RESPONSE TO REVIEWER 1

Seasonal survey of the composition and degradation state of particulate organic matter in the Rhone River using lipid tracers (written by M.-A. Galeron et al.)

- **General comments:**

This paper provides detailed data on seasonal variation of a wide variety of lipid molecules contained in suspended particulate matter in the Rhone River estuary. Using these data, the authors discussed the origins and the biological and abiotic degradation processes of the lipids.

In particular, their approach for evaluating quantitatively the influence of abiotic oxidation on the removal of a few lipid compounds seems an interesting challenge. Even though similar studies have been published for a few other ecosystems recently (e.g. Rontani et al. 2009, 2014b), I think that their study has a sufficient merit to be published in Biogeosciences after appropriate revision, because this study focused on an environment with relatively high human impacts compared to the preceding studies. The argument concerning the provenance of POM based on the lipid composition is generally convincing.

My concern is that their conclusions, particularly those for the individual degradation processes, depend on numerous assumptions that are not verified enough. Above all, the estimation of the degrees of biodegradation and auto- and photo-oxidation undergone by specific lipids such as cholesterol and sitosterol depends on the assumption that the parent lipids and all the degradation intermediates used for the estimation have similar turnover rates in river water, which seems dubious. In fact, the authors noted that some of the intermediates (e.g. Δ6-5α-hydroperoxides, p.14213, line 5) are too unstable under certain conditions to be

used as tracers. They used an alternative, apparently more stable species as a tracer; however, it seems quite difficult to confirm whether it is so stable as to conform to the above assumption. The assumption that the yield ratios of different oxidation intermediates are constant is also questionable. Although they mentioned these assumptions only briefly (p.14212, lines 2-5; p.14213, lines 1, 10-13), I would like to request them to elaborate the validity of these assumptions in more detail and discuss how the following interpretations may be changed if the turnover rates and/or the yield ratios are variable.

Authors: Although each sterol and its degradation products may be potentially totally mineralized by marine bacteria, we assume that they should exhibit similar reactivity towards bacterial degradation. This assumption is based on the fact that aerobic biodegradation of sterols generally involves initial attack on the side chain, which is similar in all the degradation tracers selected to that of the corresponding parent Δ^5-sterol. Moreover, it may be noted that 3β,5α,6β-steratriols, employed for autoxidation estimates are weakly affected by abiotic degradation processes. This is also the case for Δ^4-6α/β - hydroperoxysterols (photooxidation tracers), which are much more stable than Δ^5-7α/β - and Δ^7-5α-hydroperoxysterols (Christodoulou et al., 2009). Indeed, β-scission of the alkoxyl radicals resulting from homolytic cleavage of Δ^5-7-hydroperoxysterols and Δ^6-5-hydroperoxysterols affords secondary and tertiary radicals, respectively, more stable than the primary radical resulting from the cleavage of Δ^4-6-hydroperoxysterols (Christodoulou et al., 2009). Moreover, proton driven cleavage (Hock cleavage) of Δ^5-7-hydroperoxysterols and Δ^6-5-hydroperoxysterols involves a highly favored migration of vinyl group (Frimer, 1979), while only an unfavored migration of alkyl group is possible in the case of Δ^6-5-hydroperoxysterols (Rontani et al., 2014). These precisions will be added into the manuscript, in the sterol degradation section.

- **Specific comments:**

• The use of the terms "plant-derived organic matter" and "organic matter of human-origin" in Abstract (lines 13-16) is an overgeneralization. This study investigated the degradation processes of only two sterols, one of which (sitosterol) is surely of plant origin but should not be regarded as a representative organic matter of plants. The other, cholesterol, is not limited to human (cf. p.14206, lines 7-9).

Authors: In the abstract, we propose to replace the sentence "terrigenous contribution to the plant-derived particulate organic matter" by "terrestrial higher-plant contribution to the particulate organic matter". We also propose to replace the sentence "Plant-derived organic matter appears to be mainly affected by photo-oxidation and autoxidation" with "higher-plant-derived organic matter appears to be mainly affected by photo-oxidation and autoxidation (…) while organic matter of mammal or human origin…". Cholesterol is indeed a sterol that can have multiple sources, whether human or not, but we did include coprostanol and epicoprostanol in the degradation products studied (these 2 sterols being exclusively of mammal or human origin). The point here is mainly to point the difference in degradation patterns between these 2 sterols (and the types or organic matter they are mainly found in)

• The authors mentioned that the sampling station at Arles was an estuarine station (p.14201, line 1). If so, the mixing between seawater and river water would play an important role in the dynamics of particulate matter. But they didn't argue this point in this paper. I would request them to show the salinity data in Fig. 2 and discuss briefly possible influence of salinity on the behavior of suspended particles. Water temperature data are also worthwhile to show here.

Authors: This is a good point, and one we became aware of as well. Arles has often been

referred to as an estuarine station, when it actually is a riverine station – the salinity in Arles is 0, and it is too far to receive any seawater inputs. On p. 14201 – line 1, the term “estuarine” will be replaced by “riverine” in the manuscript. There is no salinity data to show, and the automated measurement of water temperature at the station is highly unreliable, which is why we did not include it in this study.

• Acidification with sulfuric acid (p.14201, line 13) is relatively rare for the treatment of POM samples. Can the authors refer to some reference paper? I guess excess sulfur may cause rapid deterioration of catalysts in the instrument.

Authors: Generally hydrochloric or phosphoric acids are used to remove inorganic carbon. We didn't use phosphoric acid because of the risk for phosphorus contamination. We chose sulfuric acid (4%) instead of hydrochloric acid due to the large proportion of carbonate in the suspended matter. Also, this acidification procedure is similar to the one used for the wet-oxidation method which gives excellent results. On p.14201, line 13, the following reference will be added to the manuscript: (Raimbault et al., 1999)

Raimbault P., Diaz F., Boudjellal B., Simultaneous determination of particulate forms of carbon, nitrogen and phosphorus collected on filters using a semi-automatic wet-oxidation procedure. Mar. Ecol. Progr. Ser., 180: 289-295, 1999.

• Measuring silicate using the GF/F filtrates (p.14201, lines 15-16) often leads to an overestimation due to leaching from the GF/F.

Authors: Sampling procedure for silicate has not been described in the paper. In fact, samples for silicate analysis were filtrated through a 0.45 µm polycarbonate membrane and kept at 5°C until analysis. This will be added in the Materials & Methods section of the manuscript.

• A mixture of ethyl acetate/BSTFA (p.14202, line 24): what proportion?

Authors: Dry samples were taken up in 100µL BSTFA and an appropriate amount of ethyl acetate, depending on the concentration in lipids in each sample, in order to get the best possible GC-EIMS reading. On p.14202, line 24, this will be added into the manuscript: "After evaporation to dryness under a stream of N_2, the derivatized residues were taken up in 100µL BSTFA (to avoid desilylation of fatty acids) and an appropriate amount of Ethyl Acetate, depending on the concentration in lipids in each sample, in order to get the best possible GC-EIMS reading"

• The combined DCM extracts (p.14203, line 4): Did it include also the chloroform phase of the initial phase separation, or not?

Authors: Yes, it included the chloroform phase of the initial phase separation.

• The authors mentioned the minimum and maximal daily flow rates of the Rhone River recorded on 8 Oct 2011 and 19 May 2013, respectively (p.14204), but I could not find such sampling dates in Fig. 2. In addition, the time axis in Figs. 2-6 seems a bit confusing. Please consider adopting a real time scale where it is possible.

Authors: The minimal and maximal flow rates dates mentioned are the days during the entire sampling period on which flow rates were the highest and the lowest. The original manuscript included a figure where daily flow rates were shown for the entire period, not just on sample dates. The figure has since been removed, and we will correct the dates and water flows mentioned so that they reflect minimal and maximal flow rates on actual sample days. On p. 14204, the revised manuscript will read: "During our sampling period the daily flow rate fluctuated between 680 (19 September 2012) and 4661 $m^3.s^{-1}$ (5 November 2011; Figure 2)."

Also, we did consider using a real time scale, but since our sampling dates were not regular (some months have multiple samples, and other months none), using a real time scale made the figures more confusing since we could have gaps in the data presented. We chose to be consistent in the time axis used across all figures, even though it is not a real time scale, so that readers would not be confused by missing data or multiple points on one month.

• "Typical for river systems" (p.14204, line 18): Literature should be referred to here.

Authors: p.14204, line 18: the following reference will be added into the manuscript (Jansson, 1982)

Jansson, M.B. Land erosion by water in different climates. UNGI Rapport (Sweden), 1982

• On page 14207 (lines 5-8), the authors suggested that 5 April 2011, 2 May 2013, and 4 Nov 2011 were flood dates. However, Fig. 2 shows that the river was under the base-flow conditions on 5 April 2011, and that the flow rate was not recorded on 4 Nov 2011.

Authors: That's right, there is a mistake about April 5, 2011 and we will correct that in the manuscript. However, November 4 was a flood date (this was actually a major recent flood), but on major floods, our measurement instrument is sometimes overflowed and could not record flow rates for a few days. Flow rates were only recorded again on Nov 7. On page 14207 (lines 5-8), the revised manuscript will read "The amount of cuticular waxes is variable amongst samples, between 0.02 and 3.8 $\mu g.mg^{-1}$(dry weight), with the highest in the 5/4/2011, 2/5/2013 and 4/11/2011 samples (3.8, 2.2 and 1.7 $\mu g.mg^{-1}$ respectively). Two of these sample dates (2 May 2013 and 4 November 2011) happen to be flood dates."

• What do the authors mean by "content variability" (p.14208 line 5)?

Authors: By "content variability" we meant that the amount of chlorophyll quantified on our

different samples dates was highly variable.

• Section 3.2.4 (p.14208-9): The authors may explicitly mention here that the provenance analysis depending only on the fatty acid composition likely leads to an underestimation of higher plant contribution.

Authors: In section 3.2.4 (p.14208-9), on line 18, the revised manuscript will include a comment stating that "Due to the high degradability of fatty acids, a number of them could not be quantified, potentially leading to an underestimation of higher plant contribution."

• I recommend that the definitions and calculation methods for CPPI (p.14211), biodegradation % (p.14212), and auto- and photo-oxidation % (p.14213) may be described in the Materials and Methods section.

Authors: Since all calculations methods for CPPI, biodegradation, photo- and autoxidation heavily depend on the compound descriptions preceding it, adding it all into the M&M section might make this section too long and descriptive. Adding only CPPI methods might make for too short of a dedicated section in the M&M?

• On page 14216 (lines 26-29), the authors mentioned the detection of cis and trans allylic 18,(8-11)-dihydroxyoleic acids (auto- and photo-oxidation intermediates) referring to Table 2 and Fig. 5, but no such data can be found in this figure. Table 2 only shows 18,(8-11)-dihydroxy-C16:0. They suggested a high proportion of cis isomers (line 29) and larger amounts of oxidation products than the parent ω-hydroxyoleic acid on a few sampling events (p.14217, lines 1-3), but these are not confirmed by the presented data.

Authors: Since the manuscript had to be downsized, a number of figures were removed, and it's true that there no longer is a figure presenting this data, for size and clarity purposes. On

page 14216 (lines 26-29) the “Table 2 and Fig. 5” reference shall be replaced by (Galeron & Rontani, unpublished data) in the manuscript.

• On page 14217 (lines 3-5), the authors mentioned "the previously discussed yearly variability in cuticular wax content", but it is a bit unclear what part of this manuscript they indicated by this phrase. Fig. 3b may be referred to here.

Authors: On page 14217 (lines 3-5) the reference (See section 3.2.2 and Fig. 3b) will be added in the manuscript after the sentence "the previously discussed yearly variability in cuticular wax content".

• What do the authors mean by "high compartmentalization effects" (p.14218, line 2)? Is it same as the protection by waxy materials from degradation suggested on p.14213 (lines 24-25)?

Authors: By “high compartmentalization effects” we mean that these compounds are structurally protected by rigid structures that physically protect them from degradation. This is different from the waxy material protection suggested on p. 14213.

- **Technical corrections:**

• Line 10 of page 14199: et -> and

Authors: Both instances of “et” on that line will be corrected to “and”

• Line 28 of page 14205: A -> As (?)

