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Article

Metal Ions and Hydroperoxide Content: Main Drivers of Coastal Lipid Autoxidation in Riverine Suspended Particulate Matter and Higher Plant Debris

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Abstract: Autoxidation is a complex abiotic degradation process, and while it has long been known and well studied in biological compounds, it has been widely overlooked in environmental samples and as a part of environmental processes. With recent observations showing the magnitude of the involvement of autoxidation in coastal environments, it has become critical to better understand how and why this degradative process takes place. At the riverine/marine interface, recent findings evidenced a spike in autoxidation rates upon the arrival of suspended particulate matter in seawater. In this study, we aimed at identifying autoxidation-favoring factors *in vitro* by analyzing suspended particulate matter incubated under different conditions. If metal ions have long been known to induce autoxidation in biological systems, we show that they indeed induce autoxidation in particulate matter incubated in water, but also that the content in photochemically-produced hydroperoxides in suspended particulate matter is crucial to the induction of its autoxidation in water.

Keywords: autoxidation; photo-oxidation; lipid tracers; organic matter degradation; suspended particulate matter; hydroperoxide

1. Introduction

Autoxidation is a free radical chain process. Such reactions can be divided into three stages: chain initiation, propagation, and termination. In the initiation process, enzymes, heat, light and metal ions may play a role in the generation of radicals. The metal ion-catalyzed homolytic cleavage of photochemically-produced hydroperoxides [1] has been suggested to play an important role in the initiation of autoxidation reactions in phytodetritus [2], although this has not been firmly established experimentally. The chain reaction ends when free radicals collide and exchange electrons to form a new bond, hence rendering them unreactive.

While lipid autoxidation has been extensively studied in medical and food sciences [3,4], it has been long overlooked in the environmental sciences, even though its impact on organic matter could be tremendous.

In estuarine and coastal areas, a better knowledge of the fate of river-transported particulate organic matter (POM) is essential for a better understanding of organic matter export, sedimentation rates, and ultimately coastal carbon cycles. It has been previously demonstrated that autoxidation is a major degradative process in rivers [5] and that it is enhanced upon the POM's arrival at sea [6].

While there can be a number of explanations for this phenomenon, it is critical that we understand what drives it in order to know if this is a localized phenomenon or a global occurrence.

It has been long known that transition metals can catalyze autoxidation in biological systems [7,8], but could there be other drivers in the environmental systems? Is the presence of transition metal ions in freshwater and seawater enough to kickstart autoxidative chain reactions? What are the dynamics of these reactions in suspended particulate matter (SPM) in aquatic systems?

In previous observations in the Mackenzie River (Canada), it has been demonstrated that autoxidation acts intensely on POM reaching seawater, while it is fairly limited in the river [6]. These results also made apparent the crucial role of the photodegradation state of SPM on its future autoxidation, through the production of associated autoxidation-promoting hydroperoxides. With this work we aim to look at the importance of trace metal ions and hydroperoxide contents on the degradation of POM, in order to better understand the behavior of terrestrial organic matter reaching marine environments.

2. Materials and Methods

2.1. Tracers Used

In light of recent observations made in the Rhône River [5] and during in vitro experiments [9,10], we selected a set of lipid tracers allowing us to trace both the origin of SPM and the degradative processes undergone by lipids in SPM.

We selected 24-ethylcholest-5-en-3 β -ol (sitosterol if the C-24 stereochemistry is 24 α), lup-20(29)-en-3 β ,28-diol (betulin), urs-12-en-3 β -ol (α -amyrin) and olean-12-en-3 β -ol (α -amyrin) as lipids of interest, since we are also able to quantify their oxidation products and associate them with autoxidative processes (Figure 1). Betulin and both amyrins are also unambiguous tracers of terrestrial vascular plants [9,10].

