

***Interactive comment on* “Lability of natural organic matter in freshwater: a simple method for detection using hydrogen peroxide as an indicator” by Isabela Carreira Constantino et al.**

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To: Biogeosciences – Discussion manuscript - bg-2018-122 Subject: Response to referee 2

We appreciate the valuable time and critical review done by referee 2 and certainly considered the useful comments and suggestions made to improve the manuscript. In this manuscript, our experimental approach was based on previous observations published by Jardim et al (2010), in which it was suggested that H₂O₂ could be used to distinguish

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the difference between organic matter incorporated in waters during flooding periods in Negro River (Amazon Basin), but it was not possible to quantify the amount of LOM. These authors used H₂O₂ kinetic consumption in two samples (freshwater from Negro River and water fortified with fresh leached soil organic matter). They showed a significant change in the chemical speciation of Hg coordinated by redox conditions in aquatic region studied in the presence of labile organic matter (LOM). In the rainy season, there was a great input of allochthonous natural organic matter (NOM) in aquatic bodies, and this NOM, considered fresh and reactive, would be able to scavenge H₂O₂ naturally photogenerated in the water column, influencing directly the oxidation conditions in this environment. Thus, this comprises one of the direct effects caused by the presence of LOM. Please, find ahead our answers for specific questions given in point by point below.

Referee: This makes me wonder what exactly is meant by "labile" and "recalcitrant." Do these terms refer to the bioavailability of NOM and, if so, why were no incubation experiments done to validate that material promoting rapid HOOH decay is actually consumed quickly by heterotrophic communities? (this is certainly true for pyruvate, but what about natural samples?) Or do these terms refer to lability with respect to reactive oxygen species and, if so, how would this translate to bioavailability and persistence in the environment?

Authors: We aimed at the possibility of quantifying labile and recalcitrant organic matter in freshwater samples. This objective was based on the importance that NOM plays in aquatic environment. It is known that NOM plays a relevant role in photoreactions, forming reactive species, or even scavenging these species. It is also primary source of biota and it is able to complex or adsorb other species as well. In this context, we would like to try measured the different degrees of reactivity of NOM according the characteristics and considering our definition. We denominated LOM as NOM that was few oxidized or degraded and it is still able to react as a scavenger of oxidant species in aquatic systems. On the other hand, recalcitrant organic matter (ROM) is the frac-

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tion that had already suffered oxidation, and it is less reactive towards oxidant species, such as H_2O_2 . Our approach is different from classical methods used to distinguish organic matter degraded by microorganism or chemically, such as the ones used in the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) measurements, respectively. We agree with the referee that the way of LOM and ROM were defined in this work, it could be reformulated in the new version of the manuscript. In our attempt, we led the lability and recalcitrance concepts through the chemical approach, trying to reach a simpler approach than the protocols currently used to determine labile fraction, that consider it as biodegradable fraction of NOM, hence they always include bioassays. Considering that the concept used by us to describe the lability and the points highlighted by the referee, we agree that our experimental approach was not enough to distinguish the labile fraction of NOM, but rather those part of NOM that is able to scavenge the H_2O_2 , which might to be construed as the NOM components that are resemblance to our model (i.e. pyruvate), in terms of the reactive groups present, as suggested by the referee as well. Therefore, certainly we will improve the discussion over this subject using this experimental approach and the other amendment.

Referee: Additionally, I find the kinetics of these experiments to be poorly described and poorly justified. Most importantly, I am left wondering why the kinetic order of HOOH decay depends on the chemical composition of OM added (i.e. described as zero-order for fluvic acid and lignin but first-order for pyruvate). For example, for the pyruvate case, is HOOH decay first-order with respect to itself, with respect to pyruvate concentration, or both? As this manuscript is written, I think the authors treat this as first-order with respect to itself, but this was not tested. Why was an experiment not done in which the NOM concentration was held constant and initial HOOH concentration was varied?

