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1 Characterising Ecotoxicological effects of a resinoid Natural Complex Substance using experimental
2 and *in silico* approaches

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22 Abstract

23 Natural Complex Substances (NCS) are botanical extracts of unknown or variable composition (UVCBs).
24 Among NCS, resins contain long chain substances that are very difficult or impossible to identify with
25 reasonable resources representing a challenge to characterisation and toxicity assessment. This study
26 aims to provide an innovative and relevant method to identify the constituents of *Galbanum* (*Ferula*
27 *galbaniflua* Boiss.) resinoid, experimentally assesses and accurately predicts its ecotoxicity. A
28 fractionation technique was used to separate the NCS into three phases: “volatile”, “non-volatile but
29 potentially bioavailable” and potentially “inert”. The whole substance (WS) and each fraction were
30 tested as Water Accommodated Fractions (WAFs) for acute and chronic ecotoxicity to algae and
31 daphnids. Then, the toxicity of each fraction and the WS was predicted using i) an *in silico* method
32 adapted for mixture toxicity testing and ii) the classical additivity CLP/GHS, based on the analytically
33 determined composition. The volatile fraction was more toxic than the non-volatile fraction while the
34 inert fraction was not acutely or chronically toxic to algae or daphnids. The experimental daphnid
35 chronic toxicity was higher than the acute for the WS probably because of the larger molecules not
36 toxic up to their solubility limit in acute studies. The results from the experiments substantially
37 validated the *in silico* predictions which accurately predicted the toxicity of the fractions and the WS
38 even if rather conservative for chronic daphnid toxicity. Also, the *in silico* method mechanistically
39 explained the WAF test results and attributed the observed toxicity to specific constituents. However,
40 CLP/GHS overpredicted toxicity in all cases and failed to account for observed toxicity. Experimental
41 and *in silico* approaches appear to be highly complementary to deal with NCS. As experiments are costly
42 and difficult to perform, the *in silico* methods provide a rapid and cost-effective option to obtaining
43 data for difficult to test substances.

44 Keywords: Natural Complex Substance; ecotoxicity; in silico; Water Accommodated Fraction

45 1. Introduction

46 Natural Complex Substance (NCS) (Api et al., 2022) are typically botanical extracts. NCS are substances
47 of unknown or variable composition, complex reaction products, or biological materials (UVCBs)
48 (Salvito et al., 2020). Their composition is variable due to source and process fluctuations and often
49 only partially known, making environmental risk assessment difficult (EFEO/IFRA, 2016). This contrasts
50 with multi-constituent substances, which are better defined, with main constituent concentrations
51 between 10 and 80% (w/w) of the total and impurities counting for <10% (REACH, 2006; EFEO/IFRA,
52 2015).

53 The recommended approach to perform an environmental aquatic hazard assessment for UVCB
54 substances is to use Water Accommodated Fraction (WAF) toxicity assays (OECD 2019). This method
55 leads to the separation of the UVCB to different phases, allowing the exposure of the organisms to the
56 fraction of the substance which is dissolved and bioavailable. Such methodologies have typically been
57 employed by the fragrance industry's essential oil products (termed Type 1 or NCS1, where at least 90%
58 of the substance has been characterised) and are used for regulatory purposes (Wheeler et al., 2020).
59 However, WAF lacks mechanistic interpretability, as it only provides "loading rates" without identifying
60 the activity of the constituents. Its application in hazard and risk assessment is therefore questioned.

