Responses to Reviewer #2:

This manuscript entitled "Seawater Analysis by Ambient Mass Spectrometry-Based Seaomics and Implications on Secondary Organic Aerosol Formation" by Zabalegui et al. presented a seawater "metabolomics" or "seaomics" analysis method by TM-DARTQTOF-MS. As the paper described, this method required very little sample preparation, and they were able to identify features unique to sea surface microlayer and underlying seawater. Additionally, after the untargeted chemical screening, they performed lab-to-the-field tests to look at the secondary organic aerosol potency. I appreciate they used such an experiment to add value to the untargeted chemical analysis. Based on their SOA experiment, they were able to associate certain chemical characteristics in samples with SOA formation potential. The paper presents an exciting future direction for organic characterization for a better understanding of how organic matter can impact atmospheric processes. The paper is well written, albeit some details were lacking.

We acknowledge the reviewer's feedback and helpful comments on the manuscript.
Just for clarification, lab-to-the-field experiments were performed during the field campaign at Cape Verde islands, whereas the untargeted chemical screening by TM-DART-QTOF-MS was performed after the campaign.

Additionally, I am concerned about:

(1) The organic matter concentrations for the TM-DART-QTOF-MS. Marine metabolomics used solid-phase extraction not only for desalting the samples but also for further concentrating the samples. Open ocean seawater required concentrating a large volume (e.g. > 1000 x concentration factor) to perform metabolomics study. With only a concentrating factor of 6.67, they may only see a very limited class of organic compounds. If the samples were collected in high productivity waters, then 6.67 might have been fine. But, without knowing the organic carbon concentration, it is hard to assess the performance of such a method. The authors should include organic carbon concentrations if available.

We acknowledge the suggestion provided by the reviewer. Seawater samples were collected between 500 and 1000 m away from the coastline of Bahia das Gatas. Information regarding the sampling site and dissolved organic carbon levels for SML and ULW samples are detailed in the following manuscript that provides an introduction to the MarParCloud (Marine biological production, organic aerosol Particles and marine Clouds: a process chain) campaign at the Cape Verde islands and the MARSU project, and describes the scientific content of the field campaign, the interconnection between the different facets of the project and the first findings to serve as an overview of each specific study: van Pinxteren et al., in review, 2019. As indicated in this manuscript, DOC levels varied between 1.8 and 3.2 mg L^{-1} in the SML and between 0.9 and 2.8 mg L^{-1} in the bulk water (Table S4 of the cited reference in review) and were in agreement with previous studies at this location (e.g. van Pinxteren et al., 2017).

Following the reviewer's suggestion, we will include DOC levels measured in SML and ULW samples and the cited reference at the end of section 2.2 entitled "Sample Collection at the Cape Verde Field Campaign" in the revised version of the manuscript as follows:

"DOC levels varied between 1.8 and 3.2 mg L^{-1} in the SML and between 0.9 and 2.8 mg L^{-1} in the ULW water (van Pinxteren et al. in review, 2019)."

Regarding the analytical platform used in the present study, lipophilic compounds exhibited the largest sensitivity among the different type of small molecules evaluated (Table S2). Sensitivity depends on ionization efficiency for compounds ionized with a DART source, which makes use of ionization mechanisms that predominantly follow atmospheric pressure chemical ionization (APCI)-like pathways, but in an open air format. The concentration factor selected in this study allowed organic compound extraction considering the large mass ratio between salt and organic content, and yielded 889 features (m/z) detected within samples, which were further subjected to a stringent curation process before conducting multivariate analysis. The volume of acetonitrile used for reconstituting lyophilized samples was optimized to allow enough sample volume (number of droplets and droplet volume used for depositing the sample in the mesh) that would i) allow the analysis of technical replicates, tandem MS experiments and pooled QC samples, and ii) maximize sensitivity for the maximum number of features.

It is worth noting that other examples in the literature for untargeted marine metabolomics have utilized a concentration factor close to 10 (e.g.: Sogin et al., 2019.).

(2) While APCI-like ionization may be less prone to salt issues, electrospray ionization covers a large range of polar compounds representing important cell metabolites. Some of such metabolites may play important roles in SOA chemistry. I would appreciate they can further comment on this so that other scientists can make informed decisions on analytical strategies for future studies.

