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## Humins in the environment - Early stage insights on ecotoxicological aspects

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### KEYWORDS

Humins, furanics, ecotoxicity, immunomarkers, biodegradability, byproduct valorization

### ABSTRACT

With the growing interest of a circular economy, the use of lignocellulosic residues such as lignins and humins as potential renewable feedstock for biorefining processes looks more and more promising. With humins, many challenges are still remaining for their sustainable use, starting from the need of providing reference data reflecting actual usable feedstocks of such materials. With this perspective, this paper offers a first outlook on the potential environmental fate of those materials and components, all related to furanics, a family of compounds for which toxicity is still a matter of debate.

During the assessment, the conventional OECD ecotoxicity and biodegradability tests demanded by the European REACH regulation for a primary evaluation of environmental hazards were performed in combination with fish immunomarker tests to study the possible long-term effects on aquatic ecosystems. These first results are promising as humins did not exhibit any immediate ecotoxicological concerns and hence would allow considering their use in environmental-friendly applications.

### INTRODUCTION

Furanic compounds have recently drawn noticeable techno-economic interest due to their production from non-edible parts of lignocellulosic biomass to produce fuels, chemicals and materials.<sup>1</sup> For this very reason, some of these furanic compounds have been listed by the U.S Department of Energy as one of the top 12 and top 30 high potential chemical building blocks.<sup>2</sup> In a similar context, Avantium's YXY® process produces 2,5-Furandicarboxylic acid (FDCA) for the development of bio-plastics.<sup>3</sup> FDCA production process primarily involves the dehydration of carbohydrates (C6 and C5 sugars) into alkoxymethyl furfural (RMF).<sup>1,4</sup>

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3 RMF is the furanic intermediate mixture essentially composed of methoxymethyl furfural  
4 (MMF) and 5-hydroxymethyl furfural (HMF). Besides leading to various furanics and other  
5 platform chemicals, the Acid Catalysed Dehydration (ACD) process also leads to the  
6 production of an unavoidable side stream residue called humins.<sup>5</sup>  
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9 Humins are polyfuranic macromolecule mixtures with minor quantities of furanic derivatives  
10 retained in their structure.<sup>6,7</sup> The chemical structure of humins is highly complex and largely  
11 depends on the type of feedstock, operating conditions and the functional groups associated  
12 with it.<sup>8,9</sup> Despite existing for many decades, humins, until today, have been used only as  
13 residues in low value applications such as combustion and gasification.<sup>10</sup> With the primary  
14 aim of upgrading biorefinery side streams to improve bio-based economics in the recent past,  
15 innovative potential application for humins as renewable raw materials have been identified  
16 mainly in catalysis, water purification, matrix of impregnation materials, CO<sub>2</sub> sequestration  
17 and energy storage.<sup>11–14</sup>  
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22 The complex structure of humins certainly indicates highly variable physico-chemical  
23 properties, and in turn gives an indication of the potentially varying risk profiles that need to  
24 be anticipated for their sustainable new use. Besides understanding these aspects, it is equally  
25 important to recognize the nature and properties of the most important compounds present  
26 throughout the entire production chain of humins.  
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29 Although MMF and HMF have been independently addressed by various researchers so far,  
30 their effect as a mixture has not been studied together. Also noteworthy, neither humins nor  
31 RMF is yet assessed and classified according to the international Classification and Labelling  
32 System of Chemicals such as the CLP regulation in the EU (derived from the Globally  
33 Harmonised System of Classification and Labelling of Chemicals (GHS)<sup>15</sup>) or according to  
34 the United Nations Model Regulations for the Transport of Dangerous Goods (UN TDG).<sup>16</sup>  
35 Thus, very limited or almost no safety related information of humins or RMF mixtures is  
36 available for the end user, indicating that the materials are still in the early stage research.<sup>6</sup>  
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41 Bio-based nature humins and furanic intermediates and/or platform chemicals does not  
42 necessarily provide safe attributes to these materials in all working environments.<sup>17</sup>  
43 Considering potentially the vast amounts of production and broad application routes being  
44 explored, it is important to strengthen the sustainable application pathways, for which availing  
45 information on their safety profiles plays a key role. Currently, there is a lack of information  
46 regarding the persistence of these materials in the environment and their possible effects or  
47 consequences which seems to be the main concern as perceived from the literature sources.<sup>6,18</sup>  
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51 The current study as part of the EU funded HUGS project<sup>19</sup> is focusing exclusively on  
52 addressing the ecotoxicological and biodegradation properties of humins and other key  
53 furanic intermediates namely HMF, MMF and RMF and an important side-stream methyl  
54 levulinate (ML). The proposed assessment is based on the output of a dual experimental  
55 approach combining conventional OECD ecotoxicity and biodegradability tests as demanded  
56 by REACH regulation for a first appraisal of environmental hazards and the examination of  
57 several immunomarkers in fish cells to document possible long-term effects of studied  
58 substances on the aquatic ecosystem.  
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## MATERIALS AND METHODS