61

62 In 2021, Bichere and Thomas (2021) published an *in silico* method for toxicity estimation for complex
63 mixtures using an equivalent of the WAF methodology deemed applicable to (mostly) non-polar
64 narcotic mixtures such as essential oils. This novel *in silico* method mimics WAF studies for non-polar
65 narcotic mixtures using chemical activity principles. It predicts acute and chronic ecotoxicity Lethal or
66 Effect Loading Rate (LL50 or 10/EL50 or 10/ErL50 or 10) by i) calculating solubility and chemical activity
67 of constituents, ii) summing individual toxicities up to a critical activity threshold, iii) converting this
68 back into an equivalent loading rate. The method showed strong agreement with 13 reliable
69 experimental acute WAF studies on fish, daphnids, and algae (within a factor of 2), outperforming
70 standard additive models, which tend to overestimate toxicity. The *in silico* WAF method is therefore
71 considered fit for purpose for non-polar narcotic mixtures when constituents are known >90%. The
72 main limitations of the method are that >90% of the constituents in the mixture must be known for the
73 model to function correctly. Furthermore, the method has only been successfully validated for NCS1
74 where most constituents are known to be non-polar narcotics and the model has not yet been
75 demonstrated as applicable to more compositionally uncertain NCS (NCS2). The European Chemical
76 Agency (ECHA) indicated that the properties of each component of a UVCB should be analysed and
77 identified separately for constituents as low as to 0.1% for PBT assessment purposes (ECHA 2006; ECHA
78 2017). However, as well as having the same difficult properties as other NCS (i.e., variable composition
79 and volatile and/or hydrophobic constituents), NCS2s contain a large fraction of resins, waxes, etc.
80 which makes them particularly difficult to analyse in the laboratory.

81 In this project, named "Compartmentalised Hazard Assessment for Natural Complex Extracted
82 Substances (type 2)" (CHANCES2) we describe an innovative approach that aims to use a fractionation
83 approach (i.e., methods designed to effectively separate the mixture into fractions containing
84 constituents with certain similar physico-chemical properties). An NCS2 (*Galbanum* resinoid) was
85 divided into three fractions each with similar properties and each tested as an individual block as
86 follows: i) Fraction 1 composed of low to intermediate solubility, volatile components, ii) Fraction 2
87 composed of more soluble, non/semi-volatile, potentially bioavailable components, iii) Fraction 3

88 composed of constituents that are non-volatile and insoluble, unmeasurable and expected to be
89 “Inert” (i.e. non-toxic in acute and chronic aquatic toxicity studies) and iv) the whole NCS2 substance
90 (unseparated fractions). The fractions and the WS were then tested using the following methodology.
91 First, each fraction was tested individually on aquatic organisms using both acute and chronic algae
92 and daphnid OECD Guideline studies under GLP. Then, the *in silico* WAF method was applied to each
93 fraction and compared to the experimental values. The WS toxicity was compared with the sum of the
94 three fractions. Finally, all the results were compared with CLP/GHS additivity method for classification.

95 Our research goals were to i) determine if a partially characterized NCS2 can be accurately assessed
96 experimentally, ii) evaluate if the WAF-based *in silico* method can be extended directly to mixtures with
97 different mechanisms of toxic action and iii) compare effectiveness of different calculation methods.
98 This comparison first used the published *in silico* method based uniquely on non-polar narcotic toxicity
99 regardless of the mechanism of action of the constituents, and also the mixture followed independent
100 action according to the mechanisms of action of each constituent. This approach represents a
101 potentially significant advancement in strategies for assessing and understanding complex natural
102 substances where traditional methods are insufficient due to the unknown composition of the
103 substances.

104 2. Material and Methods

105 2.1 Tested substance

106 NCS2 substance *Galbanum* resinoid (*Ferula galbaniflua* Boiss.) was tested. *Galbanum* resinoid extracts
107 were provided by dsm-firmenich. The substance was analysed and fractionated by University of Côte
108 d’Azur, France and this is fully described in an article by Canton et al. (2025).

109 2.2 Testing strategy of the NCS2

110 To characterise the overall ecotoxicity of the whole NCS2 and identify which part of the substance is
111 leading the toxicity of the whole NCS2, the substance was separated into three different fractions, as
112 follows:

113 Fraction 1 (= volatile fraction, VF): This typically represents < 10% to 40 % of the composition of NCS2
114 compounds. It is a fraction expected to be predominantly composed of terpenes, alcohols, ketones,
115 etc. which can be readily characterized by Gas Chromatography.