As we have detailed in the introduction section:

"It has been suggested that complex photoactive compounds are enhanced at the air-sea interface (Reeser et al., 2009a; Reeser et al., 2009b), inducing abiotic production of volatile organic compounds. For instance, experimental photosensitized reactions at the air-water interface using humic acids as a proxy of dissolved organic matter (DOM), have led to the chemical conversion of linear saturated fatty acids into unsaturated functionalized gas phase products (Ciuraru et al., 2015). Atmospheric photochemistry can even take place in the absence of photosensitizers if the air-water interface is coated with a fatty acid (Rossignol et al., 2016). On a global scale, interfacial photochemistry has recently been proven to serve as an abiotic source of volatile organic compounds comparable to marine biological emissions (Brüggemann et al., 2018)*."*

Based on previous experience from the research groups involved in the present work, the analytical strategy was selected to optimize a method for lipophilic compound analysis that were proven to be involved in SOA chemistry, using a DART ionization source that is less prone than ESI to ionization suppression by high salt contents as those expected in seawater samples. This information was stated in the original version of the manuscript as follows:

"The selected OM extraction method with acetonitrile as extracting solvent favored the analysis of lipophilic compounds. In addition, to enhance the detection of organic acids, the analytical method was optimized operating the DART ion source in negative ionization mode." "Thermally-desorbed analytes having typically MW<1000, are ionized following atmospheric pressure chemical ionization-like pathways (Cody et al., 2005; Song et al., 2009a; Song et al., 2009b; McEwen and Larsen, 2009). An important advantage of DART compared to electrospray ionization for seawater analysis is that it is less affected by high salt levels (Kaylor et al., 2014; Tang et al., 2004), avoiding desalinization processes that may lead to sample alteration."

The analytical strategy adopted in this work focused on minimum sample preparation and no sample desalinization, using a DART source. It is important to remark that the fraction of the marine metabolome that was covered with the implemented analytical strategy included compounds extracted in acetonitrile and subsequently ionized under APCI-like mechanisms. Regarding the selection of the ionization source, it is well known that seawater samples cannot be properly analyzed using an ESI source without a previous desalinization step. We agree with the reviewer about ESI covering a larger range of polar analytes compared to ionization sources operating under APCI-like mechanisms. Indeed, different ionization techniques are able to cover different portions of the metabolome under study. In a previous work on complex sample analysis, unique features were detected by different ionization techniques, including DART and ESI, as well as a certain degree of overlapping compounds among them (Zang et al., 2017). Accordingly, different seawater fingerprints may be obtained with different ionization techniques providing complementary information.

In addition, compared to a direct infusion ESI or APCI-MS-based method, in DART-MS there is no need of rinsing any tubing used to infuse liquid into the ion source. This makes DART more resistant to memory effects, minimizing carryover, as all parts in contact with the sample are disposable, and allows high-throughput analysis, as there is no need for cleaning parts between sample runs (Monge and Fernández, 2014). Another advantage of DART compared to ESI is that it mostly produces singly charged ionic species, which facilitates metabolite identification. On the other hand, ESI sources allow coupling mass spectrometry with a different orthogonal separation technique such as liquid chromatography, and hyphenated LC-MS systems provide the widest metabolome coverage with an additional dimension for compound identification, and are the most widely used analytical platforms in metabolomics (Kuehnbaum and Britz-McKibbin, 2013).

To further address the reviewer's comment, the following edits will be performed to the introduction section in the revised version of the manuscript (changes indicated in italics):

"Thermally-desorbed analytes having typically MW<1000, are ionized following atmospheric pressure chemical ionization-like pathways (Cody et al., 2005; Song et al., 2009a; Song et al., 2009b; McEwen and Larsen, 2009). *Therefore, a major limitation is that it requires analytes to be volatile or semi-volatile, reducing the metabolome coverage.* An important advantage of DART compared to electrospray ionization *(ESI)* for seawater analysis is that it is less affected by high salt levels (Kaylor et al., 2014; Tang et al., 2004), avoiding desalinization processes that may lead to sample alteration. *Conversely, ESI sources allow the coupling of MS to chromatographic systems that provide an additional parameter to improve confidence in compound identification when compared to an authentic chemical standard."*

(3) While I understand 11 features were the result of aggressive feature reduction after QA/QC, this is a rather small number for "omics".