Tests required for the annex VII of REACH regulation (1-10 tonnes/year) (i.e. (a) Algal growth inhibition test (OECD 201),<sup>20</sup> (b) *Daphnia magna* acute immobilisation test (OECD 202),<sup>21</sup> and (c) ready biodegradability test (OECD 301F)<sup>22</sup> have been performed for ML, HMF, MMF, RMF and humins. They are presented briefly in Table 1.

Further details on humins samples and other selected compounds have been made available in a parent work of the same team.<sup>34</sup>

The selected biodegradability test is a stringent test for which a positive result will allow concluding that the chemical will degrade ultimately and quickly in the environment. Water solubility of stock solutions was confirmed by Total Organic Carbon (TOC) analysis, by comparing the TOC measurement with theoretical organic content of the selected compound.

For the *Daphnia magna* test, results are expressed as EC<sub>50</sub> 48h (i.e., the concentration that immobilizes 50% of daphnids within 48 hours). In *Pseudokirchneriella subcapitata* test, results are expressed in terms of EC<sub>10</sub> and EC<sub>50</sub> (i.e. effective concentration of the substrate at which either 10 % or 50 % of the test population are affected respectively). The algal growth inhibition test is a multi-generation test. However, for risk assessment purposes and derivation of Predicted No-Effect Concentrations (PNECs), the EC<sub>50</sub> is treated as a short-term toxicity value. The EC<sub>10</sub> may be used as an additional long term result when other long-term data are available. Effective Concentration (EC) values have been calculated using a logistic Hill model with bootstrap estimation of confidence intervals. Calculations have been performed with REGTOX software.<sup>23</sup>

In addition to the tests detailed above, immunotoxicity tests<sup>24</sup> were also conducted to develop a first approach concerning positive or negative effects on the ecosystem. Forty adult sticklebacks (*Gasterosteus aculeatus*, 5.3 ± 0.3 cm, 2.0 ± 0.4 g), obtained from one spawn in INERIS husbandry facility, were used. Before experiments, the fish were maintained in a laboratory tank and fed daily with frozen red mosquito larvae and brine shrimp. Each fish was sacrificed by cervical dislocation, measured and weighed. Splenic leucocyte isolation was made following further protocols.<sup>25</sup> Each leucocyte suspension was adjusted at 10<sup>6</sup> cells mL<sup>-1</sup> before incubation at 4 °C for 16 h with RMF, HMF, Humins b or ML with concentration ranging from 0 to 500 mg L<sup>-1</sup>.<sup>26</sup> Then, leucocyte distribution, cellular mortality (apoptotic and necrotic leucocytes), leucocyte respiratory burst,<sup>27</sup> lysosomal presence<sup>25</sup> and phagocytosis activity<sup>28</sup> analyses were carried out on all leucocytes, using a Cyan<sup>TM</sup>ADP flow cytometer (Beckman Coulter). Statistical analyses were performed using XLStat 2008 (Addinsoft). After verification of normality (Anderson–Darling test) and of homogeneity of covariance matrices (Bartlett test), a one-way ANOVA and a Student Newman-Keuls post-hoc tests were performed to assess the effect of each pollutant at each concentration in relation to control values. All hypotheses were tested for statistical significance at the level of  $p \leq 0.05$ .