Authors: Indeed, we will correct the “A” into “As”

• Line 16 of page 14214: please remove "a" from McCalley et al., 1981a.

Authors: This will be corrected in the manuscript

• Cauwet et al. (1990) that appears in the reference list (p.14220) does not appear in the main text.

Authors: Although we carefully checked references after downsizing, this one must have slipped through the cracks! It will be removed from the reference list in the manuscript.

• Please remove "a" from Kolattukudy, P.E. 1980a (p.14222).

Authors: This will be corrected in the manuscript

RESPONSE TO REVIEWER 2

Seasonal survey of the composition and degradation state of particulate organic matter in the Rhone River using lipid tracers by Galeron et al.

This manuscript couples lipid and lipid degradation byproducts to explore the underlying dynamics to determine the origin of particulate organic matter in a major mixed-use river system. The authors sampled near the river outlet repeatedly throughout the year to explore the seasonal dynamics and change in the source and origin of POM sources.
There is relatively little preexisting work exploring these dynamics using lipids in mixed use rivers, and the seasonal aspect to this work is particularly valuable. The manuscript will be a welcome contribution to the literature after some minor revisions and considerations. Overall, the manuscript is fairly well written, although it contains some loose and colloquial language and could use some additional tightening. The paper could also be improved by creating a table of lipid tracer names, origins, and associated degradation products discussed in the study. However, these issues are minor, and my two main areas of concern are as follows:

The merit of the manuscript is presenting all of the data available in POM at the Rhone estuary. However, the conclusions made from this information ignore the significant gap in knowledge about the breakdown rates of these byproducts. Since sampling was only conducted at the mouth of the river, diverse breakdown or uptake rates of these compounds may heavily bias the interpretation of these results. The conclusions should be qualified with this concern and discuss the gap in knowledge and its implications.

The presentation and interpretation of fatty acid data is underdeveloped. There is unclear

usage of fatty acid nomenclature conventions, particularly in regards to PUFA classifications. This is particularly important due to different potential origins of 16-18 C PUFA in green plants and 20+ C forms only found in algae. See Taipale et al. 2013 regarding fatty acid profiles of various algal groups, and related literature for information about terrestrial plants. PUFA should be considered to be split between 16-18 C and 20+ C forms. In addition, fatty acid profile (percentage relative to sum of all fatty acids quantified) should be considered in the tables instead of simple fatty acid content by weight. More detailed comments are contained as annotations in the attached document.

Authors: In the discussion with reviewer #1, we addressed the assumption that the removal rate of the tracers used was similar to that of the associated parent sterol. In section 3.3.2 (p. 14212, lines 4-6), we added the following precisions: "This assumption is based on the fact that aerobic biodegradation of sterols generally involves initial attack on the side chain, which is similar in all the degradation tracers selected to that of the corresponding parent Δ^5-sterol. Moreover, it may be noted that 3β,5α,6β-steratriols, employed for autoxidation estimates are weakly affected by abiotic degradation processes. This is also the case for Δ^4-6α/β - hydroperoxysterols (photooxidation tracers), which are much more stable than Δ^5-7α/β - and Δ^7-5α-hydroperoxysterols (Christodoulou et al., 2009). Indeed, β-scission of the alkoxyl radicals resulting from homolytic cleavage of Δ^5-7-hydroperoxysterols and Δ^6-5-hydroperoxysterols affords secondary and tertiary radicals, respectively, more stable than the primary radical resulting from the cleavage of Δ^4-6-hydroperoxysterols (Christodoulou et al., 2009). Moreover, proton driven cleavage (Hock cleavage) of Δ^5-7-hydroperoxysterols and Δ^6-5-hydroperoxysterols involves a highly favored migration of vinyl group (Frimer, 1979), while only an unfavored migration of alkyl group is possible in the case of Δ^6-5-hydroperoxysterols (Rontani et al., 2014).". These precisions on removal rates of degradation

products should eliminate all interpretation bias.

Comments on fatty acid profiles and on the interpretation of their origins have been added (see next comments), but their determination and quantification was not done with enough detail to be able to clearly identify a unique source.

Specific comments:

- P 14198: "likely dominated by diatoms" - This could be more directly addressed by the fatty acid profiles

Authors: Additional comments will be added in the manuscript in the fatty acid section regarding the fatty acid profiles and their potential sources: "More recently. $C_{16:1\omega7}$ and $C_{20:5\omega3}$ (eicosapentaenoic acid) were identified as 2 of the main fatty acids in Bacillariophyceae (Taipale et al., 2013). These 2 markers are present in our samples, and together, form 40.5 and 34.1% of the 6 March 2012 and 12 March 2013 samples (versus an average of 13,2% across all samples), which, when coupled with our sterol analysis, concurs with our hypothesis that diatoms are major contributors in the algal blooms identified. It is worth noting that in the 3 October 2012 sample, $C_{16:1\omega7}$ forms 53.6% of all quantified fatty acids, while $C_{20:5\omega3}$ is completely absent (0%), and therefore these markers alone cannot be considered to be specific enough in natural river water samples which can contain a number of fatty acids from various sources."

- p. 14200 : "it would be logical" - Casual language. Why?

Authors: On page 14200 line 8, this will be rephrased in the revised manuscript and changed to " we propose to confirm the hypothesis according to which the POM sampled in the Rhone is mainly constituted of terrestrially-produced particulate organic matter travelling with the

runoff, while also identifying other sources of OM, sometimes significantly aquatic/planktonic.

- p. 14201 : "40 km upstream from the river mouth" - Is there strong tidal influences and what is salinity range?

Authors: We had identified an issue here, where the sampling station was qualified as estuarine, which is something that was also picked up by reviewer #1: This will be corrected in the manuscript, and "riverine" will replace "estuarine" in the revised manuscript, as the sampling station sees no tidal influences, and the salinity is constant and at 0.

- p. 14203 : "standards" - Need detail about what standards were used. Singles/FAME mix?

Authors: Standards used were single standards. This precision will be added in the revised manuscript on page 14203,line 27: "calibration with external single standards"

- p. 14205: "A we" - Grammar/typo? Should this be "As we also see an increase..."

Authors: Yes, this will be corrected in the revised manuscript

- p14205: "less important" - Less important how? Rather subjective and loose wording

Authors: This refers to figure 3a, where a slight increase in planktonic sterols is visible but is much less important than the one in march 2012. We wonder here if this slight increase is important enough to consider these potential blooms a yearly event.
On page 14205, this will be revised and will read: "As we see an increase, although less important in the proportion of planktonic sterols in the Spring 2013 samples (20 times less in quantity when compared to the 6 March 2012 sample, but still constituting 31 and 27% of the

total of all sterols quantified in the 12 March and 21 March 2013 samples respectively)"

- p. 14208 : "Polyunsaturated fatty acids" - Was this defined here or in the methods? Including both sum of 16-18 and 20+ C polyunsaturated fatty acids? See Galloway et al and Taipale et al 2013 for the different origins of various carbon length PUFA and HUFA.

Authors: The manuscript did not provide details on how the proportion of PUFA was calculated. We have added the information on p.14208 with "(quantified using the total of all PUFA quantified between C_{14} and C_{26})". As discussed previously, we have added a comment discussing the origin of different fatty acid profile.

- p. 14208: "These high contributions support the presence of a high proportion of fresh algal material in these samples." - Examination of total fatty acid profile and indicator FAs could give additional detailed information into fatty acid origin (algal class, terrestrial, bacterial)

Authors: See previous comment

- p. 14209: "harmless" - Rather strong statement. Harmless in what way?

Authors: "Harmless" was added when we were concerned that readers would tend to conclude that this industrial input was dangerous to the environment. This is also why we use the word contamination and not pollution. Since we do not have sufficient data to prove that it is harmful, or harmless for that matter, we will remove the word "harmless" from the revised manuscript leaving only "This industrial contamination" on p. 14209.

- p. 14212: Would be useful to have a singular table that summarizes all examined lipid

products, their origins, and degradation byproducts products by process

Authors: Upon inital submission, we were required to reduce the length of the manuscript, along with the number of figures and table. We agree this could be a useful table, but if we include all quantified lipids, degradation products and origins, this could become a rather large table. If deemed necessary by both reviewers, we can add it into the manuscript.

- p. 14215: "we will logically be only looking at unsaturated fatty acids here." - Loose and colloquial language.

Authors: This will be corrected in the revised manuscript on p. 14215 into "We will only be looking at unsaturated fatty acids here"

- p. 14218: "suspect diatoms to be major contributors." - This can be more closely examined using the fatty acid profiles. See Taipale and Galloway.

Authors: See previous comments. We have not been able to determine the double bond position with enough precision on all quantified FA to precisely identify contributors using the references provided. They have however been discussed in the revised manuscript (see previous comments).

- p. 14218: " Using specific lipidic degradation products, we were able to identify for the first time the part that bacterial degradation, autoxidation and photo-oxidation play in organic matter degradation in a Mediterranean river." - Discussion needed re: differing degradation rates and its implications

Authors: See first comment. Precisions have been added regarding the removal rates of these degradation products, according to reviewer 1 comments as well.

- p. 14218: "underestimated" - Needs more and clearer explanation

Authors: The underestimation of photo-oxidation was discussed in section 3.3.2

- Table 2a: Consider using fatty acid profile as a percentage - No $C_{22:6\omega3}$?

Authors: The table has been modified to show Fatty Acid and Hydroxy Acid contents in percentage of the total FA and HA quantified. $C_{22:6\omega3}$ has not been detected.

- Figure 2: X axis should be standardized in time rather than equally display sampling dates

Authors: This has also been discussed with reviewer #1. We chose to represent the data this way since a real time scale would mean months with multiple data points and months with no data points at all. This would make for confusing figures with gaps in the data, and would make it difficult for the reader to get a clear picture. We have tried to make the X-axis as readable as possible, with a font size as large as possible.

- Figure 3a: Same comment about X axis as Fig 2. X-axis labels are small and hard to read. Y axis in Fig 3A appears bold and non-consistent with previous. Y-axis lines in figure are inconsistent.

Authors: See previous comment for x-axis. We will alter figure 3 in order to make the x-axis labels larger and easier to read. We will also fix the y-axis, which shouldn't appear bold. We are not sure what is meant by " Y-axis lines in figure are inconsistent". If this is about the horizontal grid lines, they are actually all present but for some reason sometimes only appear when the PDF file is zoomed in.

- Figure 3C: If March 6th 2012 is associated with the algal bloom and evidence in

sterols, what about the PUFA spike in March 2013?

Authors: On p. 14208 (lines 21-22) we mentioned that "Polyunsaturated fatty acids are present in very low proportions in our samples (quantified using the total of all PUFA quantified between C14 and C26), apart from the 06/03/2012 and 12/03/2013 samples where they contributed to 44 and 40% of total fatty acids (Figure 3C). These high contributions support the presence of a high proportion of fresh algal material in these samples. " We previously emitted "the hypothesis of a yearly phytoplanktonic spring bloom, with a larger magnitude for the 2012 event" and the PUFA data goes towards such an hypothesis, but as previously said, we did not go into enough detail in the PUFA analysis to be able to clearly identify contributors on PUFA data alone.

- Figure 4a: No error or replicate samples for Chlorophyll-a? - Is this supposed to be Jan 22nd? Or was this just sampled on a different date than everything else, including its photodegradation?

Authors: This data has been collected by the MOOSE observation system, and there is no replicate sample data, hence no error calculated. The data represented here is all the available data during our entire sampling period (no measurements were made in 2011), and if a number of points fall on the same dates as our own samples, not all of them do (Jan 21 is an example). This only applies to chlA quantification, as the data in Fig. 4b was calculated using our own samples.

- Figure 4b: Could this be lined up so that the dates are in the same position for both?

Authors: See previous comment. Since the dates are not the same, we chose not to align these 2 figures. There are dates in figure 4a that do not appear on figure 4b and aligning them could be misleading as readers might make a parallel between different dates.

Seasonal survey of the composition and degradation state of particulate organic matter in the Rhone River using lipid tracers.