116 Fraction 2 (= non-volatile and bioavailable fraction, NVF-baf): This fraction was expected to include the
117 constituents which are not extractable in the previous phase (e.g. not volatile enough) but still be
118 bioavailable as at least slightly soluble (i.e., between 0.1 and 10 mg/L and characterisable using Liquid
119 Chromatography-Mass Spectrometry.

120 Fraction 3 (= inert fraction): This fraction is expected to comprise waxes, high molecular weight tannins,
121 alkanes and alkenes which are expected to be almost completely insoluble in water and therefore not
122 bioavailable.

123 After separation, the ecotoxicity of each fraction and of the whole substance (WS) was experimentally
124 assessed by Laboratoire des Pyrénées et des Landes, France, using the WAF approach (Fig. 1). An *in*
125 *silico* assessment of ecotoxicity was performed by KREATiS using their iSafeRat® software. In practice,
126 fractions 2 and 3 were tested together for simplicity to obtain information on the bioavailable fraction

127 as the inert fraction was not expected to influence the result. The basic principles of the extraction of
128 the fractions performed from a sample of the WS are explained in the next section.

129

130 2.3 Characterization of the different fractions

131 The volatile fraction (VF) was separated from the non-volatile fraction (NVF-baf) by distillation under
132 reduced pressure. The VF was then characterized by gas chromatography coupled with mass
133 spectroscopy (GC/MS) and by gas chromatography coupled with flame ionization detector (GC-FID). β -
134 Pinene was identified as the main compound of the fraction (25.0%) ahead of bulnesol (7.2%) and
135 guaiol (6.9%). The NVF-baf was studied by high performance liquid chromatography (HPLC). Major
136 compounds were first targeted by HPLC coupled with mass spectrometry (HPLC-MS), evaporative light
137 scattering detector (HPLC-ELSD) and diode array detector (HPLC-DAD). The NVF-baf was then purified
138 by semi-preparative HPLC to isolate and identify the main components by one and two-dimensional
139 nuclear magnetic resonance (1D and 2D NMR). Finally, they were quantified in the NVF and in the
140 *Galbanum* resinoid extract by external calibration. Specific constituent peaks found in the three
141 fractions of the NCS2 were analysed. Due to the complexity of the test substance, it was not possible
142 to determine the complete substance profile, but the techniques allowed analytical determination and
143 production of analytical methods to follow-up on critical peaks (which were identified as major
144 constituents) and structurally similar substances, within the bioavailable phases. Two types of
145 information were available: 1) specifically attributed peaks linked quantitatively (as a percentage of the
146 fraction) to an identified substance (e.g., $C_{10}H_{16}$, terpinolene 0.3% with retention time of 13.64
147 minutes); 2) Peaks related to an unidentified substance but with a retention time and a percentage
148 (e.g., unknown, 0.2% with retention time of 15.54 minutes). In this latter case the structural formulae
149 of the nearest neighbours were used to identify the most probable chemical formula of the unknown
150 constituent, and these substances were pooled and attributed to the most similar profile (e.g., " β -
151 pinene-like") for use in the *in silico* calculations. The main intention was to demonstrate relative
152 presence of these substances in the tested fractions to allow their attribution to mechanistic, toxic
153 profiles for *in silico* predictions.

154

155 2.4 Ecotoxicology experimental methods

156

157 To assess the acute and chronic toxicity of the WS, the VF, inert fraction and the NVF-baf to daphnids
158 and algae was tested following OECD Guidelines (201: 72 hour-ErC10/ErC50 algal growth rate; 202: 48
159 hour-EC50 daphnids immobilization; 211: 21 day-EC10 daphnids reprotoxicity) and under Good
160 Laboratory Practice principles. As the *in silico* method WAF calculation for the VF (Bichere & Thomas,
161 2021) is considered as fully valid, and WAF chronic tests are complicated to perform on such
162 substances, no chronic toxicity assessment of the VF fraction was performed on daphnids.