## RESULTS AND DISCUSSION

The results of the ecotoxicity and biodegradability tests required for the annex VII of REACH regulation are summarized in Table 2. Among all the tested compounds, median effective concentrations highlighted the lack of short-term toxicity of ML, towards *Daphnia magna* and *Pseudokirchneriella subcapitata* tests (EC50s > 500 mg.L<sup>-1</sup>). These results are consistent with the daphnia test results conducted by Lomba *et al.*,<sup>29</sup> where the EC50 24h was 2,761±512 mg.L<sup>-1</sup>. Irrespective of the duration of exposure, these values confirm that ML is a chemical causing no short-term toxicity effects. Regarding humins, the range of EC50 48h values for the 2 batches (Ha and Hb with compositions C= 53.4 %, H= 5.9 %, O= 39.7% and C= 53.59 %, H= 5.88 % and O= 40.2 % respectively) highlighted no short-term toxicity effects towards *Daphnia magna*. Despite containing varying quantities of furanics in their structure, humins in general resulted in lack of short-term toxicity compared to other furanics tested independently. Whereas, EC50 value for HMF, MMF and RMF ranged from 13 to 36.5 mg.L<sup>-1</sup>, globally indicating the same range of toxic effects for compounds with a similar chemical structure (i.e. presence of a furan ring and aldehyde as one of the common functional groups). Remarkably, for these three compounds a significant increase in inhibitory effects between 24 hours and 48 hours of exposure is observed. This reveals that comparison of EC50 values, in particular for such compounds, must be based on the same duration of exposure.

In contrast with *D. magna* mobility test, the EC50 values for *P. subcapitata* ranged in a significantly narrower interval between the highest and lowest toxic compound (i.e. 51.5 – 110.2 mg.L<sup>-1</sup>), making it difficult to draw firm conclusions on the respective toxicity of tested compounds. Such findings were observed also by Lomba *et al.*,<sup>29</sup> and Roberts *et al.*,<sup>30</sup> where similar compounds tested with different species resulted in varying levels of observed toxicity. Variations in structural and functional properties of chemicals, alkyl chain lengths, responses of similar chemicals between different species were some of the common observations made from their results, which reasonably correlate in our case as well. From this it is evident that, the toxicity profiles of tested compounds can be both species and exposure dependent.

Regarding immune responses of humins and related compounds, major effects concerned inflammatory processes<sup>31</sup> whereas the innate immune response seems to be weakly impacted. In the present work, for whatever immune parameters tested in stickleback splenocytes, no effect was detected, regardless of products and test concentrations (from 0 to 500 mg.L<sup>-1</sup>). Moreover, the immune values were in line with classic proportions of granulocytes-monocytes (24.4±6.5 %) and lymphocytes (75.6 ± 6.5 %) and to basal phagocytic activity (47.9 ± 4.5 % of phagocytic capacity; 20.2±6.5 % of phagocytic capacity; 1.2 ± 0.8 for respiratory burst index; 4.3 ± 0.7 MFI for lysosomal presence) previously observed.<sup>24,28,32</sup>

As shown in Figure 1, biodegradability data made it possible to distinguish three groups of compounds: *i*) ML and 5-HMF which can be classified as readily biodegradable as they reached the pass level (i.e. 60% theoretical oxygen demand in a 10-day window within the 28-day period of the test); *ii*) the two batches of humins (Ha and Hb) for which some biodegradation was observed (% biodegradation ranging between 35 and 40%) and *iii*) MMF and RMF which showed no significant sign of biodegradation. For RMF, the inhibition