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Abstract:

Lipid tracers including fatty acids, hydroxyacids, *n*-alkanols, sterols and triterpenoids were used to determine the origin and fate of suspended particulate organic matter (POM) collected in the Rhone River (France). This seasonal survey (April 2011 to May 2013) revealed a year-round strong terrestrial higher-plant contribution to the particulate organic matter (POM), with significant algal inputs observed in March and attributed to phytoplanktonic blooms likely dominated by diatoms. Major terrigenous contributors to our samples are gymnosperms, and more precisely their roots and stems, as evidenced by the presence of high proportions of ω-hydroxydocosanoic acid (a suberin biomarker). The high amounts of coprostanol detected clearly show that the Rhone River is significantly affected by sewage waters.

Specific sterol degradation products were quantified and used to assess the part of biotic and abiotic degradation of POM within the river. Higher-plant-derived organic matter appears to be mainly affected by photo-oxidation and autoxidation (free radical oxidation), while organic matter of mammal or human origin, evidenced by the presence of coprostanol, is clearly more prone to bacterial degradation. Despite the involvement of an intense autoxidation-inducing homolytic cleavage of peroxy bonds, a significant proportion of hydroperoxides is still intact in higher plant debris. These compounds could affect the degradation of terrestrial material by inducing an intense autoxidation upon its arrival at sea.

Key words: Rhône River: Rhone; Particulate organic matter; Lipid tracers; Biotic and abiotic degradation; Autoxidation; Photo-oxidation; Hydroperoxides.

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Marie-Aimée Galeron 8/1/y 09:05
Supprimé: Plant-derived organic matter

Marie-Aimée Galeron 8/1/y 09:06
Supprimé: while organic matter of human origin

Marie-Aimée Galeron 8/1/y 09:06
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1. Introduction

With an average water discharge of 1700 m^3 s^{-1} (Ludwig et al., 2009), the Rhone is the main freshwater contributor to the Mediterranean Sea. Based on various studies, the mean annual flux of suspended matter is about 6.7 x 10^6t yr^{-1} over the period 1967-2008 (Eyrolle et al., 2012), and the annual particulate organic carbon (POC) flux was estimated to be 1.94±1.09 x 10^5 t yr^{-1} (Sempéré et al., 2000). The labile POC fraction, calculated as the carbon contribution from sugars and amino acids to Total POC, was estimated to be between 3 and 27% depending on the amount of SPM (Sempéré et al., 2000). The Rhone River represents 14% and 10% of fresh water and particulate carbon input to the Mediterranean Sea, respectively (Ludwig et al., 2009). Given the importance of such a contribution, it is crucial to study the provenance and composition of the organic matter found in the Rhone, prior to its arrival in the Mediterranean Sea.

Up until recently, it was widely considered that the terrestrial particulate organic matter (TPOM) flowing into oceans through rivers was refractory to degradation (biotic and abiotic), since it was constituted of terrestrial plant debris previously degraded during transport (de Leeuw and Largeau, 1993; Wakeham and Canuel, 2006). However, coastal sediments have shown very little trace of a terrestrial OM signature (Hedges and Keil, 1995; McKee et al., 2004), which implies that either global carbon fluxes and budgets are wrong, or, and it is more likely, that terrestrial POM undergoes a rapid and intense degradation upon its arrival at sea (Hedges et al., 1997). The belief that terrestrial POM is refractory to all sorts of decomposition has been recently challenged through studies in the Mediterranean Sea (Bourgeois et al., 2011) and Northern Canada (Rontani et al., 2014b), showing that well-preserved TPOM is heavily degraded upon leaving river systems. This is why we intend to study the chemical structure and degradation state of TPOM in the Rhone River, in order to better understand how such an intense degradation can be possible at sea, while it wasn't during freshwater transport.

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In comparison with bulk geochemical analyses, where $\delta^{13}C$ terrestrial end-member determination is precarious, $\delta^{13}C$ signatures of organic matter at a molecular level can be more specific (Tolosa et al., 2013). Unfortunately, carbon isotopic data in freshwater ecosystems is not always source-specific, because freshwater phytoplankton and terrestrial plants can often produce similar $\delta^{13}C$ signatures (Cloern et al., 2002). Many studies used biomarkers such as lipids, cutins or waxes in sediment or soil samples in order to determine the specific contribution of plants in organic matter (Amelung et al., 2008; Simpson et al., 2008). Sterols (steroidal alcohols) have specific structural features that can be linked to a restricted number of organisms (Volkman, 1986), and can be used to determine the main contributors to a pool of organic matter. Their degradation products, such as stanols, 3,6-diols, or triols, can also inform on the type of degradation undergone (Christodoulou et al., 2009; Rontani et al., 2009). On the other hand, cuticular waxes and cutins can be linked to relatively specific higher plant groups (Mueller et al., 2012), allowing an estimation of the contribution of terrestrial plants to the OM.

There are no studies dealing with the composition of the plant-derived particulate organic matter carried along the Rhone River in the literature. We propose to confirm the hypothesis according to which the POM sampled in the Rhone is mainly constituted of terrestrially-produced organic matter travelling with the runoff, while also identifying other sources of OM, sometimes significantly aquatic/planktonic. Although limited research has been done on freshwater plankton dynamics in the Rhone, it has been found that phytoplankton communities in the middle Rhone suffer a high turnover (Fruget et al., 2001) implying an important potamoplankton input in the particulate organic matter flowing down the river.

Using specific lipids and their degradation products, here we report for the first time on the different sources of OM in the Rhone River and discuss the main degradation processes affecting this OM.

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2. Materials and methods

2.1. Study area and Sampling

The Rhone River is 816 km long and has a drainage area of 97,800 km^2 (Figure 1). The Rhone being a river prone to seasonal flooding, samples span a wide range of water flow conditions in order to be as representative as possible (Figure 2).

To monitor temporal variations of suspended particulate matter (SPM), POC, nutrients and lipidic biomarkers in the Rhone River, 30 surface water and particle samples were collected between April 2011 and May 2013 at the Rhone River reference riverine station of Arles, 40 km upstream from the river mouth (Figure 1).

The high frequency study of the nutrients and particulate matter input by the Rhone to the Mediterranean Sea has been carried out since 2010 in the framework of the national program MOOSE (Mediterranean Ocean Observing System for the Environment). Monitoring was undertaken at the Arles station (Figure 1). Sampling included filtering between 100 and 400 mL of water on GF/F glass fiber filters, as well as particles collection using a high-speed centrifuge device (CEPA Z61) coated with teflon to avoid metal contamination. For suspended matter determination, water samples were filtered on pre-weighted GF/F filters. After drying, filters were weighted to determine the suspended particulate matter (SPM) content (in $mg.l^{-1}$). Non-weighted filters were used to quantify Particulate Organic Carbon (POC) contents: the filters were acidified with 50 - 100µl 0.5N sulfuric acid and dried overnight at 60°c (Raimbault et al., 1999). POC contents were determined using high combustion (900°C) procedure on a CN Integra mass spectrometer (Sercon). Filtrates were used to analyze inorganic nutrients (nitrate, nitrite, phosphate) using the automated colorimetric method described in Aminot and Kérouel (2007), while samples for silicate analysis were filtrated through a 0.45µm polycarbonate membrane and kept at 5°C until analysis. Uncertainty of SPM is estimated to be 0.05 $mg.l^{-1}$ while the analytical error for POC determination is 5 µM (both errors were calculated during method validation, using replicates, and take into

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account scale and spectrometer precision and detection limits). Chlorophyll concentration was quantified using methanol extraction according to Raimbault et al. (2004). Samples (filters, filtrates and particles) were immediately frozen at -20°C until analysis.

2.2. Chemical treatment of the samples

Prior to analysis all samples were thawed and their water content was measured through the weighting of humid versus oven-dried particles: Dry particles (g)/Humid particles (g) *100. The suspended particles collected (between 200 and 450 mg per sample) first needed to be reduced with $NaBH_4$ and saponified. $NaBH_4$-reduction of hydroperoxides to alcohols that are amenable to gas chromatography-electron impact mass spectrometry (GC-EIMS) is essential for estimating the importance of photo-oxidative and autoxidative degradation in natural samples (Marchand and Rontani, 2001). Without this preliminary treatment, these labile compounds can be thermally cleaved during alkaline hydrolysis or GC analysis and thus be overlooked during conventional organic geochemical studies. Lipids and their degradation products in the resulting total lipid extracts (TLEs) were then quantified by GC-EIMS. All manipulations were carried out using foil-covered vessels in order to exclude photochemical artifacts. It is well known that metal ions can promote autoxidation during hot saponification (Pokorny, 1987). The prior reduction of hydroperoxides with $NaBH_4$ allowed us to avoid such autoxidation artifacts during the alkaline hydrolysis.

Particles were placed in methanol (MeOH) (15 ml) and hydroperoxides were reduced to the corresponding alcohols with excess $NaBH_4$ (70 mg; 30 min at 20 °C). During this treatment, ketones are also reduced and the possibility of some ester cleavage cannot be excluded.

Saponification was carried out on reduced samples. After $NaBH_4$ reduction, 15 ml water and 2.24 g KOH were added and the mixture directly saponified by refluxing for 2 h. After cooling, the content of the flask was acidified with HCl (pH 1) and extracted (3 x) with dichloromethane (DCM). The

combined DCM extracts were concentrated to give the TLE. After solvent evaporation, residues were taken up in 300 µl of pyridine/N,O-bis(trimethysilyl)trifluoroacetamide (BSTFA; Supelco; 2:1, v:v) and silylated for 1 h at 50 °C to convert OH-containing compounds to TMSi-ether or ester derivatives. After evaporation to dryness under a stream of N_2, the derivatized residues were taken up in 100µL BSTFA (to avoid desilylation of fatty acids) and an appropriate amount of ethyl acetate, depending on the concentration in lipids in each sample, in order to get the best possible GC-EIMS reading. It should be noted that under these conditions steran-3β,5α,6β-triols were silylated only at C3 and C6 and thus need to be analyzed with great care (Rontani et al., 2014b).

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A different treatment was used to quantify hydroperoxides and their ketonic and alcoholic degradation products. The samples were extracted three times with chloroform-MeOH-H_2O (1 : 2 : 0.8, v/v/v) using ultrasonication. The supernatant was separated by centrifugation at 3500G for 9 min. To initiate phase separation, purified H_2O was added to the combined extracts to give a final volume ratio of 1 : 1 (v/v). The upper aqueous phase was extracted three times with DCM and the combined DCM extracts were filtered and the solvent removed via rotary evaporation. The residue obtained after extraction was dissolved in 4 ml of DCM and separated in two equal subsamples. After evaporation of the solvent, degradation products were obtained for the first subsample after acetylation (inducing complete conversion of hydroperoxides to the corresponding ketones, Mihara and Tateba, 1986) and saponification and for the second after reduction with $NaBD_4$ and saponification. Comparison of the amounts of alcohols present after acetylation and $NaBD_4$ reduction made it possible to estimate the proportion of hydroperoxides and alcohols present in the samples, while after $NaBD_4$-reduction deuterium labelling allowed to estimate the proportion of ketones really present in the samples (Marchand and Rontani, 2003).

2.3. GC-EIMS analyses

Lipids and their oxidation products were quantified using an Agilent 7850-A gas chromatograph connected to an Agilent 7000-QQQ mass spectrometer. The following conditions were employed:

30 m x 0.25 mm (i.d.) fused silica column coated with HP-5MS (Agilent; film thickness: 0.25 µm); oven programmed from 70 to 130 °C at 20 °C/min, then to 250 °C at 5 °C/min and then to 300 °C at 3 °C/min; carrier gas (He), 1.0 bar; injector (splitless), 250 °C; injector (on column), 50 °C; electron energy, 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; m/z 40-700; collision energy, ranging from 5 to 15 eV; collision flow, 1.5 ml/min (N_2); quench flow, 2.25 ml/min (He); cycle time, 0.2 s. An on-column injector was used for the analysis of sterol degradation products and a splitless injector for the analysis of FA degradation products. Compounds were assigned by comparison of retention times and mass spectra with those of standards and quantified (calibration with external single standards) with GC-EIMS. For low concentrations, or in the case of co-elutions, quantification was achieved using selected ion monitoring (SIM) or Multiple Reaction Monitoring (MRM). The main characteristic mass fragment ions used to quantify degradation products of sterols have been described previously (Christodoulou et al., 2009; Rontani et al., 2011). Using replicates, the analytical standard error for lipid quantification (from preparation to integration) was estimated to be 14% (Standard Error = Standard Deviation / √(n) for n replicates).