163

164 All fractions were obtained from the same batch as the WS. The three fractions and the WS were tested
165 following the WAF approach to accommodate the hydrophobic compounds in the aqueous medium.
166 The experimental loading rates for the WAF tests were prepared as follows:

167 Stock solution bottle kept closed under nitrogen and in darkness to prevent oxidation/photolysis of the
168 compounds.

169

170 Solutions were prepared using 1 to 5 L cylindrical glass bottles closed with screw caps and fitted with a
171 drain port near the bottom for drawing off the WAFs. A magnetic stirring bar was placed in each mixing
172 vessel completely filled with test water (with a minimum headspace). The loading rates of the test item
173 were either weighed on glass slides and then placed under the surface of the test water contained in
174 the mixing vessels held by fishing line (e.g. for the VF) or in glass vials (approximate volume: 100 mL)
175 subsequently filled with approx. 100 mL of test water and sealed. If vials were used each glass vial was
176 placed in an ultrasonic bath at ambient temperature and sonicated for 40 minutes. The mixing vessels
177 were filled with the contents of the glass vials and closed. After approximately 24 hours of gentle

178 stirring in the dark, at room temperature, the WAFs were allowed to stand undisturbed for at least 1
179 hour before use. The first 100 mL were discarded via the drain port.

180 The WAFs were thereafter added into test vessels.

181 The NVF-baf, due to inclusion of the inert fraction in the WAF preparation was treated in different ways
182 according to the test performed:

- 183 • In the acute daphnid study no separation of NVF-baf from the inert fraction was performed;
- 184 • In the algae study on NVF-baf, the highest test loading rate only of the NVF-baf was filtered
185 (0.45 µm) to test this fraction without residual inert resins. Vessels were immediately closed
186 after filling with a minimum headspace. At the start of the test, the test solutions were
187 observed to be clear and colourless (Tyndall effect, checked via laser beam). The pH of the test
188 solutions was not adjusted;
- 189 • In the daphnid chronic study, all WAFs were filtered (0.45 µm) to remove the insoluble fraction
190 (residual inert resins) before addition into test vessels.

191

192 For the inert fraction a supplementary step was performed: the contents of the vial of the NVF-baf
193 were filtered through a 0.4 µm polycarbonate membrane filter to separate the soluble part from the
194 insoluble fraction retained on the filter. The membrane filter was immersed in 10 mL test water,
195 followed by sonication for 10 min. Then the contents of the vial, including the membrane filter, were
196 added in the 1 L mixing vessel pre-filled with test water (see above). Additional nitrogen was injected
197 into the headspace before closing the mixing vessel. After 5 days of gentle stirring) in the dark at room
198 temperature, the contents of the vessel were allowed to stand undisturbed for approx. 30 min before
199 use. The first 100 mL were discarded via the drain port.

200

201 The aquatic toxicity of NCS2 WAF was tested on the above fractions using a closed set up and static
202 conditions for the OECD 201 test and semi-static conditions for the OECD 202 test and the OECD 211.

203

204 In the first step, *Galbanum* resinoid was tested as range-finding studies on daphnids and algae. Various
205 test conditions (e.g., semi-static) and varying conditions of test solution preparation were tested prior
206 to initiating the definitive tests, e.g., with and without solvent, slow-stir preparation with different
207 stirring and/or settling times, differing separation techniques etc.

208

209 2.4.1 Monitored substances

210 A small number of substances identified by Canton et al, as “sentinel” constituents were analysed
211 during the studies for stability and ultimately quantification. Table 1 provides an overview of the
212 constituents monitored in each test.