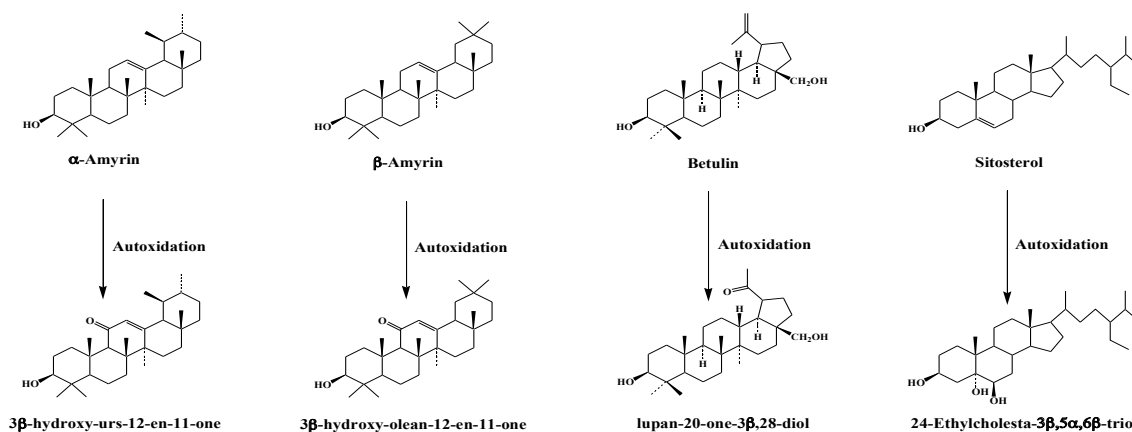


Figure 1. Proposed tracers of autoxidative damages in higher-plant material.

2.2. Experimental

2.2.1. Rhône SPM Samples

Samples of SPM were collected in the Rhône River (South of France), 30 km upstream from the river mouth (Figure 2), using a CEPA Z61 Teflon-coated centrifuge directly connected to the water stream. On the date sampled, the SPM Particulate Organic Carbon (POC) content was 6.8% (MOOSE data, www.moose-network.fr). Wet samples were finely ground and conserved at 4 °C prior to the experiments. SPM samples were incubated for 49 days under different conditions:

- Seawater
- Ultra-pure water

- Ultra-pure water with added Cu^{2+} (2.4 μM of CuSO_4)
- Ultra-pure water with added Fe^{2+} (1.0 mM of Mohr's salt)

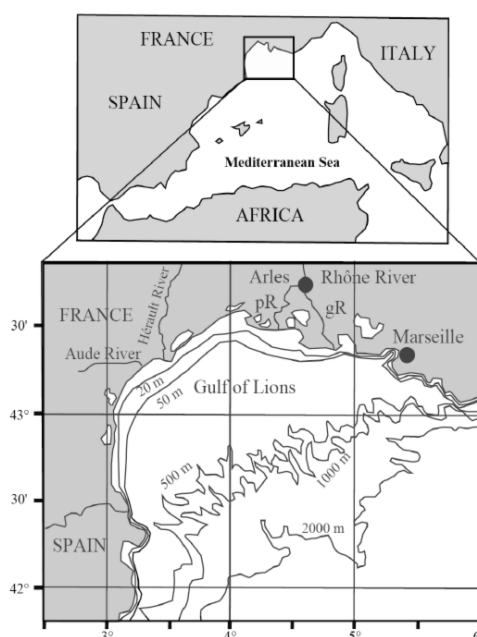


Figure 2. Rhone River mouth area and sampling locations: “Arles” and “Marseille”. gR and pR mean grand Rhone and petit Rhone which are the two arms of the river.

These two metals were chosen because of their potential influence on autoxidation processes [7,8] and the choice of metal ion concentrations was based on observations made by Ollivier et al. [11] in the Rhone River. Seawater (38 g/L) was collected in the Mediterranean Sea, three miles offshore from Marseille.

2.2.2. Terrestrial Vascular Plant Samples

Senescent leaves of *Quercus ilex* and *Smilax aspera*—both widespread species in Mediterranean ecosystems—were collected on the ground near Marseille, France. Samples were freeze-dried, ground to a fine powder, and kept at 4 °C. Samples were incubated in ultra pure water with or without added Fe^{2+} ions, and analyzed after 39 and 103 days.

2.2.3. Chemical Treatment of the Samples

After filtration on GF/F glass fiber filters, samples were treated with excess NaBH_4 in MeOH (25 mL; 30 min) to reduce labile hydroperoxides (resulting from photo- or autoxidation) to alcohols that were more amenable to analysis using GC–EIMS [12]. After NaBH_4 reduction, water (25 mL) and KOH (2.8 g) were added and the resulting mixtures saponified under reflux (2 h). After cooling, the resulting solutions were acidified (HCl, 2N) to pH 1 and extracted with dichloromethane (DCM) (3×20 mL). The combined DCM extracts were dried over anhydrous Na_2SO_4 , filtered, and concentrated by rotary evaporation (40 °C).

A different treatment was undergone by SPM sample (wet, or freeze-dried) in order to quantify the hydroperoxides present in the suspended particulate matter used for incubation [13]. 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 $3500 \times g$ 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

5 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 [14]) and saponification and for the second after reduction with NaBD₄ and saponification. After saponification, the samples were then acidified, extracted and concentrated as previously described.