2. Results and discussion

3.1 Water discharge, Suspended Particulate Matter (SPM) and Particulate Organic Carbon (POC)

Water flow patterns in the Rhone show pronounced seasonal variations predominantly influenced by 3 factors that can induce maximum discharge (Vivian, 1989): snow melting (May-June), intense rains in the south basin (October-November) and rains over the entire basin (January-march). During our sampling period the daily flow rate fluctuated between 680 (19 September 2012) and 4661 $m^3.s^{-1}$ (5 November 2011; Figure 2). Suspended Particulate Matter (SPM) and POC concentrations (Figure 2) ranged from 6.5 to 1381.3 $mg.l^{-1}$ and from 17.9 to 1383.3 µM C, respectively.

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The temporal evolution of suspended particulate matter (SPM) clearly followed the same pattern than water discharge (Figure 2), which is typical for river systems (Jansson, 1982). The samples collected on flood dates (November 2011, May 2013, liquid discharges above 3000 $m^3.s^{-1}$) display the highest SPM content and POC values (Figure 2). POC expressed as a percentage of SPM, ranged from 0.8 to 11.6%, and tends to be lower when water flows are highest (Sempéré et al., 2000)

3.2. Use of lipid tracers to estimate the composition and origin of POM collected in the Rhone River

3.2.1. Sterols

Based on a literature review, cholest-5-en-3β-ol (cholesterol), 24-ethylcholest-5-en-3β-ol (sitosterol if the C-24 stereochemistry is 24α), cholesta-5,24-dien-3β-ol (desmosterol), 24-methylcholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol), 24-methylcholesta-5,22-dien-3β-ol (brassicasterol and/or epi-brassicasterol depending on C-24 stereochemistry) and 24-methylcholest-5-en-3β-ol (campesterol) have been selected as tracers of the origin of POM. Apart from cholesterol, which can originate from a wide number of sources, all the other quantified sterols are relatively source-specific. Sitosterol constitutes the major sterol in higher plants, even though it can also be found in diatoms (Volkman, 1986), and is often used to trace terrestrial organic matter in lacustrine and marine systems (e.g. Meyers and Ishiwatari, 1993). Desmosterol is mainly found in algae (Volkman, 1986). 24-Methylenecholesterol is mainly found in diatoms, more particularly in the *Thalassiosira* and *Skeletonema* genera in the marine realm (Volkman, 2003). Epi-brassicasterol is mostly found in algae (Volkman, 1986).

All samples are dominated by sitosterol and cholesterol, with proportions being on average three times higher than those of the other sterols, apart from the March 6, 2012 sample (Table 1). This sample exhibits a rather different profile, dominated by desmosterol (0.122 $\mu g.mg^{-1}$ (dry weight))

and 24-methylenecholesterol (0.096 µg.mg^{-1} (dry weight)), with a strong contribution from brassicasterol (0.026 µg.mg^{-1} (dry weight)) compared to all other samples. All these sterols have been considered to be planktonic markers, and have been summed to compose the planktonic sterol fraction shown in Figure 3A. This fraction forms the major part of the total sterol fraction of the March 2012 sample, evidencing a phytoplanktonic bloom event. Given the nature of the sterols involved (desmosterol, methylene-cholesterol), diatoms seem to be major contributors (Rampen et al., 2010). This concurs with the drops in silica observed at the same period (MOOSE data, unpublished). As we see an increase, although less important in the proportion of planktonic sterols in the Spring 2013 samples (20 times less in quantity when compared to the 6 March 2012 sample, but still constituting 31 and 27% of the total of all sterols quantified in the 12 March and 21 March 2013 samples respectively), this type of planktonic event is probably a yearly spring occurrence but our sampling frequency was not adapted to study blooms that can appear and disappear in a matter of days. Sitosterol has been previously identified in marine algae, and more widely as the major sterol in higher terrestrial plants (Volkman, 1986; 2003). However the clear increase observed here in March 2012 demonstrates that it is also present in potamoplankton (Figure 3A). The presence of cholesterol can indicate a zooplankton contribution (Volkman, 1986), but it is also often found in freshwater algae (Volkman et al., 1981; Gagosian et al., 1983), and can also evidence human impacts (Sicre et al., 1993). The relatively high proportions of 5β(H)-cholestan-3β-ol (coprostanol) detected in the samples investigated (see section 3.3.2) clearly show that in the Rhone River cholesterol mainly arises from waste water inputs (Brown and Wade, 1984).

We also looked at the ratio sitosterol/campesterol (Table 1) as an indicator of the terrigenous versus diatom origin of our POM. While many plants have a sitosterol/campesterol ratio of less than 4 (Volkman 1986), Nishimura and Koyama (1977) reported values ranging from 11.5 (*Pinus densiflora*) to 31 (holly *Ilex pedunculosa*). Dachs et al. (1998) identified the threshold of 1 as the limit under which samples are dominated by OM of aquatic origin, and above which sources of OM are terrigenous. In our case, all the samples present a sitosterol/campesterol ratio above 1, mainly

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between 2 and 8 (up to 12). Three samples show a ratio near 1 (July 2011 and 2012 and March 2012; Table 1), a low value which confirms the presence of diatoms in the plankton since freshwater diatoms exhibit sitosterol/campesterol ratios close to 1.0 (Ponomarenko et al., 2004).

3.2.2. Terrestrial vascular plant biomarkers

The contribution of terrestrial vascular plants to our samples is also evidenced by the presence of the triterpenoids betulin (lup-20(29)-ene-3β,28-diol), oleanolic (3β-hydroxyolean-12-en-28-oic) and ursolic (3β-hydroxyurs-12-en-28-oic) acids (Razboršek et al., 2008) as well as components of cuticular waxes (16,8-11-dihydroxyhexadecanoic, ω-hydroxyhexadecanoic, ω-hydroxyoleic and 18-hydroxyoctadec-9-enoic acids) (Kolattukudy, 1980) (Figure 3B). Betulin has been proposed as a tracer for paper birch (Fine et al., 2001), a common species along the Rhone River, while oleanolic and ursulic acids are widely distributed in terrestrial higher plants (Liu, 1995). The amount of cuticular waxes is variable amongst samples, between 0.02 and 3.8 $\mu g.mg^{-1}$(dry weight), with the highest in the 5/4/2011, 2/5/2013 and 4/11/2011 samples (3.8, 2.2 and 1.7 $\mu g.mg^{-1}$ respectively). Two of these sample dates (2 May 2013 and 4 November 2011) happen to be flood dates. It is clear that floods, during which higher water flows are coupled to surface runoff, collect and carry more terrestrial plant leaf debris, and hence increase the amount of cuticular waxes found in our samples. The yearly variations in quantity are probably due to the fact that waxes (linear compounds) are more easily degraded by bacteria than cyclic structures such as sterols or triterpenoids (Atlas and Bartha, 1992).

The betulin and sitosterol concentrations are significantly correlated in most of our samples (r=0,67 between sitosterol and betulin, on 29 samples, p-value = 3.10^{-5}, excluding the March, 6, 2012 sample due to its out-of-range phytoplanktonic profile), thus reinforcing the idea that in the Rhone

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River sitosterol mainly results from terrestrial higher plant inputs. However, at the time of the spring bloom a significant part of this sterol seems to derive from potamoplankton (Figure 3A).

Another ratio commonly used to attest to the terrigenous origin of compounds is the Terrigenous-to-Aquatic ratio (TAR, Bourbonnière & Meyers, 1996). Here we used the $TAR_{(AL)}$ as calculated by Van Dongen et al. (2008) for *n*-alkanols: $(C_{26}+C_{28})/(C_{16}+C_{18})$. The $TAR_{(AL)}$ in our samples is always above 1, and clearly indicates a strong terrigenous contribution to the suspended particulate matter found in the lower Rhone. The Average Chain Length of *n*-alkanols, a proxy positively correlated to the abundance of higher plant debris (Van Dongen et al., 2008), ranged from 26 to 22 across all samples, also attesting to the strong contribution of terrestrial vascular plants. The long-chain even-numbered *n*-alkanol profiles show a strong contribution of C_{22} and C_{28} *n*-alkanols. Compared with those previously described in the literature (Diefendorf et al., 2011), this characteristic suggests a strong gymnosperm contribution, which concurs with the low amounts of long-chain *n*-alkanes detected.

3.2.3. Chlorophyll

The available data on chlorophyll *a* (MOOSE database, only available for 2012 and 2013) shows a content variability between 0.9 (10 October and 6 November, 2012) and 14.0 (3 April 2012) $mg.m^{-3}$ (Figure 4A). Chlorophyll *a* is frequently used as a proxy for photosynthetic organisms and the variation observed here is consistent with the hypothesis of a yearly phytoplanktonic spring bloom, with a larger magnitude for the 2012 event.

3.2.4. Fatty acids

A number of saturated linear fatty acids have been found in our samples, with $C_{16:0}$ and $C_{18:0}$ being the most abundant (Table 2). These fatty acids are not specific, and can stem from a number of sources including terrestrial vascular plants, algae and bacteria (Volkman et al., 1981). More

316 recently. $C_{16:1\omega7}$ and $C_{20:5\omega3}$ (eicosapentaenoic acid) were identified as 2 of the main fatty acids in
317 Bacillariophyceae (Taipale et al., 2013). These 2 markers are present in our samples, and together,
318 form 40.5 and 34.1% of the 6 March 2012 and 12 March 2013 samples (versus an average of 13,2%
319 across all samples), which, when coupled with our sterol analysis, concurs with our hypothesis that
320 diatoms are major contributors in the algal blooms identified. It is worth noting that in the 3
321 October 2012 sample, $C_{16:1\omega7}$ forms 53.6% of all quantified fatty acids, while $C_{20:5\omega3}$ is completely
322 absent (0%), and therefore these markers alone cannot be considered to be specific enough in
323 natural river water samples which can contain a number of fatty acids from various sources.
324 Longer-chain saturated fatty acids (between C_{20} and C_{28}) with a strong even-chain predominance,
325 which are specific to the epicuticular waxes of vascular plants (Kunst and Samuels, 2003), are
326 scarcely present in our samples, with C_{20} and C_{22} being the most abundant relative to the others:
327 over 98% (on average) of the total of long-chain (C_{20}-C_{28}) saturated fatty acids across all samples.
328 Due to the high degradability of fatty acids, a number of them could not be quantified, potentially
329 leading to an underestimation of higher plant contribution.
330 Polyunsaturated fatty acids are present in very low proportions in our samples, apart from the
331 06/03/2012 and 12/03/2013 samples where they contributed to 44 and 40% of total fatty acids
332 (Figure 3C - quantified using the total of all PUFA quantified between C_{14} and C_{26}). These high
333 contributions support the presence of a high proportion of fresh algal material in these samples.
334 If we compare the average chain length (ACL) of fatty acids in our samples with that of *n*-alkanols,
335 it appears clearly that the ACL of fatty acids is lower, with an average of 16.7 across samples,
336 against 23.9 for alcohols. It is widely accepted that fatty acids are more prone to bacterial
337 degradation than other lipids (Wakeham, 1995), and long-chain fatty acids tend to be degraded
338 more efficiently by a number of bacteria (Novak and Carlson, 1970). Such a bacterial degradation
339 could explain the ACL difference between *n*-alkanols and fatty acids, and the lack of terrestrial
340 higher-plant fatty acids in our samples, while other markers for higher plants (such as waxes or
341 betulin) are present in large quantities. This is reinforced by the fact that we also find a relatively

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high proportion of vaccenic acid in our samples, a specific marker for bacterial activity (Sicre et al., 1988)

3.2.5. Hydroxyacids

Hydroxyacids contents were low during the period studied but some samples (18/07/2011, 4/11/2011 and 16/01/2012) exhibited high amounts of C_{22} ω-hydroxyacid and small quantities of C_{20} and C_{24} homologues (Table 2). These compounds are generally considered to be suberin markers when found in soil (Nierop, 1998; Otto et al., 2005), even though they have been found in leaves and stems of higher plants as well (Mendez-Millan et al., 2010). Suberin is a cell wall component of cork cells, and is mainly found in bark, woody stems, and roots (Kolattukudy, 1980). Given the geographical location of our sampling point, we can consider this compound to be a marker of industrial activity, probably associated to a paper paste mill being implanted less than 3 km upstream. The mill uses mainly local conifers (collected within a 250 km radius according to the company, Paper Excellence (2014)), more particularly *Pinus halepensis*, *Pinus nigra*, and *Pinus sylvestris* (Etude AGRESTE, 2011) and is authorized to reject a certain amount of waste water in the river. This is consistent with our findings that the POM is dominated by gymnosperms. *Pinus* species also display a sitosterol/campesterol ratio comprised between 5 and 10 (Conner et al., 1980) in line with most of our samples as well. This industrial contamination could explain the large contribution of gymnosperms to our POM, and implies that we consider the extra input of terrestrial plant matter that will be released, and degraded, at sea.