As we have stated in the original version of the manuscript, in the section 2.6 entitled Seaomics Data Analysis: "Spectral features (*m/z* values) were further extracted from TM-DART-QTOF-MS data using Progenesis QI version 2.1 (Nonlinear Dynamics, Waters Corp., Milford, MA, USA). An absolute ion intensity filter was applied in the peak picking process for integration, defining a threshold for the aggregate run. Only SML and ULW samples were considered for peak picking. This process yielded 889 features (*m/z*) detected within samples."

The subsequent curating process, using QC samples and different filtering criteria, which are also described in section 2.6 of the manuscript, yielded a 51-feature matrix. The size of the curated feature matrix exhibited a similar size as other DART-MS-based untargeted metabolomics studies focused on complex sample analysis such as aqueous samples comprised of exhaled breath condensate (e.g.: Zang et al., 2017).

A feature selection process was then applied to find a sub-panel of features that would allow sample classification and class membership prediction. This information was also indicated in section 2.6 of the manuscript as follows:

"Orthogonal projection to latent structures-discriminant analysis (oPLS-DA) (Trygg et al., 2007; Bylesjö et al., 2006; Trygg and Wold, 2002; Shrestha and Vertes, 2010) coupled with a genetic algorithm (GA) variable selection method was applied to find a feature panel that maximized classification accuracy for the binary comparison of SML and ULW samples. The selected group of discriminant features had the lowest root-mean-square error of cross-validation (RMSECV) at the conclusion of the GA variable selection process. This process was performed five different times and the selected panel yielded the lowest RMSECV and exhibited largest feature overlap with the other four panels."

In the genetic algorithm feature selection process, the maximum and minimum number of features used for isolating the discriminant panel was fixed to 15 and 5, respectively; among other parameters described in section 2.6. Metabolite identification was subsequently attempted for the 11 discriminant features resulting from the GA variable selection process.

The data processing, classification, prediction and analysis pipeline used in the present work is a possible strategy utilized in untargeted metabolomic studies (Clendinen et al., 2017; González-Riano et al., 2020; Broadhurst et al., 2018).

Please see the specific comments below.
I recommend publication of this manuscript in ACP after major revision and after the major concerns are addressed. Further specific comments are listed below.

We acknowledge the reviewer's recommendation.

Specific Comments:

Line 40: N = 22 is rather low. The authors discussed this in the Conclusion section, which I appreciate, but probably it should be discussed earlier.

The Conclusions section does not address limitations associated with the size of the sample cohort. The abstract of the work, however, indicates that the results obtained using a lab-to-the-field approach that were compared with those obtained using the TM-DART-QTOF-MS-based metabolomics strategy provide a proof of concept that organic compounds play a key role in aerosol formation processes at the water/air interface. In addition, the last paragraph of section 3.4 entitled "Discriminant Compound Identification & Role in Aerosol Particle Formation" addresses also the limitation associated to the low number of samples that were simultaneously analyzed by both strategies as follows:

"Further analysis on samples analyzed by both TM-DART-QTOF-MS and the lab-to-the-field approach suggest differences in compound concentration levels between SML samples that led to SOA formation from those that did not (Fig. S8, Table S5). Figure S8A shows that PC2 clearly separates samples according to SOA formation. Those features that mainly contribute to sample class separation with largest absolute values in the loadings plot associated to PC2, and illustrated in Fig. S8B, were putatively identified as boron-containing organic compounds (Table S5). Despite the limitations associated with the low number of samples used to perform statistical analysis, results suggest that SML samples that led to particle formation were enriched on boron-containing organic compounds and other unidentified molecules (Table S5). These results provide a proof of concept that organic compounds play a key role in aerosol formation process at the water/air interface."