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3 control, containing both the test compound and aniline (reference compound) confirmed that  
4 RMF was not toxic for the activated sludge used as inoculum. This finding allowed  
5 concluding that the lack of biodegradation could not be attributed to an inhibitory effect on  
6 the activated sludge. The measurements of TOC removal at the end of the test confirmed the  
7 results calculated from the oxygen consumption for the whole set of compounds.  
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10 Interestingly, similar results have been obtained for each type of ecotoxicity and  
11 biodegradation test conducted for the two different batches of humins (Ha and Hb). These  
12 findings reveal that the slight variations observed in the composition of humins do not result  
13 in a significant variation of their ecotoxicological profile.  
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16 Eventually, an attempt was made to summarize the most significant and discriminating results  
17 of our study in Figure 2 (i.e. *Daphnia magna* immobilisation test and biodegradation test).  
18 They highlight that MMF and RMF (left part of the figure) could be the compounds of  
19 concern as they showed some toxicity for aquatic organisms and a lack of ready  
20 biodegradability. Our results are in accordance with the data publicly available in the ECHA's  
21 chemicals database for MMF where this compound is classified as toxic to aquatic life with  
22 long lasting effects (H411, acute chronic 2).<sup>33</sup> Accordingly, given the fact that these  
23 compounds of concern are generally present as impurities in humins<sup>34</sup>, better environmental  
24 footprint might be targeted by minimizing their presence in crude humins. In this manner,  
25 larger portfolio of sustainable uses of these biobased feedstocks could be developed.  
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## 34 CONCLUSIONS

35 Limited knowledge of the characteristics and physico-chemical properties of humins probably  
36 caused them to be used in the past only in low value applications and visibly no efforts were  
37 made in providing enough data regarding their environmental attributes. With promises of  
38 future commercial products emerging from humins, fulfilling the existing data gaps is  
39 therefore a priority from the production, regulatory and end user perspectives. The  
40 preliminary assessment shows that industrial humins produced by YXY® process do not  
41 display any immediate ecotoxicological concerns so far, thereby displaying some safer  
42 margins for further applications. As a word of caution, environmental fate of chemicals is  
43 purely species and exposure dependent, as is evidenced in the case of HMF, MMF and RMF.  
44 Therefore, due care must be taken before generalizing the hazards of a specific chemical  
45 tested with various species. Nevertheless, in the long run, there is a definite scope for in-depth  
46 safety-oriented research with further optimization of humins production pathways and newly  
47 targeted applications. In this context, research has already started to address thermal and fire  
48 hazards of these chemicals to complete the overall assessment of their safety profiles.  
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## 56 Conflicts of interest

57 There are no conflicts to declare.  
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For Peer Review

Table 1: Summary of tests performed for regulatory purposes (REACH regulation Annex VII)

Organisms	Effect	Endpoints	Expression of results	Test duration	Test methods
<b>Micro-algae</b> <i>Pseudokirchneriella subcapitata</i>	Chronic	Growth	EC10 EC50	72 hours	OECD 201, 2011
<b>Micro-crustaceans</b> <i>Daphnia magna</i>	Acute	Mobility	EC50	48 hours	OECD 202, 2004
Activated sludge receiving predominantly domestic sewage	Ready Biodegradability	Oxygen consumption	% biodegradation	28 days	OECD 301F, 1992

Table 2: Results of ecotoxicity and biodegradability tests

Compounds	<i>Daphnia magna</i>			<i>Pseudokirchneriella subcapitata</i>				Ready Biodegradability OECD 301F
	OECD 202			OECD 201				
	EC50 24h (mg.L <sup>-1</sup> )	EC50 48h (mg.L <sup>-1</sup> )	95% confidence interval	EC10 72h (mg.L <sup>-1</sup> )	95% confidence interval	EC50 72h (mg.L <sup>-1</sup> )	95% confidence interval	% biodegradation after 28 days (mean value)
<b>ML</b>	> 500*	> 500*	-	≈ 500*	-	> 500*	-	81
<b>HMF</b>	> 500*	36.5	30.0 – 45.1	28.0	26.0 – 30.5	110.2	106.8 – 115.5	82
<b>MMF</b>	> 200*	29.6	28.5 – 30.5	N.T**	N.T**	N.T**	N.T**	3.9
<b>RMF (crude MMF)</b>	≈ 500*	13.0	10.7 – 15.7	29.6	26.6 – 32.4	79.2	80.6 – 108.8	12.4
<b>Humins (a)</b>	> 500*	203.2	180.1 – 225	37.6	34.9 – 43.7	51.5	49.0 – 53.0	36.4
<b>Humins (b)</b>	> 500*	213.4	108.6 – 214	50.4	47.9 – 51.7	59.7	56.3 – 69.3	37.2