213

214 2.5 *in silico* Water Accommodated Fraction prediction

215 The *in silico* ecotoxicity prediction of the WS and VF and NVF-baf fractions was carried out by KREATIS
216 SAS, France and was based on two steps. First, the acute and chronic toxicity to daphnids and algae of
217 each constituent identified in the WS and representing at least 1% of the composition was predicted
218 using the Quantitative Structure Activity Relationship (QSAR) modelling approach (Thomas et al., 2019)
219 as detailed in section 2.5.1. Then, each predicted value was used in a WAF calculation based on activity
220 principle as described in Bichere & Thomas (2021) designed for calculating WAF toxicity for “baseline”
221 toxicants (commonly known as non-polar narcotics) and giving an Effect Loading leading to 10 (EL10)
222 or 50% (EL50) of effect for chronic and acute toxicity respectively as described in section 2.5.2. The
223 workflow is presented in Fig. 2. The KREATIS QSARs and WAF calculation were used as they represent
224 the only accurate *in silico* method for assessment of mixture toxicity of essential oils using the WAF
225 approach currently available, other methods proposing uniquely a CLP/GHS additivity type approach.

226

227 2.5.1 Single-compound ecotoxicity prediction

228 The ecotoxicological values (e.g. EC_x, ErC_x) of each constituent to both daphnids and algae were
229 predicted using iSaferat[®] ecotoxicity model (algErC10 v2.4, algErC50 v2.1, daphEC50 v2.1 and
230 daphEC10 v2.3). The starting point for ecotoxicity prediction using iSaferat[®] QSARs is the allocation of
231 a mechanism of toxic action (MechoA) as described in Bauer et al. (2018). In brief, MechoA is a
232 structural alert scheme containing 6 main classes and 26 sub-classes that designates a specific toxic
233 mechanism according to the chemical structure of each constituent. The sub-classes are linked to
234 QSARs containing substances relevant to that mechanistic sub-class. iSaferat[®] ecotoxicological models
235 consist of a single-linear relationship between the subcooled liquid water solubility (SLWS, model
236 descriptor) and the ecotoxicity (model outcome). Each regression is specific to a MechoA, the trophic
237 level (e.g. daphnids, fish) and exposure (acute, chronic).

238 The mechanism of toxic action (MechoA) (Bauer et al., 2018) of each constituent was classified using
239 the MechoA Premium SAR v1.3. Once the MechoA was allocated, the ecotoxicological model descriptor
240 (SLWS), used as input was predicted for each constituent value using the iSaferat[®] WatSol model v2.1.

241

242 2.5.2 VF, NVF-baf and WS ecotoxicity calculation

243 The WAF calculation was performed following methodologies outlined in Bichere and Thomas (2021).
244 Predictions of individual constituents using iSaferat[®] software were compared to experimental data
245 on constituents when available. In the case of Galbanum resinoid, the ecotoxicity of the VF, the NVF-
246 baf and the WS was predicted according two different methods: The first method based on the
247 hypothesis that if a complex substance contains a large fraction of non-polar narcotics, then all
248 substances will fall under the WAF calculation method proposed by Bichere & Thomas (2021), (i.e.,
249 that all substances are non-polar narcotics, typically related to “baseline toxicity”). Initially developed
250 for NCS1 only, this approach calculates an effect loading (ELX), based on a series of calculations that
251 mimic the WAF experimental approach. The method is based on the chemical activity of each
252 compound, (i.e., the activity of a molecule is its effective concentration taking into account
253 intermolecular interactions modifying the water solubility). Briefly, the thermodynamic influence of
254 each constituent on the solubility of the others within the mixture (i.e., the concentration of each
255 constituent in the “loading rate”) is determined. Then, the non-bioavailable fraction is estimated
256 (bioavailability factor < 1) and removed to calculate the free concentration of each constituent exerting
257 toxicity. As this hypothesis was validated for non-polar narcotic compounds only (mechoA 1.1), each
258 substance related to a different MechoA was considered the same way. Finally, the loading rate was
259 adjusted until the sum of activities of the bioavailable fractions is equal to the fraction-weighted
260 average of toxic activity of each constituent. All the different steps of calculation are fully explained in
261 (Bichere & Thomas, 2021).