3. Results

Because sitosterol alone cannot be unambiguously attributed to organic matter of terrestrial origin (possible production by freshwater plankton [5]), we have compared its autoxidation rates to those of other compounds of a more clearly terrestrial origin. In all incubations, we determined autoxidative degradation rates for three families of lipid tracers: sitosterol, α -/ β -amyrins, and betulin.

3.1. Rhone SPM Incubations

In SPM incubations, it appears clear that the degradation rates of the compounds studied vary greatly. In the Rhone SPM, sitosterol is particularly reactive, and the addition of metal ions into the incubations has a marked effect on its autoxidation rates, going from $2.6\% \pm 0.4\%$ at the beginning of the experiment to $8.0\% \pm 1.2\%$ after 49 days in ultrapure water with added Cu²⁺ ions, $10.6\% \pm 0.7\%$ in seawater, and even $18.2\% \pm 2.1\%$ in ultrapure water with added Fe²⁺ ions (Figure 3A).

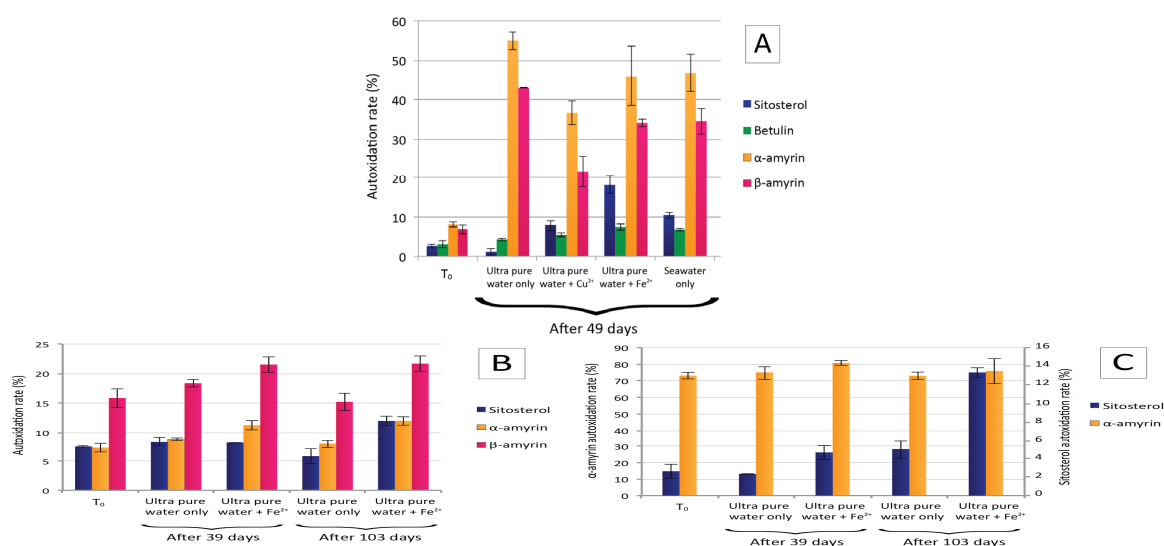


Figure 3. Autoxidation rates (calculated using the following ratio: oxidation product/(parent compound + total of oxidation products quantified) of the studied compounds in SPM incubations for the Rhone River (A); and in *Quercus ilex* (B) and *Smilax aspera* (C) plant debris incubations.

Betulin, on the other hand, is slower to react and the observed increase of autoxidation rates with added metal ions is not as dramatic as that of sitosterol. The addition of metal ions still had a significant effect on its autoxidation rates. The addition of Fe²⁺ brought its starting autoxidation rate of $3.1\% \pm 0.8\%$ to $7.5\% \pm 0.8\%$ after 49 days (compared to the $5.5\% \pm 0.4\%$ observed in ultrapure water with added Cu²⁺ ions, and $6.8\% \pm 0.3\%$ in seawater after 49 days; Figure 3A). In fact, the addition of Fe²⁺ seems to bring up betulin autoxidation rates to a level close to the one they are at in seawater.

The α - and β -amyrins, however, showed variable autoxidation rates. In incubated SPM from the Rhône, autoxidation rates for both amyrins seem to be going down after 49 days (regardless of the conditions), with rates when extra Cu²⁺ was added being the lowest with $36.7\% \pm 2.7\%$ and $21.8\% \pm 3.6\%$ for α - and β -amyrins, respectively (compared to their T₀ levels of autoxidation of $8.3\% \pm 0.7\%$ and $7.0\% \pm 1\%$, respectively). It is important to note that the starting autoxidation rates of amyrins were higher than those of sitosterol and betulin.