As a summary, the overall lipid composition of the Rhone River SPM is characterized by major terrestrial higher plant components (mainly derived from gymnosperms) with episodic, but significant, contributions from freshwater algal material (probably dominated by diatoms) in the spring. Despite the strong concentration of industries along this river, SPM appears to be very

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370 weakly contaminated by petroleum hydrocarbons, but is strongly impacted by the local paper mill
371 and wastewater discharges (see section 3.2.2).
372
373
374 ### 3.3. Use of lipid tracers to estimate the degradation state of POM from the
375 ### Rhone River

376 If they can inform us on the origin of organic matter in natural environments, lipid biomarkers are
377 also invaluable in helping us estimate biotic and abiotic alterations of organic matter and determine
378 what are the main processes involved in its degradation. Products resulting from the degradation of
379 sterols, chlorophyll, monounsaturated fatty acids and hydroxyacids are among the most useful and
380 specific tracers.
381
382 #### 3.3.1. Chlorophyll

383 The absorption of light by some compounds, called photosensitizers, in the presence of oxygen
384 (regardless of these compounds being endogenous or not) causes an oxidation whose effects,
385 chemical or biological, are mostly adverse (Spikes and Straight, 1967). Photosensitizers induce
386 chemical reactions via the absorption of light that would not occur in their absence. Photosensitizers
387 (Sens) are involved in indirect photo-oxidative processes: they have 2 systems of electronically
388 excited states, ^{1}Sens and ^{3}Sens. The triplet state is much longer lived than the singlet state, which is
389 the initial product issued from light absorption. Indirect photo-oxidation (photo-sensitized
390 oxidation) can be intense during the senescence of phototrophic organisms (Rontani, 2012) due to
391 the presence of chlorophyll, which is a very efficient photosensitizer (Foote, 1976) capable of
392 generating singlet oxygen particularly reactive towards unsaturated cellular components (Type II
393 photoprocesses). Chlorophyll may be also directly photodegraded by solar light (Nelson, 1993).
394 Direct photodegradation of chlorophyll and Type II photo-oxidation of unsaturated cellular
395 components can be thus considered two competitive photo-processes.

In the photic layer of aquatic environments, photo-oxidation has long been considered a major degradation process for phytoplankton chlorophyll pigments (Lorenzen, 1967; Vernet, 1991). Since we have no marker stable and specific enough for chlorophyll tetrapyrrolic ring photodegradation, we used the CPPI (Chlorophyll Phytyl side chain Photodegradation Index) for the in-situ determination of the rate of photodegradation of chlorophyll (Cuny et al., 1999). Indeed, the photodegradation of the chlorophyll phytyl side chain produces 3-methylidene-7,11,15-trimethylhexadecan-1,2-diol (phytyldiol), specific of Type II chlorophyll photodegradation and widespread in the environment (Cuny and Rontani, 1999). The CPPI, (phytyldiol:phytol molar ratio) can be linked, through a mathematical model, to the global quantity of photodegraded chlorophyll (Cuny et al., 1999),

The photodegradation rate of chlorophyll fluctuates greatly (Figure 4B). The 06/03/2012 and 12/03/2013 samples display very low rates (2.6 and 9.8% respectively), in line with our identification of planktonic blooms in March 2012 and 2013. Such blooms result in an increase of fresh chlorophyll inputs, with intact phytyl side chains, and thus in a decrease of CPPI. The dips in chlorophyll photodegradation rates can help us identify blooms, or at least periods when the input of chlorophyll is higher. The summer 2012 samples (July and September) also display a low photodegradation rate ranging from 8.8 to 13.9% while their amount of planktonic sterols increases slightly.

3.3.2. Δ^5-sterols

Δ^5-sterols possess structural features that can be restricted to a limited number of organisms (Volkman, 1986; 2003). Moreover, biotic and abiotic degradation processes result to specific functionalizations of their cyclic skeleton (De Leeuw and Baas, 1986), which are very useful to estimate the relative importance of these processes (Christodoulou et al., 2009; Rontani et al., 2009). Consequently, degradation products of Δ^5-sterols constitute excellent biomarkers for tracing

diagenetic transformations of specific organisms (Mackenzie et al., 1982). Using these tracers to evaluate the relative influence of different degradation processes requires that their removal rate (by further degradation) is comparable to that of the parent Δ^5-sterol. Although each sterol and its degradation products may be potentially totally mineralized by marine bacteria, we assume that they should exhibit similar reactivity towards bacterial degradation. This assumption is based on the fact that aerobic biodegradation of sterols generally involves initial attack on the side chain, which is similar in all the degradation tracers selected to that of the corresponding parent Δ^5-sterol. Moreover, it may be noted that 3β,5α,6β-steratriols, employed for autoxidation estimates are weakly affected by abiotic degradation processes. This is also the case for Δ^4-6α/β -hydroperoxysterols (photooxidation tracers), which are much more stable than Δ^5-7α/β - and Δ^7-5α-hydroperoxysterols (Christodoulou et al., 2009). Indeed, β-scission of the alkoxyl radicals resulting from homolytic cleavage of Δ^5-7-hydroperoxysterols and Δ^6-5-hydroperoxysterols affords secondary and tertiary radicals, respectively, more stable than the primary radical resulting from the cleavage of Δ^4-6-hydroperoxysterols (Christodoulou et al., 2009). Moreover, proton driven cleavage (Hock cleavage) of Δ^5-7-hydroperoxysterols and Δ^6-5-hydroperoxysterols involves a highly favored migration of vinyl group (Frimer, 1979), while only an unfavored migration of alkyl group is possible in the case of Δ^6-5-hydroperoxysterols (Rontani et al., 2014).

Aerobic bacterial hydrogenation may convert Δ^5-sterols to 5α(H)-stanols, 5α(H)-stanones and ster-4-en-3-ones (Gagosian et al., 1982; De Leeuw and Baas, 1986; Wakeham, 1989). During the treatment undergone by our samples ($NaBH_4$ reduction), 5α(H)-stanones and ster-4-en-3-ones are respectively converted in 5α(H)-stanols and ster-4-en-3-ols; these compounds hence constitute useful markers of bacterial degradation of sterols. To evaluate the proportion of biological degradation of cholesterol, and to better trace human impacts on the OM found in the Rhone, we also included coprostanol and epicoprostanol in its biodegradation products. Sterol biodegradation percentages were estimated using Eq. (1a) and (1b). Coprostanol (5β(H)-cholestan-3β-ol) is a stanol that arises from the anaerobic microbial degradation of cholesterol in the digestive tracts of higher

land mammals, including man (Martin et al., 1973). Epicoprostanol (5β(H)-cholestan-3α-ol) is not a major sterol in human faeces, but it is often used as an indicator of sewage treatment (McCalley et al., 1981).

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Sitosterol biodegradation % = [sitostanol] / [sitosterol] * 100 (1a)

Cholesterol biodegradation % = [cholestanol + coprostanol + epicoprostanol]/ [cholesterol] * 100 (1b)

Free radical autoxidation yields mainly Δ^5-3β-7α/β-hydroperoxides and in smaller quantities 5,6-epoxysterols and 3β,5α,6β-trihydroxysterols. 3β,5α,6β-trihydroxysterols were chosen as tracers of autoxidation (Christodoulou et al., 2009; Rontani et al., 2009) and the sterol autoxidation percentage was estimated using Eq. (2) based on autoxidation rate constants calculated by Morrissey and Kiely (2006).

Sterol autoxidation % = ([3β,5α,6β-trihydroxysterols] * 2.4) / [sitosterol or cholesterol] * 100 (2)

$^1O_2$-mediated photo-oxidation (Type II photoprocesses) yields mainly Δ^6-5α-hydroperoxides and to a lower extent Δ^4-3β-6α/β-hydroperoxides. Δ^6-5α-hydroperoxides are unstable and are converted very easily to the non-specific 7-hydroperoxides, so they were discarded as markers of photo-oxidation. Although produced in lesser amounts, Δ^4-3β-6α/β-hydroperoxides, which are relatively stable and highly specific, have been chosen as tracers of photo-oxidation processes and quantified after $NaBH_4$ reduction to the corresponding diols. The percentage of sterol photo-oxidation was estimated using Eq. (3) (Christodoulou et al., 2009), based on the ratio Δ^4-6α/β-hydroperoxides/Δ^6-5α-hydroperoxides found in biological membranes (0.30) (Korytowski et al., 1992).

Sterol photo-oxidation % = ([Δ^4-3β-6α/β-dihydroxysterols] * (1+0.3)/0.3) / [sitosterol or cholesterol] * 100 (3)

Here, values are expressed in proportions relative to the amount of remaining parent sterol in the sample. A total percentage of over 100% hence only means that degradation products were present in larger quantities than their associated parent sterol.

The results of the evaluation of sitosterol and cholesterol degradation processes are shown in Figure 5. The most highly degraded samples sitosterol-wise were the ones from 18/07/2011, 26/06/2012 and 22/01/2013. Interestingly, cholesterol degradation shows a completely different trend. When looking at the type of degradation undergone by Δ^5-sterols, it also appears clearly that, if auto- and photo-oxidation processes are the major drivers of sitosterol oxidation, biodegradation is the major player in cholesterol degradation. Hedges and Keil (1995) hinted that sterols associated with waxy higher plant material might not be as prone to enzymatic degradation as other sterols, which would explain why sitosterol is only weakly biodegraded in our samples. Indeed, even though we showed that sitosterol is also produced during spring phytoplanktonic bloom, it is mainly derived from higher plants in most of the samples investigated. The dominance of coprostanol relative to cholestanol attests to a strong contribution of human or animal faeces to the cholesterol present in the Rhone River. Here we used two different ratios in order to better understand of how human activities and waste waters affect the Rhone waters. The first ratio, used to determine if waters are affected by waste water inputs and runoff has been previously used by Writer et al. (1995), and is calculated as follows: coprostanol/(cholesterol+cholestanol). These authors have defined a threshold of 0.06 above which samples are considered affected by wastewater inputs and runoff from pastures and feedlots, and below which they can be considered pristine. The second ratio is the epicoprostanol:coprostanol ratio proposed by Mudge and Seguel (1999), in order to illustrate the level of treatment undergone by wastewaters. The higher the ratio, the more treated the waters are, as epicoprostanol is only present in traces in primary sludge, while it is present in much higher quantities in treated sludge (McCalley et al., 1981). Both ratios are represented in Figure 6 and they show that the Rhone is undeniably affected by wastewaters and, to a much lower extent given the

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historical land use around the Rhone, pasture runoff. However, we noted that both the ratio and the threshold used are unofficial and in no way are proof of a large-scale contamination or pollution. We only highlight here the non-pristine state of the Rhone waters, which is logical given the level of urbanization along the river. The epicoprostanol:coprostanol ratio also shows fluctuations in the level of treatment of wastewaters, and fluctuates with flow rates and precipitation levels.

During senescence, unsaturated higher plant lipids (and notably Δ^5-sterols) may be photodegraded (type II photo-oxidation), with chlorophyll acting as a sensitizer (Rontani et al., 1996). Sitosterol present in higher plant phytodetritus should thus have been intensely photodegraded on land. However, the photo-oxidation rate estimates appeared to be relatively low compared to that of autoxidation and biodegradation (Figure 5A). This is probably due to an intense free-radical-driven breakdown of hydroperoxides produced during photo-oxidation (Rontani et al., 2003). The photo-oxidation percentages displayed here are thus certainly underestimated.