Authors: On this questioning about the kinetic of decay of H_2O_2 , we agree that is feasible to realize an experiment using a range of H_2O_2 and exceed of pyruvate to

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confirm the kinetic order.

Referee: I recommend the authors provide a sound, theoretical justification for their choice of kinetic model and include a mathematical derivation starting from first principles, in addition to the results of any test experiments needed to verify their choices. [. . .] If I am interpreting this correctly, then why would HOOH decay be first-order with respect to itself when pyruvate is added but zero-order with respect to itself when lignin or fluvic acid is added? How could this be translated to a natural sample that contains a complex mixture of compounds? Reaction order would need to be known a priori. Rather, it seems to me like a more reasonable kinetic model would be one that is zero order with respect to HOOH concentration and first-order with respect to oxidizable functional groups present in NOM (although the abovementioned test would need to be performed to validate this). If this is true, then HOOH decay could be described as something like: $k_0 + k_1[\text{NOM}]$ (see comments of referee 2) where k_0 is the "intrinsic" zero-order decay rate without NOM present (termed "control" throughout this manuscript) and k_1 describes the additional HOOH decay promoted by the presences of NOM and is dependent on NOM chemical composition. This would result in a HOOH half-life that scales inversely with NOM concentration (i.e. $t_{1/2} = \ln 2 / (k_0 + k_1[\text{NOM}])$). This relationship fits the data reported in Table 1 significantly better than does the model described in Fig. 2 and Eq. 1-2. Of course, the model I describe here might not be the best one to describe these data, but as this manuscript is currently written I'm not convinced that it's any worse than the model presented in the text. I recommend the authors provide a sound, theoretical justification for their choice of kinetic model and include a mathematical derivation starting from first principles, in addition to the results of any test experiments needed to verify their choices.

Authors: It is important to add that kinetic models employed here to determine the order and consequently half-lives of H₂O₂ were based on the mathematical strict sense of the classical kinetic laws, as an exponential decay formula, as to fit the data, and it was not our attempt with this experimental approach discuss about any specific chemical

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mechanism behind these reactions, once our focus is to apply the mathematical formalism, only in its empirical form, to general and more complex systems such as natural aquatic samples. We have chosen the order of the exponential function, for each case, using the correlation of the fit as the mandatory parameter, so we could decide the best order for each model compound used. Empirically, we found that in presence of pyruvate, peroxide reacts in a first order kinetic decay. In truth, in those cases, we have an excess of pyruvate allowing a treatment as pseudo-first order kinetics. We also have found that in the absence of pyruvate the systems decay in a zero order fashion which, as we have observed, is characteristic of the HOOH spontaneous decay in the system condition, and it is quite slow compared to the reactions in presence of pyruvate, so for that reason it should be neglected from the equation. Our first conclusion was that all those models used (lignin, fulvic acid, and others not mentioned in this work), except pyruvate, cannot be used as a LOM model, neither as recalcitrant model, once what we see in the zero order behavior, probably is the spontaneous decomposition of HOOH in water. Once we have realized that, we start to look at the reason why pyruvate could behave as a model for LOM. From that we came with follow interpretation based in a solely chemical selection approach:

Figure 1.

In the basis of the mechanism shown in Figure 1 we have made ab initio calculations for roughly (Hartree-Fock level – HF/6-31++G(d,p) – of theory using C-PCM as solvation model to simulate aqueous system) estimate the Gibbs Free Energy of Activation for the reaction ($\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O}$) and we have found a value of ~ 24 kcal mol⁻¹ (at 25 °C) for this main barrier (rate determining step).

Figure 2.