The α -amyrin is more degraded than its β counterpart after 49 days, but individual rates are similar whether particulate matter was incubated in ultrapure water with added Fe^{2+} ions or in seawater.

Analyzed lipid extracts obtained from SPM from the Rhone River allowed us to calculate the proportion of sitosterol-derived hydroperoxides in the SPM used for the incubations. In the Rhone SPM, these hydroperoxides represent $2.96\% \pm 0.19\%$ of sitosterol (Figure 4).

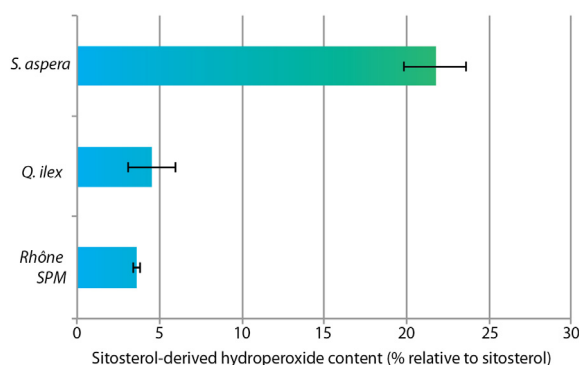


Figure 4. Relative percentages of intact sitosterol-derived hydroperoxides (relative to residual sitosterol) in the different materials used for incubation at T_0 : *Smilax aspera* (*S. aspera*) or *Quercus ilex* (*Q. ilex*) senescent leaves, and Rhône SPM.

3.2. Plant Debris Incubations

Since SPM collected in rivers has a partially terrestrial origin, we also collected senescent plant material (leaf material from *Quercus ilex* and *Smilax aspera*) in order to have it undergo the same incubation treatment. Based on the previous results, with this experiment we aimed to show the role of metal ions and hydroperoxide content in the induction of autoxidative reactions in larger-sized organic matter of terrestrial origin.

While betulin and its oxidation products were below detection levels, we were able to quantify autoxidation rates for sitosterol, α -amyrin and β -amyrin. However, in *S. aspera* samples, β -amyrin seemed to be co-eluted with an unknown compound; for that reason and in order to be as precise as possible, we will not be looking at its degradation products in *S. aspera* incubations.

The effect of metal ions on autoxidation rates appeared to be more evident in the case of *S. aspera*, where the autoxidation rate of sitosterol went from $2.6\% \pm 1\%$ at the beginning of the experiment to $4.7\% \pm 0.9\%$ after 39 days and $13.3\% \pm 0.6\%$ after 103 days (versus $2.3\% \pm 0.1$ and $5.0\% \pm 1.1\%$, respectively, without added Fe^{2+} ions; Figure 3C). This effect can also be seen in the autoxidation rates of α -amyrin (going from $72.7\% \pm 2\%$ at the beginning of the experiment to $75.1\% \pm 4\%$ after 39 days incubated in ultrapure water and $80.1\% \pm 1.5\%$ when Fe^{2+} ions were added).

When *Q. ilex* leaves were incubated, metal ions seemed to take longer to have an effect on autoxidation, with a starting autoxidation rate of $7.5\% \pm 0.1\%$ for sitosterol only increasing to $8.2\% \pm 0.1\%$ after 39 days, but climbing to $12.0\% \pm 0.8\%$ after 103 days (Figure 3B). For α - and β -amyrins, the effect of added Fe^{2+} ions was significant on their autoxidation rates, with α -amyrin autoxidation rates going from $7.3\% \pm 0.7\%$ to $11.3\% \pm 0.8\%$ and $12.0\% \pm 0.7\%$ after 39 and 103 days, respectively, when Fe^{2+} ions were added to the incubation. The β -amyrin, on the other hand, saw its autoxidation rate increasing from $15.9\% \pm 1.5\%$ to $21.5\% \pm 1.3\%$ and $21.7\% \pm 1.3\%$ after incubation for 39 and 103 days, respectively, when Fe^{2+} ions were added.

As previously explained, we also quantified sitosterol-related hydroperoxides. The hydroperoxide content of the leaf debris sampled also seemed to greatly influence the level of autoxidation observed in the incubations. *S. aspera* had the highest hydroperoxide content, representing $18.07\% \pm 1.56\%$ of sitosterol, while they represented only $3.74\% \pm 1.2\%$ of sitosterol in *Q. ilex* leaf debris.