262 The second method hypothesises that each constituent acts in accordance with its own MechoA. Thus,
263 constituents were classified into respective MechoA sub-Classes and the toxicity of each constituent
264 was predicted in accordance with its specific MechoA. Also, compounds with a MechoA other than 1.1
265 were related to a bioavailable factor of 1 (i.e., that the constituent is entirely bioavailable), representing
266 the worst-case situation. Under method two, the WAF calculation was used for MechoA 1.1
267 constituents and the additivity approach was used for fractions composed of constituents belonging to
268 other MechoA classes. This is an important step as using two different regressions (two different
269 MechoAs) for the same compound may result in substantially different ecotoxicity values, leading to

270 significant changes to the resulting WAF calculation. The different strategies of calculations are
271 summarised in Table 2.

272 2.6 CLP/GHS mixture calculation

273 The standard regulatory method for additivity used for assessing mixture toxicity in the CLP/GHS
274 regulations (CLP 2008, add GHS) was applied to compare with the in silico WAF mixture predictions.
275 The iSAFeRat predictions for each constituent was used based on the actual MechoA of each
276 constituent.

277

278 3. Results

279 3.1 Analytical results of the fractions

280 A brief overview of analytical results for each fraction is outlined below.

281 First, fraction 1 (VF) represented 34.5% of the WS, while fraction 2 (including both NVF-baf and the
282 inert fraction) represented 65.5% of the WS. For fraction 1 (VF), 99.8% of the constituents of the
283 composition were detected in the analysis, however Canton et al. (2025) identified only 21 substances
284 (representing 76.7% of the fraction) above 1%. Major constituents were terpenes, notably β -pinene
285 (representing 25.4% of the VF), bulnesol (7.2%) and guaiol (6.9%). Other minor constituents above 1%
286 were eudesmol isomers (8.9%), germacrene B (2.5%), elemene (2.1%), δ -cadinene (1.9%) which are
287 sesquiterpenes with a larger carbon skeleton. For fraction 2 (NVF-baf), 7 constituents above 1% were
288 identified. The major constituent was galbanic acid (representing 14.3% of the fraction) which was
289 titrated at 11.9% in the raw material of *Galbanum* resinoid. The other main compounds in the NVF-baf
290 were farnesiferol A (10.9%), badrakemin (6.85%), colladonin (5.83%), feselol (2.31%) and kellerin
291 (2.87%). Umbelliferone was one of the minor compounds of NVF-baf and a raw material of *Galbanum*
292 resinoid.

293 For fraction 3 (“inert”), no identification of constituents was undertaken, all bioavailable parts of the
294 substance having been leached out by extensive washing (and included in the other fractions).

295 For detailed information on analytical characterisation of the fractions and the WS, refer to Canton et
296 al. (2025).

297

298 3.2 Acute and chronic ecotoxicity testing

299 All studies performed (under GLP) during this project were valid and met the OECD Guideline validity
300 criteria. Experimental results of ecotoxicity testing for each tested entity (VF, NVF-baf, inert fraction,
301 WS) and both trophic levels (daphnids, algae) are presented in Table 3. Acute toxicity was observed for
302 the VF and the WS for both daphnids and algae. The VF was the most toxic fraction to algae and
303 daphnids in both acute and chronic tests. The NVF-baf was less toxic in acute studies with
304 concentrations greater than the highest loading rate tested (50 mg/L, as loading rates were originally
305 100 mg/L but this included the inert fraction of approximately 50% of the NVF-baf). In chronic studies
306 the endpoint values were lower and chronic toxicity for daphnids was observed for all fractions except
307 the “inert” fraction. In this fraction, there was no statistical difference observed compared to the
308 control algae and daphnid studies. The largest differences in ecotoxicity were observed between acute
309 and chronic experimental daphnid toxicity studies on the WS with a 48h-EL50 of 23.5 mg/L and a 21d-
310 EL10 of 0.8 mg/L resulting in an acute to chronic ratio of 30.