Reflecting on the basis of the referee's questions, in our hypothesis, any specie which could react with HOOH with an activation barrier lower or equal to this value (24 kcal mol⁻¹) should be considered LOM, and that should be reached only for "easily" organic

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oxidable matter. That should install a chemical definition for LOM, it means that once we have that amount of energy, a threshold stipulated on the basis of the pyruvate reaction, any specie that react faster or in equal rate should be considered LOM. For this reason, pyruvate has a behavior that we could correlate to the LOM in the environmental samples analyzed. It follows the Cartesian coordinates of the transition state, pyruvate and HOO⁻ used in the calculation CPCM/HF/6-31++G(d,p) performed in GAMESS-US version 1 May 2013 (R1).

```
Transition State C 6.0 1.6816350116 0.1010684975 -0.2277477680 O 8.0
2.1332403247 -0.4627711238 0.7839124009 O 8.0 2.3445793973 0.7031280298
-1.0817602132 C 6.0 0.1467425077 0.0145001255 -0.4409410642 O 8.0 -
0.3233234672 0.2048176881 -1.5749461202 C 6.0 -0.6180775921 -0.8880991464
0.5001816824 O 8.0 -0.1290602750 1.6670036824 0.5265217665 O 8.0 -
1.1608590282 2.3091250331 -0.2028123400 H 1.0 -1.6690724085 -0.6319647769
0.4508054964 H 1.0 -0.2642339719 -0.8189532239 1.5155537825 H 1.0 -
0.4969221131 -1.9142434582 0.1576182172 H 1.0 -1.1713883854 1.8101786728 -
1.0157058403
```

```
Pyruvate C 6.0 1.6935563717 0.0186054902 -0.2705855508 O 8.0 2.0945133541 -
0.0529859412 0.8957945494 O 8.0 2.3570246488 0.1042454662 -1.3071337138 C
6.0 0.1555022243 0.0193979230 -0.4143599551 O 8.0 -0.3833560760 0.8081793314
-1.1419747000 C 6.0 -0.6146609989 -0.9645362969 0.4225977542 H 1.0 -
1.6730939503 -0.8819106367 0.2158895184 H 1.0 -0.4215626761 -0.7698612741
1.4705418768 H 1.0 -0.2733428976 -1.9736340619 0.2119002211
```

```
HOO- O 8.0 -0.1123690206 1.6640975373 0.5502444474 O 8.0 -1.1627181376
2.3027578858 -0.2161034494 H 1.0 -1.1862228418 1.8194445769 -1.0261409980
```

Thank you. Sincerely yours, Isabela C. Constantino and co-workers.

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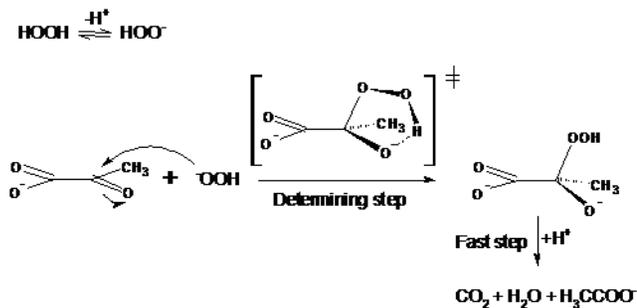


Fig. 1. Suggested mechanism for the decarboxylation of pyruvate from HOOH. Inspired by Phys.Chem.Chem.Phys.,2017, 19, 19316 and Lopalco et al., J. Pharm. Sci..2015.DOI 10.1002/jps.24653

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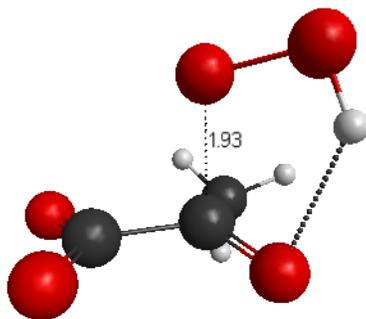


Fig. 2. Transition State for the determining step of the decarboxylation of pyruvate by HOOH, found using HF/6-31+G(d,p) and C-PCM for simulating the aqueous environment. 666.

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