Regarding the size of the sample cohort (n=22, 10 paired samples), the design of the study prioritized the analysis of collected paired samples over a larger number of non-paired samples due to variability associated with different weather conditions along the field campaign. Based on this design, ULW GP5 and ULW GP7 samples were excluded from the statistical analysis. Samples were collected during the field campaign with 2 different devices for a large number of studies that involved different analytical platforms and instrumentation both on-site at Cape Verde and after sample transportation to the different laboratories of the research groups involved in the project. We agree with the reviewer that a larger number of samples would have been desirable but the size of the sample cohort was limited by the length of the campaign, the challenges associated to sample collection, and the different types of studies that were also planned in the frame of the field campaign with these samples. More details regarding the different studies involved in the field campaign can be found in van Pinxteren, et al., in review, 2019.

Line 42 and lines 126 to 127: 11 species are also on the low end for untargeted. See the comments below.

The TM-DART-QTOF-MS-based untargeted metabolomics approach designed in the present study allowed extracting 889 features with unknown identity. Out of this initial matrix, 51 features were retained after the data curation process (noise filtering, LOESS correction, blank filter, CV filter in QC samples). A panel of 11 features was obtained after the feature selection process using a genetic algorithm variable selection method coupled to a cross validated oPLS-DA model, aimed at classifying and predicting samples according to their classes (SML and ULW).

As discussed in the Introduction section of the manuscript, targeted experiments aim to detect and quantify a predefined group of compounds with known identity. On the other hand, the untargeted metabolomics approach attempts to characterize all detectable analytes in a system, and focuses on the analysis of changes in relative abundances that generate patterns or class fingerprints without a priori knowing compound identities. The untargeted strategy utilizes multivariate statistical techniques that make use of all variables (compound features) simultaneously and deal with the relationship among them to reduce the data dimensionality, find underlying trends, and isolate those features relevant to class discrimination. Multivariate statistical methods can be supervised or unsupervised if class membership is provided or not, respectively. The chemical identification of discriminant variables contributes to the understanding of complex systems.

Additional details to address the reviewer's comment were already provided in the response to comment #3.

Lines 78 to 80: Some of these are derived from biota, consider re-structure the sentence.

We acknowledge the reviewer's remark. The statement will be modified in the revised manuscript as follows:

"The sea surface microlayer (SML) covers up to 70 % of the Earth's surface and is enriched in DOM, including organic compounds such as fatty acids, fatty alcohols, sterols, amines, amino acids, proteins, lipids, phenolic compounds and UV-absorbing humic-like substances derived from oceanic biota; particulate matter; microorganisms (Liss and Duce, 2009; Donaldson and George, 2012); colloids and phytoplankton-exuded aggregates, mainly constituted by lipopolysaccharides, (Liss and Duce, 1997; Hunter and Liss, 1977; Bayliss and Bucat, 1975; Liss, 1986; Hardy, 1982; Garabetian et al., 1993; Williams et al., 1986; Schneider and Gagosian, 1985;Gershy, 1983; Guitart et al., 2004; Facchini et al., 2008; Kovac et al., 2002)."

Lines 176 to 177: Some of the particles need to be filtered. Centrifugation is usually not sufficient to remove all particulate matter. Please address this.

We appreciate the reviewers' comment. In this study, the strategy was to use seawater samples with as little modification as possible. We did not intend to remove particles from the collected SML samples. Centrifugation was mainly conducted to concentrate the surface microlayer from the sea water. As expected, this step partly removed large particulate matter and colloids. But, we underline that centrifugation was aimed only at concentrating SML samples as a condition for aerosol formation. SML samples collected in the field are expected to contain different type of particles (Cunliffe et al., 2013). We agree with the reviewer that not all particles that may have been present in SML samples would have been removed with the implemented centrifugation step. The effect of particle filtration on interfacial photochemistry will be investigated in the future.

The revised version of the manuscript will include the following statement for clarification:

"Centrifugation was aimed at concentrating SML samples as a condition for aerosol formation."

Line 178: "Extracted" may not be the correct word here. Based on the text, I assumed they meant removed. Extracted would make the reader think they have performed certain extraction protocols. Please rephrase.

We agree with the reviewer's remark. The sentence will be modified in the revised manuscript as follows:

"Subsequently, 2 mL of surface solution was collected from each centrifugal vessel to isolate closer representations of SML samples considering the dilution factor inherent to the collection process, i.e., SML diluted with ULW contribution, and leading to a total sample volume of 24 mL for subsequent experiments."