The presence of large amounts of 24-ethylcholestan-3β,5α,6β-triol in most of the samples indicates that autoxidation plays an important role in the degradation of sitosterol (Figure 5A). Autoxidation (spontaneous free radical reaction of organic compounds with O_2), which has been largely ignored until now in the environment, seems to play a key role in the degradation of sitosterol (Figure 5A) and thus of higher plant material carried by the Rhone River. This assumption was well supported by the detection of significant proportions of compounds deriving from betulin autoxidation (Data not shown). Recently, it has been demonstrated that autoxidation plays a key role in the degradation of terrestrial (Rontani et al., 2014b) and marine (Rontani et al., 2014a) vascular plant debris in seawater. There is clearly a growing body of evidence suggesting that autoxidation reactions can strongly impact the preservation of particulate organic matter in the environment and should be considered carefully alongside other removal processes such as biodegradation when constructing carbon cycles and evaluating carbon budgets. The lowest autoxidation rates observed in samples from 06/03/2012 and 12/03/2013 may be attributed to the phytoplanktonic bloom events, with high inputs of fresh material. While there is variability in the amount and type of degradation undergone

by sterols in the sampled particulate matter, it is evident that sitosterol and cholesterol behave very differently when being degraded.

3.3.3. Unsaturated fatty acids and cuticular waxes

Fatty-acid sensitivity to photo- and autoxidation is intrinsically linked to their number of double bonds (Frankel, 1998), and we will only be looking at unsaturated fatty acids here. Unfortunately, oxidation products of polyunsaturated fatty acids (PUFA) are not stable enough to be used to monitor PUFA degradation. In contrast, photo- and autoxidation products of mono-unsaturated fatty acids (allylic hydroperoxyacids) are much more stable, and can be used (after $NaBH_4$-reduction to the corresponding hydroxyacids) as tracers of the abiotic oxidation processes affecting POM (Marchand and Rontani, 2001). Free-radical-mediated oxidation (autoxidation) processes can be easily discriminated against photo-oxidation processes thanks to the specific *cis* allylic hydroperoxyacids specifically produced by autoxidative processes (Marchand and Rontani, 2001). Samples only displayed small amounts of oxidation products of oleic acid (not quantified), probably due to the fact that unsaturated fatty acids and their degradation products are very labile and easily metabolized by bacteria (Marchand et al., 2005). Despite this degradation, the profiles obtained by GC-MS (exhibiting relatively high proportions of *cis* oxidation products) allowed us to confirm the important role played by autoxidation in the degradation of POM in the Rhone River.

Cutin is present in cuticles covering all aerial parts of higher plants. It is constituted of biopolyesters mainly composed of hydroxy fatty acids. Long-chain *n*-alkanoic, ω-hydroxy, dihydroxy, trihydroxy and epoxy-hydroxy acids constitute the major aliphatic monomers (Kolattukudy, 1980). It was previously demonstrated that Type II photooxidation processes act on some unsaturated cutin monomers such as ω-hydroxyoctadec-9-enoic acid (ω-hydroxyoleic acid) during the senescence of higher plants (Rontani et al., 2005). $^1O_2$ reacts with the carbon-carbon double bond, and leads to the formation of a hydroperoxide at each unsaturated carbon. Due to the involvement of allylic rearrangements, Type II photosensitized oxidation of ω-hydroxyoleic acid results (after $NaBH_4$-

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555 reduction of hydroperoxides to the corresponding alcohols) in the formation of isomeric allylic
556 18,(8-11)-dihydroxyoctadecanoic acids, with a *trans* double bond. These compounds constitute
557 interesting specific tracers of higher plant material photo-oxidation. Autoxidation of this compound
558 was never studied, but by analogy with oleic acid oxidation the autoxidative formation of specific
559 *cis* allylic hydroperoxyacids was expected.
560 Significant amounts of allylic 18,(8-11)-dihydroxyoleic acid, with *cis* and *trans* double bonds have
561 been effectively detected in some (not all) samples analyzed attesting to the involvement of auto-
562 and photo-oxidation of higher plant material (Galeron & Rontani, unpublished data). The high
563 proportions of *cis* isomers observed confirmed the dominance of autoxidation processes. Some
564 samples (16/1/2012, 26/6/2012 and 18/7/2011) even displayed larger amounts of oxidation products
565 than ω-hydroxyoleic acid, which evidences the importance of degradative processes on this
566 compound. The previously discussed yearly variability in cuticular wax content in our samples (see
567 section 3.2.2 and Fig. 3b) explains some of these results.
568
569 **3.3.4. Hydroperoxide stability in SPM**

570 It was previously proposed that photochemically-produced hydroperoxides could induce intense
571 autoxidation processes in the marine environment (Rontani et al., 2014a). Hydroperoxides resulting
572 from photo-oxidation processes may undergo: (i) heterolytic cleavage catalyzed by protons (Frimer,
573 1979) and (ii) homolytic cleavage induced by transition metal ions (Pokorny, 1987) or UVR
574 (Horspool and Armesto, 1992). Homolytic cleavage of hydroperoxides would lead to the formation
575 of alkoxyl radicals, which can then: (i) abstract a hydrogen atom from another molecule to give
576 alcohols, (ii) lose a hydrogen atom to yield ketones, or (iii) undergo β-cleavage reactions affording
577 volatile products. During the $NaBH_4$-reduction, hydroperoxides and ketones were reduced to the
578 corresponding alcohols. The sum of the corresponding hydroperoxides, ketones and alcohols was
579 evaluated under the form of alcohols. Application of a different treatment allowed us to specifically

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quantify hydroperoxides, alcohols and ketones (remaining in cuticular waxes, phytol, oleic acid, sitosterol and cholesterol oxidation products) (Figure 7).

Clearly, the proportion of remaining hydroperoxides was highest in the case of sterols, with 49.4 and 31.3% respectively for 3,6- and 3,7-diols of sitosterol, and 51.5 and 33.5% for 3,6- and 3,7-diols of cholesterol, against less than 20% (17.3%) for cutins, 12.0% for oleic acid, and 6.6% for phytol. Standard error was calculated based on all the results obtained (Standard Error = Standard Deviation / √(n) for n samples). These results clearly indicate that despite the involvement of an intense free radical oxidation (autoxidation) inducing homolytic cleavage of peroxy bonds, a significant proportion of hydroperoxides is still intact in POM of the Rhone River. This proportion reaches 10% of the parent residual compound in the case of sitosterol and 5% in the case of cholesterol. Probably due to high compartmentalization effects, preservation of these compounds seems to be enhanced in higher plant debris. It was recently proposed that homolytic cleavage of photochemically-produced hydroperoxides in riverine POM could be catalyzed by some redox-active metal ions released from SPM in the mixing zone of riverine and marine waters (Rontani et al., 2014b). Due to the presence of significant amounts of hydroperoxides in higher plant residues, the involvement of intensive autoxidation of this material in the Rhone estuary is thus likely.

4. Conclusions

Based on a two-year study of the particulate organic matter content of the Rhone River, the main freshwater supplier to the Mediterranean Sea, lipid analyses evidenced a strong contribution from terrestrial vascular plants, most notably gymnosperms. Phytoplanktonic cyclical inputs were also observed and identified as seasonal blooms. The observed amounts of sterols of diatom origin and steep drops in silica observed at the time of the blooms led us to suspect diatoms to be major contributors. Specific compounds like suberin markers, coprostanol and epicoprostanol allowed us to identify anthropic impacts on POM composition (paper mill discharging wastewater and treated waste water discharge).

609 Using specific lipidic degradation products, we were able to identify for the first time the part that
610 bacterial degradation, autoxidation and photo-oxidation play in organic matter degradation in a
611 Mediterranean river. The study of lipid oxidation products showed that autoxidation, which has
612 been largely underestimated until now in the environment, plays a major role in the degradation of
613 plant-derived organic matter: autoxidation-produced degradation products are nearly half as
614 important as the remaining amount of sitosterol (25% on average across all samples), while
615 biodegradation-produced compounds only represent about a quarter (15% on average across all
616 samples). Photo-oxidation degradation products, although probably underestimated, only stand at
617 10% (on average across all samples) of the remaining sitosterol amounts. While autoxidation
618 processes appeared to play only a minor role during the degradation of sitosterol in the cold
619 Mackenzie River (Arctic) (Rontani et al., 2014b), it is worth questioning the role that temperature
620 plays in OM degradation, and wondering if it influences one degradation process over the others: do
621 the temperatures found in the Rhone River, warmer than those of the Mackenzie, favor
622 autoxidation? However, the low autoxidation state in the Mackenzie River could also be attributed
623 to the presence of significant proportions of fresh sitosterol-producing phytoplanktonic species
624 (Tolosa et al., 2013). Due to the lack of specificity of this sterol, it is clear that identification of
625 autoxidation products of more specific tracers (such as betulin or amyrins) is absolutely necessary
626 to monitor the degradation of higher plant material in rivers and oceans more precisely.
627 We also noted that cholesterol offers a very different degradation profile, undergoing little
628 autoxidation, but mainly influenced by biotic degradation processes. Cholesterol is non-specific and
629 can come from a number of sources, which is why it is not used as a tracer of the origin or the
630 general degradation state of POM. However, its bacterial degradation products, including
631 coprostanol and epicoprostanol, are good markers of sewage contamination and sewage treatment,
632 and clearly indicated here that the Rhone waters were affected by wastewaters.

Finally, we have shown that even through the degradative processes impacting the TPOM flowing down the Rhone, there is still an important quantity of hydroperoxides remaining in the OM, which in turn should favor autoxidation upon the OM arrival at sea.

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Marie-Aimée Galeron 8/1/y 11:19
Mis en forme: Avec coupure mots

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Figure Captions

- Figure 1. Rhone River mouth area and sampling location: “Arles”. gR and pR mean grand Rhone and petit Rhone which are the two arms of the river.

- Figure 2: Water Flow ($m^3.s^{-1}$), Suspended Particulate Matter ($mg.l^{-1}$) and Particulate Organic Carbon (POC, µM) of Rhone waters on our sampling dates (MOOSE data). Samples span a wide range of water flow conditions.

- Figure 3: Sitosterol and planktonic sterols (A), cuticular waxes and betulin (B), and Poly-Unsaturated Fatty Acid (PUFA) (C) contents of the different samples. Standard error shown was estimated to be 14% (see section 2.3). Phytoplanktonic blooms are evidenced by the spike in planktonic sterols, and while the other tracers show the terrigenous origin of the POM sampled, the ubiquitous nature of sitosterol is made clear.

- Figure 4: Chlorophyll a levels (MOOSE data) and percentage of photodegradation of chlorophyll (calculated using CPPI (Cuny et al., 1999) in the Rhone waters on and around sample dates

- Figure 5: Biotic and abiotic degradation of sitosterol (A) and cholesterol (B) in the different samples. Full error shown here incorporates the 14% analytical standard error estimated for lipid quantification for all terms of the equations used. Sitosterol and cholesterol clearly have very different degradation patterns.

- Figure 6: Coprostanol:(cholesterol+cholestanol) and epicoprostanol:coprostanol ratios of the different samples. Full error shown here incorporates the 14% analytical standard error estimated for lipid quantification for all terms of the ratios. Contamination threshold is 0.06 (See section 3.3.2).

- Figure 7: Relative percentages of intact hydroperoxides and their ketonic and alcoholic degradation products measured in the case of ω-hydroxyoleic (cuticular waxes) and oleic acids, phytol, sitosterol and cholesterol oxidation products. Standard error was calculated based on the results obtained for all samples.

Table 1: Percentages of sterols (relative to the sum of sterols quantified), and sitosterol:campesterol ratio in the samples investigated.

