiment was discarded.

To answer these types of questions in a quantitative way our research group has already implemented exactly the kind of positive control (100% cell lysis) suggested by the Referee. The implementation began after the conclusion of this work, however, so it cannot be applied to these data.

Referee's Comment: - On p.5078 L 15-16 the authors state that it was determined that the presence of PM did not interfere with the ELISA Kit of the IL-8 assay. How was this

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determined? Was this also investigated for the LDH assay? - Please report the number of independent exposures (n = ?) and the number of LDH and IL-8 measurements for a given exposure (multiple determination) which were performed.

Author's reply: Number of independent exposures included in averages (n): Exposure Clean Air Clean air w/ MOA VOC VOC w/ MOA GIVES TOLALD 12 10 6 6 ACRO 17 10 6 6 EAVES TOLALD 20 6 3 4 ACRO 20 6 4 4

(This table is clear in attached pdf version of the authors' response) This information can be added to the manuscript if desired by the editor.

All ELISA and LDH assays were performed in triplicate on each exposure sample (each sample's supernatant)

In our experience with diesel exhaust particles we observed that, when PM was interfering with assays for our biological endpoints, the results within an exposure set (as well as within replicate measurements of the same exposure sample) were erratic.

We examined replicate measurements of each exposure as well as each member of an exposure set for outliers and for deviation from the mean value. As the data reported here had low deviation and agreed across multiple exposures, we determined that the PM material used in this study did not interfere with either the ELISA or LDH assays.

Referee's Comment: 5) The y-label in Figure 2 reads "Frequency cm-3 / Bin Width / 100". What are the values (and units) of "bin width"? Without this information the reader can not interpret the number concentration quantitatively. Please present your data in terms of dN/dlogD as is typically done in aerosol science.

Author's reply: We understand that this label may not be standard. To some on our team the "dN/dlogD" label is meaningless. The data is, in fact, displayed as the Referee requested. We attempted to be explicit in our labeling to help some of the non-aerosol team members, but, as this has inadvertently caused confusion, we have changed the axis label.

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Referee's Comment Typos/Wording etc.: - P.5070: Please use the units "âŮęC" instead of "âŮę" - Section 2.4 is entitles "Gas phase compositional analysis", but it also contains information on the condensed phase analysis - P.5078 L 25: "0.82" should read "0.83" (see line above)

Author's reply: All of these changes have been made (in addition to others that were introduced during typesetting). We thank the Referee for his diligence in finding these errors.

Referee's Comment: - P.5070 L 20: When giving the dew point temperatures, please also give a range of corresponding relative humidities.

Author's reply: The relative humidities are all reported in Table 1.

Please also note the supplement to this comment:
http://www.atmos-chem-phys-discuss.net/12/C5611/2012/acpd-12-C5611-2012-supplement.pdf

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Interactive comment on Atmos. Chem. Phys. Discuss., 12, 5065, 2012.