Line 206 to 207: The repeat thawing and freezing process may affect the organic matter composition. What is the rationale for the thawing and re-freezing?

This procedure was necessary to generate sample aliquots of exact volume and pooled QC samples for further lyophilization, transportation and analysis by TM-DART-QTOF-MS at CIBION-CONICET (Argentina). As indicated in the original version of the manuscript: "Quality control (QC) samples were prepared by mixing equal volumes of all samples including both collection methods before sample lyophilization (QC_{ALL})".

It is important to note that SML and ULW samples collected during the campaign were aliquoted on-site in bottles for the different experiments that were planned by different collaborators in the frame of the MarParCloud and the MARSU projects. Samples were stored at -20 °C at Cape Verde and cooled below -20 °C during transportation to the laboratories at TROPOS (Germany), where they were stored at -20 °C until they were prepared for lyophilization. A detailed description of water sampling for the different studies conducted in the frame of the campaign can be found in van Pinxteren et al., in review, 2019.

Lines 225 to 228: More details on sample extractions should be provided.

Following the reviewer's suggestion, more details will be included in the sample preparation description of the revised manuscript as follows:

"Lyophilized residues were reconstituted in 1200 µL of acetonitrile, yielding a concentration factor of 6.67. Reconstituted samples were vortex-mixed during 5 min for metabolite extraction, and centrifuged during 10 min at 4861 × g and 20 °C to favor the formation of a salt pellet. For each sample, 500 µL of supernatant was collected for further analysis."

Line 345 and Figure 1: Based on the text, it reads like they extracted seawater. But in Figure 1, it looked like they extracted some kinds of solid (white cluster in the centrifuge tube). So, it is unclear to readers how they extract the samples.

The whitish solid shown in Figure 1 illustrates the residues obtained after the lyophilization process due to the high salt content of seawater samples. Acetonitrile was the solvent selected for metabolite extraction; and a vortex-mixed step was performed to favor that process. Since a minimum amount of salt dissolves in the organic solvent, a white suspension of salt in

acetonitrile is formed. After centrifugation; the supernatant containing the extracted metabolites was collected without touching the salt pellet placed at the bottom of the tube, which is illustrated in the scheme of Figure 1. Supernatants were subsequently seeded on the stainless steel mesh.

Based on the reviewer's comment, the legend in Figure 1 will be modified in the revised version of the manuscript for clarification as follows (changes indicated in italics):

"Scheme illustrating the analytical strategy implemented at CIBION-CONICET for the analysis of *lyophilized* seawater samples using TM-DART-QTOF-MS."

Lines 226 and 345 to 363: Is a concentration factor of 6.67 enough? It might if the water samples were collected from high productivity water. Please include organic carbon concentration to justify this, if available.

We have already addressed this question in the response to comment #1.

Lines 365 to 405: The authors performed a large feature reduction, which is necessary to QA/QC untargeted data. However, from hundreds of total features down to 11 seems to be a bit aggressive. It would be nice to see how the PCA model changes at each step of feature reduction. In many untargeted environmental "omics" hundreds of features are typical even after substantial feature reduction. Therefore, it would be good to see a more detailed narrative and interpretations based on various levels of feature reduction.

A PCA score plot built with the 889 features that were initially extracted using Progenesis QI would lead to incorrect results and misunderstanding, since a large percentage of features would not follow the rigorous filters established based on signal-to-noise ratio, reproducibility and prevalence. The curation process that also included removal of signals present in blanks and signals that did not exhibit an isotopic pattern is aimed at retaining only robust signals that would increase confidence in compound annotation and subsequent data analysis in the research study (Broadhurst et al., 2018).

The 51-feature matrix that was retained after the curation process is comprised of the most robust features to subsequently perform multivariate statistical analysis. Figure 3A shows the score plot for the PCA model built with the 51-feature matrix obtained after the data curation process. No sample clustering was observed by using this matrix to build the model. However, the PCA model built with the 11 selected features by the genetic algorithm (Figure 3C), exhibited a certain degree of sample separation in the PC3 direction, with two sample clusters according to seawater sample collection depth, i.e., SML or ULW.

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