\* highest tested concentration

\*\* not tested

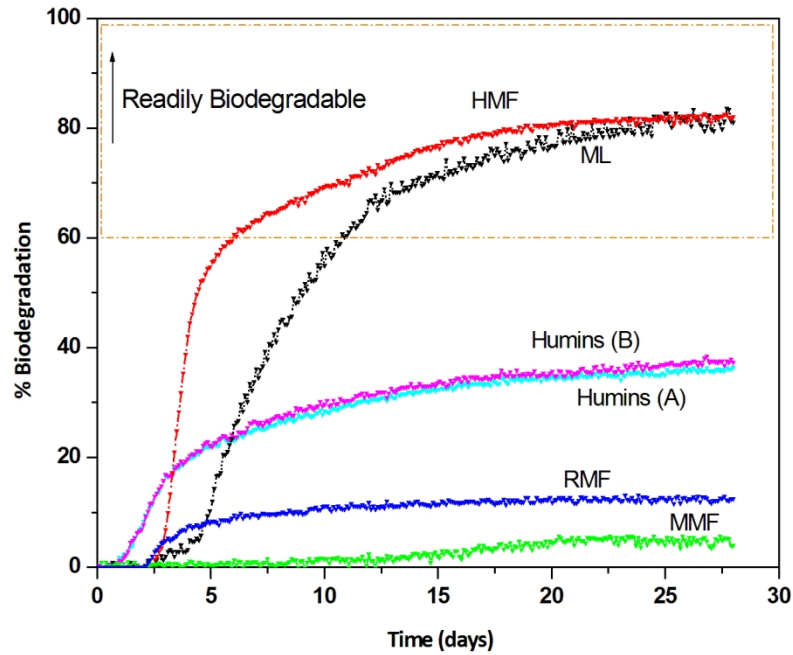


Figure 1: Biodegradability profile of Humins a, Humins b, ML, HMF, MMF and RMF.

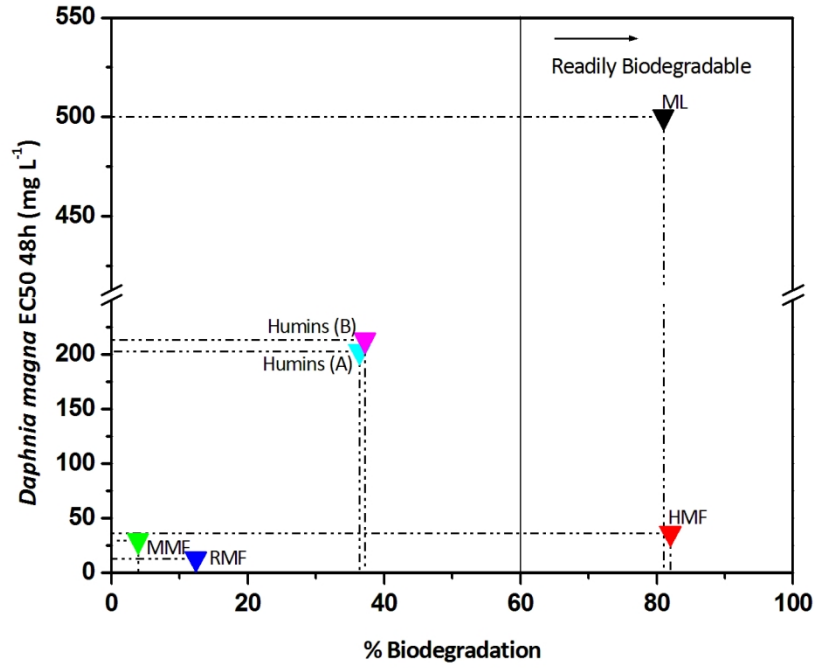


Figure 2: Relationship between biodegradability and toxicity profiles (EC50 48h) of all tested compounds.