4. Discussion

4.1. Rhone River SPM Incubations

With the recent observations made in situ on both the Rhône and the Mackenzie Rivers, where it has been demonstrated that autoxidation is a major degradative process [5,6], we expected betulin, sitosterol and α - and β -amyrins to be fairly autoxidized, even at the start of our experiment. The observed state of degradation of the incubated SPM is in line with those results, clearly indicating that whether we are looking at particles or leaf debris, autoxidation has been involved in their degradation, whether on land or during riverine transport.

Also, because of the set of lipid tracers used, it is clear that the autoxidative processes observed acted on SPM of terrestrial origin [9,10]. It has been demonstrated that autoxidation is induced when particulate matter is incubated in seawater, and this was explained by the potential desorption of metallic ions upon contact with seawater [6].

The observed effect of metal ions on the induction of autoxidation seems to confirm this hypothesis. It appears that the presence of Fe^{2+} and Cu^{2+} ions has a significant effect on the autoxidation rates of suspended particulate matter.

Previous results obtained in the Rhône [5] and Mackenzie [6] Rivers clearly showed that the SPM of terrestrial origin found in major coastal rivers has been intensely autoxidized even before reaching the ocean, but the results obtained in this study confirm that metal ions present in the water have favored this autoxidation, and will favor it in seawater. In coastal environments, it is possible that the metal ions necessary to induce this autoxidation could be desorbed from SPM upon its arrival at sea [15,16]. To confirm this hypothesis, in situ samples and metal ion concentrations are currently being studied.

4.2. Leaf Debris Incubations

Similar results were obtained when incubating leaf debris from *Q. ilex* and *S. aspera*. While it is clear that leaves underwent photo-oxidation while on land, the debris were also significantly autoxidized, whether this happened while still on land, or once in riverine waters. When incubated with added Fe^{2+} ions, autoxidation was clearly favored when compared to samples without the added metal ions. Here again, the results are similar to those obtained with incubated SPM, confirming the hypothesis that metal ions favor autoxidation in incubated plant debris.

4.3. Hydroperoxide Content

Even if our observations confirm the effect of metal ions on autoxidation, it appears they are not the sole driver of autoxidative processes. Indeed, the quantity of hydroperoxides present in SPM plays a major role in the induction of autoxidative processes, as previously reported by Rontani et al. [6].

Our quantification of hydroperoxides deriving from sitosterol oxidation products (7 α -Hydroperoxy-24-ethylcholest-5-en-3 β -ol, 6 β -Hydroperoxy-24-ethylcholest-4-en-3 β -ol, 7 β -Hydroperoxy-24-ethylcholest-5-en-3 β -ol and 6 α -Hydroperoxy-24-ethylcholest-4-en-3 β -ol) showed that very different hydroperoxide profiles are observed for the different materials sampled and incubated, whether they are leaf debris or riverine SPM. The starting hydroperoxide content and sitosterol's final autoxidative rates (with added metal ions) are well correlated ($r^2 = 0.87$, $p < 0.05$), implying that the presence of metal ions can indeed be a factor inducing autoxidation, but it seems very well coupled with the SPM hydroperoxide content.

4.4. Environmental Implications

Scientists have understood the implications of carbon behavior in natural environments very early on, and visualized carbon dynamics as early as 1956 [17]. Through the years, carbon cycles have been completed, reservoirs and fluxes were better estimated, and their implications on global change well defined [18–21]. However, in the official carbon cycle representation used by the IPCC (Intergovernmental Panel on Climate Change), river to ocean fluxes and coastal exports to the deep

ocean have not been re-estimated since 2002 [21,22]. Our findings, if they can be evidenced in natural environments on a global scale, could help redefine these fluxes and would lead to a better estimation of carbon exports and coastal burial.

5. Conclusions

The fact that metal ions can induce autoxidation has been discussed in the past [7,8], but the interest of this hypothesis has never been questioned for environmental samples. Due to the observation that SPM autoxidation rates spike when SPM is incubated in seawater [6], this hypothesis was again formulated for natural environments, and, in this study, tested *in vitro*. Our study confirms that metal ions do indeed favor autoxidation on SPM of riverine origin, but also that the presence of hydroperoxides resulting from photo-oxidative processes is another major driver of autoxidation in SPM. Moreover, our results on leaf debris validate the role of metals and the initial photo-oxidation state (i.e., hydroperoxide content) of the organic matter in the initiation of autoxidation processes. A better consideration of these abiotic degradative processes is a major issue in the understanding of the fate of terrestrial organic matter in coastal environments. In order to confirm the effect of this conjunction of factors in the environment, further tests and *in situ* sampling will be necessary.

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Conflicts of Interest: The authors declare no conflict of interest.

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