Date	Sitosterol	Cholesterol	Desmosterol	Brassicasterol	Methylene-Cholesterol	Campesterol	Sitosterol:Campesterol ratio
5 April 2011	74.4	12.7	0.4	4.9	1.2	6.4	11.6
18 July 2011	21.2	45.5	2.6	16.1	3.3	11.3	1.9
4 November 2011	54.1	23.8	1.9	10.0	2.2	8.0	6.7
5 November 2011	60.2	21.4	1.2	7.7	1.7	7.9	7.6
7 November 2011	56.7	28.6	0.4	6.1	1.3	6.9	8.2
14 November 2011	34.6	45.7	0.9	10.0	1.5	7.4	4.7
19 December 2011	52.5	29.1	0.5	7.5	1.9	8.5	6.2
16 January 2012	39.2	43.6	1.3	6.7	1.8	7.4	5.3
6 March 2012	17.6	12.4	28.8	6.2	22.6	12.4	1.4
17 April 2012	36.5	39.3	4.7	8.2	2.9	8.4	4.4
2 May 2012	38.4	41.4	2.1	9.5	1.9	6.7	5.7
22 May 2012	32.5	43.6	4.4	10.3	2.6	6.5	5.0
11 June 2012	35.0	43.5	2.5	9.6	1.6	7.7	4.5
26 June 2012	24.7	54.9	2.6	10.0	1.8	6.0	4.1
25 July 2012	21.6	37.5	3.9	20.8	4.4	11.7	1.8
5 September 2012	22.6	46.6	3.4	15.4	3.3	8.6	2.6
19 September 2012	21.1	44.3	3.8	19.1	3.0	8.7	2.4
3 October 2012	27.4	46.7	2.3	11.9	1.9	9.8	2.8
16 October 2012	42.9	38.9	1.4	7.8	1.4	7.6	5.7
6 November 2012	44.9	36.9	2.0	7.3	1.8	7.1	6.3
17 December 2012	66.3	22.8	1.0	3.5	1.2	5.2	12.7
10 January 2013	46.9	34.8	2.5	7.1	1.5	7.2	6.5
22 January 2013	36.0	48.4	1.4	6.9	1.3	6.1	5.9
4 February 2013	50.0	39.2	0.8	4.0	0.8	5.1	9.8
13 February 2013	55.4	30.7	2.0	4.8	1.3	5.9	9.4
12 March 2013	35.1	27.6	14.5	9.0	7.6	6.2	5.7
21 March 2013	37.4	29.0	11.1	8.9	6.6	7.2	5.2
17 April 2013	35.6	45.6	2.6	7.0	2.5	6.7	5.3
2 May 2013	59.0	24.5	1.1	6.8	1.7	6.8	8.7
13 May 2013	49.7	38.6	0.0	4.6	1.1	6.1	8.2
Average	41.0	35.9	3.6	8.9	3.0	7.6	5.9
Standard deviation	14.6	10.7	5.6	4.2	4.0	1.8	2.8

Table 2: Fatty and Hydroxy Acid (FA/HA) content of the different samples (% of total quantified FA/HA) (PUFA: Poly-Unsaturated Fatty Acids)*double bond position undetermined

	2011							2012															2013								
	5/4	18/7	4/11	5/11	7/11	14/11	19/12	16/1	6/3	17/4	2/5	22/5	11/6	26/6	25/7	5/9	19/9	3/10	16/10	6/11	17/12	10/1	22/1	4/2	13/2	12/3	21/3	17/4	2/5	13/5
$C_{14:0}$	4.4	8.4	5.4	3.5	1.6	4.2	5.1	6.1	11.4	6.8	2.9	2.2	3.8	6.0	2.2	8.2	1.6	1.2	1.5	1.2	1.1	3.9	2.0	1.3	2.1	3.5	5.3	3.6	1.6	4.6
$C_{15:0}$	2.6	2.1	2.1	2.7	1.0	2.1	1.9	2.0	0.4	2.2	2.1	1.1	2.1	2.3	1.1	1.3	0.9	0.9	1.3	1.0	1.0	1.9	2.3	1.1	1.5	0.7	0.8	1.7	1.2	2.5
$C_{15:1}$	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0
Iso-$C_{15:0}$	2.1	4.3	2.8	1.6	0.2	3.2	2.3	3.1	0.1	1.5	1.2	0.4	1.4	1.1	0.7	2.9	0.5	0.9	0.9	0.3	0.2	1.5	0.3	0.2	0.4	0.2	0.5	1.2	0.7	0.5
Anté-iso-$C_{15:0}$	1.4	2.1	2.1	1.3	0.2	2.1	2.3	2.9	0.1	1.3	1.0	0.5	1.2	0.9	0.4	1.4	0.4	0.7	0.9	0.4	0.2	1.5	0.4	0.2	0.5	0.2	0.5	1.1	0.6	0.7
$C_{16:0}$	40.7	42.9	42.6	57.3	40.4	47.2	42.2	46.0	17.9	46.5	58.7	45.3	56.5	64.3	46.2	46.5	46.1	4.8	5.1	50.3	51.8	45.3	63.9	53.7	51.9	25.5	27.2	40.5	54.8	71.0
$C_{16:1\omega7}$	5.5	14.6	12.3	5.7	1.2	14.0	7.6	10.7	23.2	11.9	7.0	6.3	5.2	7.0	11.4	12.9	8.5	53.6	6.6	2.4	1.0	6.9	3.8	2.6	3.9	14.1	13.6	8.8	4.1	2.3
$C_{16:2*}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.1	0.0	0.0	0.0	0.0	0.0	2.4	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.2	0.8	0.0	0.0
$C_{16:3*}$	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	11.7	0.0	0.0	0.0	0.0	0.0	0.0	2.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.9	6.0	1.2	0.0	0.0
$C_{16:4*}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.1	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5	3.0	0.0	0.0	0.0
$C_{17:0}$	0.9	1.0	0.8	0.9	0.9	1.0	0.7	0.7	0.0	0.6	0.6	0.7	0.7	0.8	0.8	0.6	0.7	1.4	1.2	0.9	0.7	0.8	0.8	0.8	0.6	0.3	0.3	0.9	0.0	0.0
$C_{18:0}$	9.3	9.1	7.5	12.4	20.6	11.0	10.2	12.2	1.2	10.5	13.5	20.3	14.6	13.5	10.7	6.9	12.8	15.3	21.1	24.3	20.5	16.5	16.7	21.3	17.7	5.6	7.3	13.2	18.6	13.2
$C_{18:1\omega9}$ (Oleic)	8.1	6.6	10.6	0.8	6.9	6.3	9.1	8.2	0.9	7.9	6.2	9.5	5.2	0.7	11.8	6.5	14.0	10.0	11.7	9.8	6.4	2.5	4.9	12.3	13.1	7.5	9.0	11.9	10.8	3.5
$C_{18:1\omega7}$ (Vaccenic)	3.3	4.9	4.6	3.7	5.1	5.3	5.7	4.2	0.0	3.6	3.5	6.2	3.5	2.1	6.4	3.2	6.5	7.2	10.1	5.1	2.3	10.5	1.8	3.4	3.9	2.0	3.2	4.8	3.4	0.9
$C_{18:2\omega6}$	3.3	1.2	4.0	0.1	1.5	1.2	3.4	1.7	0.0	2.2	0.9	2.1	0.9	0.0	2.2	1.0	2.8	2.1	2.3	1.9	11.4	4.5	0.9	1.6	2.4	1.9	2.7	2.5	0.0	0.0
$C_{18:3\omega6}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$C_{18:4\omega3}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.4	3.6	0.0	0.0	0.0
$C_{20:0}$	2.2	0.9	2.0	1.4	3.6	0.9	1.6	1.8	0.1	0.9	1.1	1.6	1.2	0.0	0.8	0.5	1.1	1.3	33.1	1.7	1.7	1.6	0.9	0.7	1.0	0.4	0.7	1.6	1.6	0.4
$C_{20:5\omega3}$	12.2	0.6	0.8	7.0	11.7	0.1	4.4	0.0	17.3	0.9	0.0	2.0	1.7	0.4	2.1	1.3	2.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	20.0	12.1	3.9	0.0	0.0
$C_{22:0}$	3.0	0.8	2.0	1.3	3.9	0.8	2.1	0.2	0.0	0.6	1.0	1.4	1.3	0.7	0.6	1.3	1.0	0.6	3.2	0.0	1.6	1.9	0.9	0.5	0.8	0.2	0.6	1.8	2.3	0.3
$C_{22:1}$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$C_{23:0}$	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$C_{24:0}$	1.1	0.1	0.4	0.3	1.3	0.1	0.9	0.1	0.0	0.2	0.4	0.4	0.3	0.3	0.2	0.1	0.3	0.1	1.0	0.5	0.3	0.6	0.2	0.2	0.3	0.1	0.1	0.5	0.4	0.1
$C_{26:0}$	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% PUFA	15.5	2.3	4.9	7.1	13.2	1.3	7.8	1.7	44.6	5.4	0.9	4.1	2.6	0.4	6.8	7.7	5.6	2.1	2.3	1.9	11.4	4.5	0.9	1.6	2.4	39.7	30.7	8.4	0.0	0.0
ω-hydroxy-$C_{16:0}$	1.0	0.3	1.1	1.3	0.4	0.1	0.4	0.4	15.8	1.3	0.1	0.4	0.1	0.1	0.4	0.2	0.2	1.3	0.2	0.5	0.3	0.3	0.1	0.3	0.4	0.8	0.4	0.5	2.7	0.6
ω-hydroxy-$C_{18:1\omega9}$	0.8	0.0	0.5	0.5	0.2	0.0	0.4	0.0	0.0	0.4	0.0	0.1	0.0	0.0	0.2	0.1	0.1	0.2	0.1	0.1	0.2	0.2	0.0	0.1	0.3	1.1	0.4	0.2	0.7	0.2
18.(8-11)-dihydroxy-	5.0	1.0	9.3	7.2	1.5	0.2	2.3	1.9	7.8	7.2	0.1	2.9	0.9	0.2	1.1	0.6	0.8	3.0	1.7	3.7	2.3	1.9	0.0	1.3	2.5	2.5	2.5	1.7	3.1	0.9
ω-$C_{18:0}$ diacid	0.3	0.0	0.3	0.2	0.0	0.0	0.1	0.0	0.0	0.2	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.1	0.0
ω-hydroxy-$C_{22:0}$	92.9	98.6	88.8	90.8	97.9	99.7	96.9	97.6	76.4	91.0	99.8	96.5	98.9	99.7	98.3	99.1	98.8	95.5	97.9	95.6	97.2	97.5	99.9	98.3	96.7	95.6	96.6	97.5	93.5	98.3

Marie-Aimée Galeron 9/1/y 10:30
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Marie-Aimée Galeron 9/1/y 10:32
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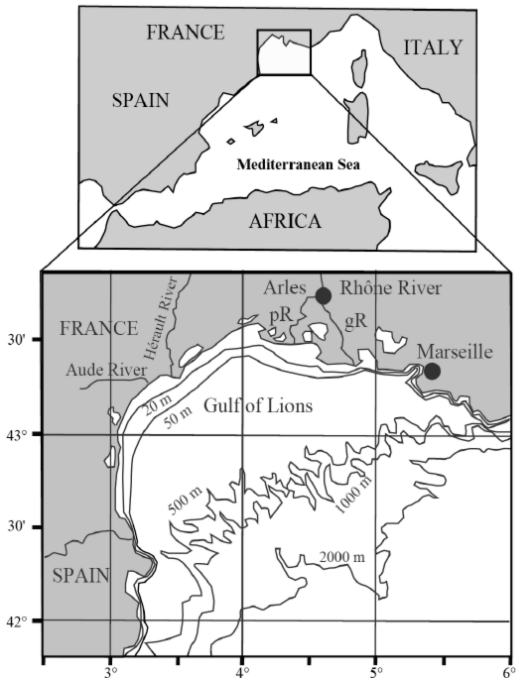

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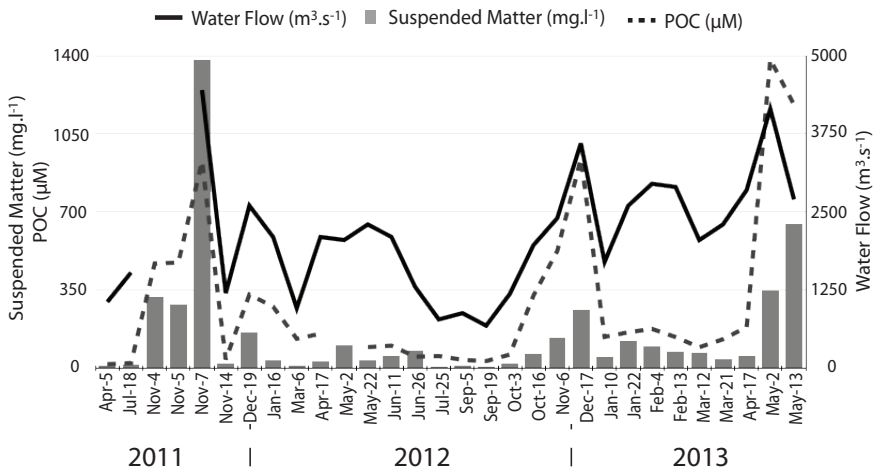

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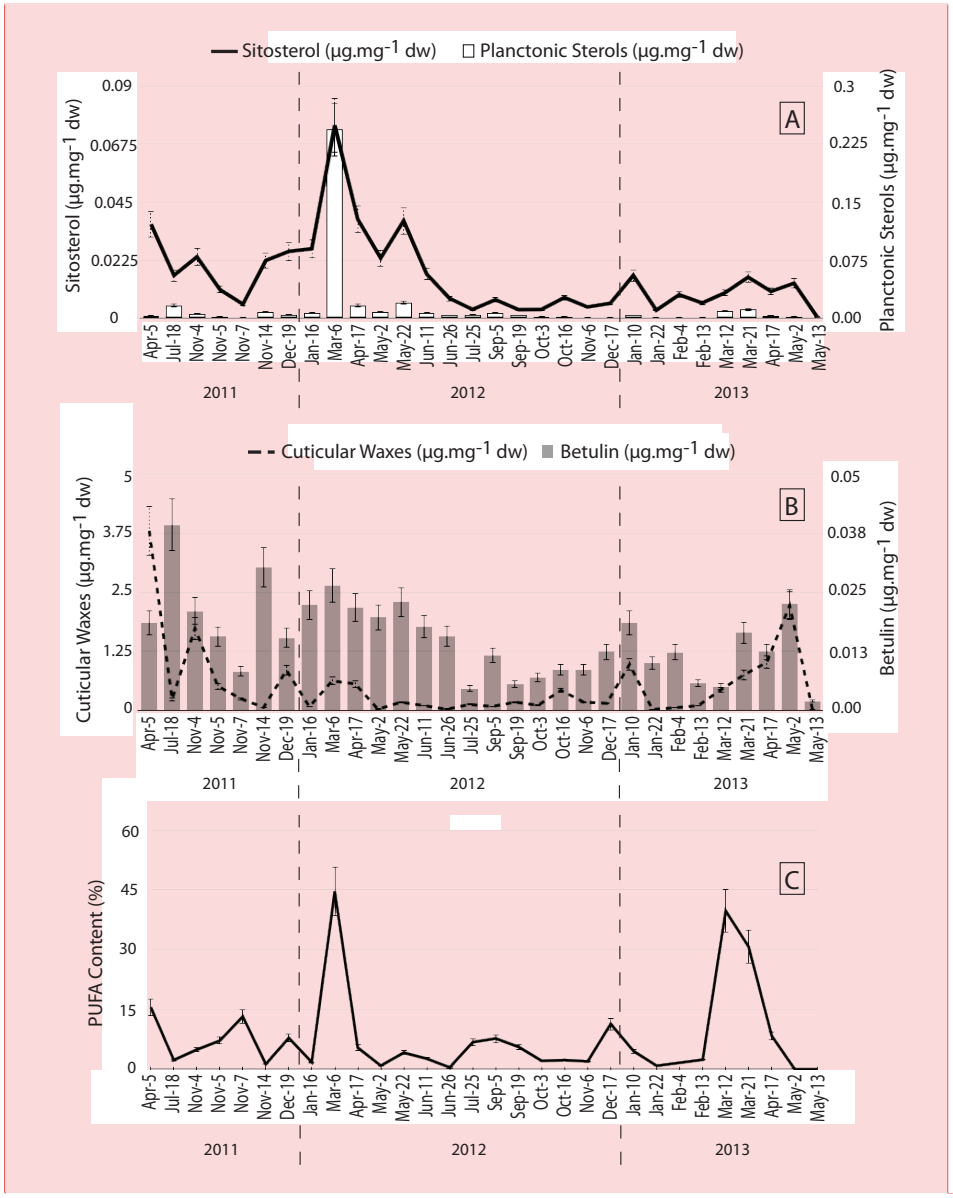

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Marie-Aimée Galeron 9/1/y 10:41
Commentaire [1]: Y-axis of figure 3A was fixed (appeared bold), X-axis labels were made bigger on all 3 figures

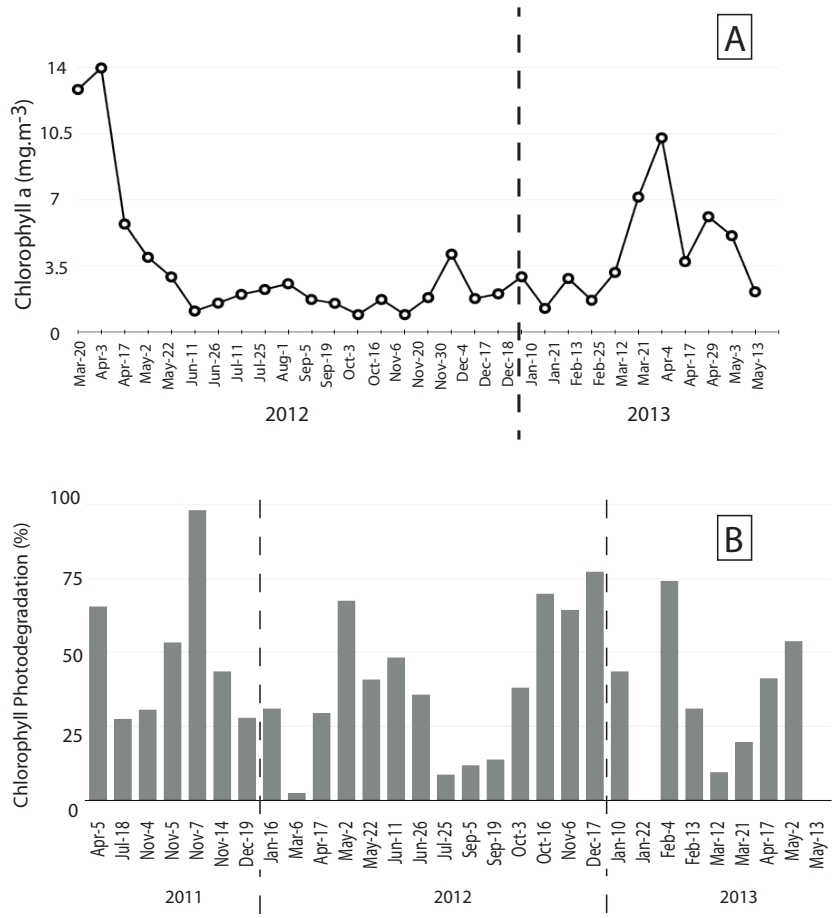

Figure 4: Chlorophyll a levels (MOOSE data) and percentage of photodegradation of chlorophyll (calculated using CPPI (Cuny et al., 1999) in the Rhone waters on and around sample dates

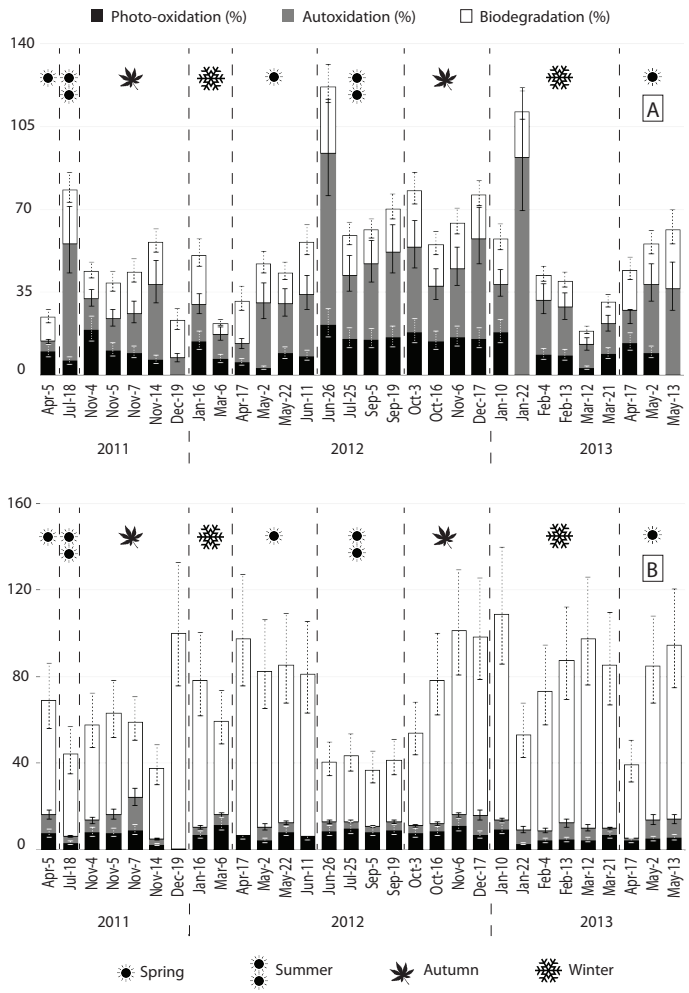

Figure 5: Biotic and abiotic degradation of sitosterol (A) and cholesterol (B) in the different samples. Full error shown here incorporates the 14% analytical standard error estimated for lipid quantification for all terms of the equations used. Sitosterol and cholesterol clearly have very different degradation patterns.

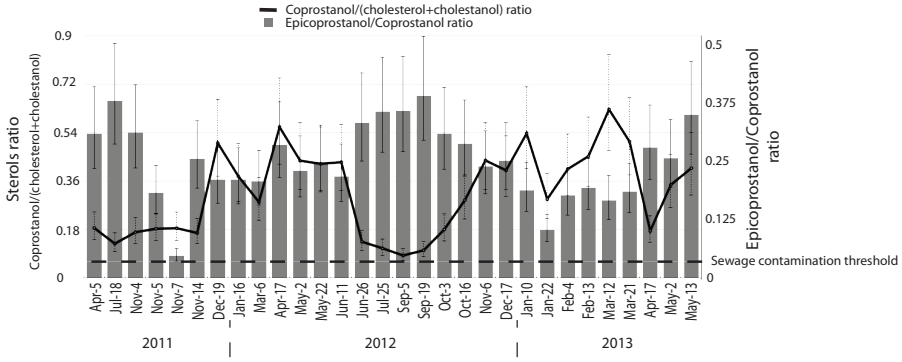

Figure 6: Coprostanol:(cholesterol+cholestanol) and epicoprostanol:coprostanol ratios of the different samples. Full error shown here incorporates the 14% analytical standard error estimated for lipid quantification for all terms of the ratios. Contamination threshold is 0.06 (See section 3.3.2).

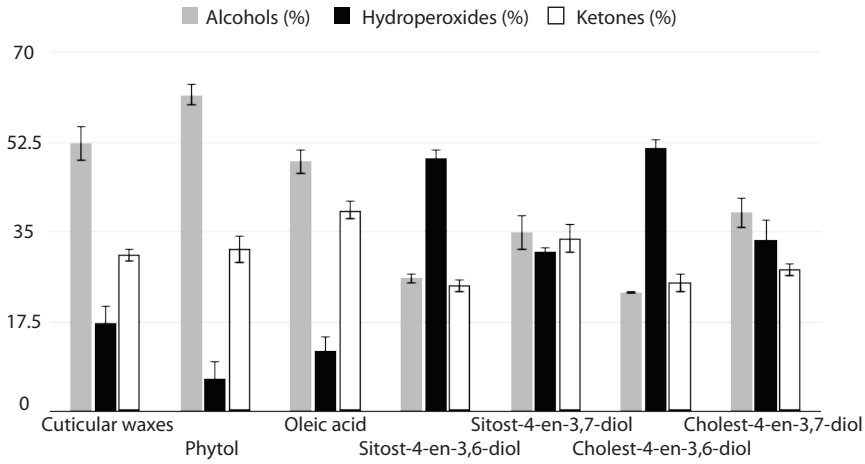

Figure 7: Relative percentages of intact hydroperoxides and their ketonic and alcoholic degradation products measured in the case of w-hydroxyoleic (cuticular waxes) and oleic acids, phytol, sitosterol and cholesterol oxidation products. Standard error was calculated based on the results obtained for all samples.