311

312 3.2.1 Focus on the acute and chronic daphnid tests on the WS

313 Acute toxicity was observed with a median Effect loading rate (EL50) of 23.5 mg/L in the 48h daphnid
314 study suggesting limited toxicity of the WAF to these organisms (equivalent to “harmful” under

315 GHS/CLP classification). Five constituents (β -pinene, guaiol, bulnesol, galbanic acid and farnesiferol A)
316 were measured on 4 occasions during this semi-static WAF test, as sentinel substances to be tracked
317 during the test as an indication of stability over the test. Substance levels at the start of the test were
318 2-5 times below the concentrations measured by Canton et al. (2025) in the WS sample, in fresh
319 solutions which may be due to their water solubility and their thermodynamic relationship with other
320 substances in the mixture when placed in aqueous solutions. These measured concentrations were
321 therefore not used to adjust the loading rates, but these concentrations were observed to be well
322 conserved in the old solutions. Based on these “nominal” loading rates, a clear concentration response
323 was observed between 9 and 50 mg/L (from 0 to 100% immobilisation) and were therefore the best
324 indicator of toxicity used for the *in silico* predictions. Use of nominal loading rates is also recommended
325 in OECD GD 23.

326 Chronic toxicity was observed at 0.8 mg/L in the 21-day *Daphnia* reprotoxicity study suggesting much
327 higher toxicity of the long-term exposure to the WAF (equivalent to “very toxic” under GHS/CLP
328 classification) than in the acute study. β -pinene, germacrene B and galbanic acid were monitored
329 during the 21 days of the test to WS. The results, presented in Tables 1 to 3 in Supplementary Material,
330 revealed that substance levels at test start were 10 times below the concentrations measured by
331 Canton et al. (2025) for β -pinene and lower still for Germacrene B. Once again, this reduction compared
332 to the original constituent concentration reported for the same sample of the substance in Canton et
333 al. is considered as normal in a WAF test. While the concentrations were not used to recalculate the
334 original loading rates values of the ecotoxicity studies, they provided useful information on stability of
335 test solutions during the study. The concentrations of β -pinene and galbanic acid were observed to be
336 well conserved in the old solutions with losses between 10 and 30%. Conversely, germacrene B was
337 very poorly conserved between fresh and old solutions with losses around 70%, although every effort
338 was made to maintain the concentrations (daily renewal of test solutions, closed conditions with
339 minimum headspace). Based on the original loading rates as analysed by Canton et al. (2025), a clear
340 concentration response was observed between 0.6 and 2 mg/L loading rates (with 4 to 66% reduction
341 in offspring compared to the control). Despite the interest of inclusion of sentinel substances to follow
342 stability throughout WAF tests, it is considerably more practical and useful to base results on nominal
343 loading rates due to the potential occurrence of unexplainable differences between nominal and
344 measured constituent concentrations.

345

346 **3.3 In silico Analysis**

347 First, a MechoA was classified for each of the constituents. The predicted and, when available,
348 experimentally determined ecotoxicity of constituents were compared and correlate well. The data are
349 included in the SI. All the identified constituents of the VF were assigned to MechoA 1.1 (non-polar
350 narcosis). In the NVF-baf, the major constituent (galbanic acid) was assigned to MechoA 5.2 (release of
351 protons in the cytosol of cells for all species) while the other constituents matched an alert, because
352 of the coumarin fragment, relating to metabolism in mammals, which fails to describe the mechanism
353 and toxicity in aquatic species. After literature research, the coumarin fragment was manually related
354 to MechoA 2.1 (hydrolysis to non-polar narcotic products), based on a few ecotoxicological datapoints
355 that were proved to fit with the QSAR regressions for MechoA 2.1. No excess toxicity could be identified
356 in these studies, while the metabolism described by MechoA Premium for mammals would lead to
357 higher toxicity if this occurred in aquatic species too. Following assignment, an E(r)C10 or E(r)C50 value
358 was obtained for each constituent and trophic level except for galbanic acid chronic toxicity to algae
359 where no model is available yet under iSafeRat[®] for that endpoint and carboxylic acid compound. All
360 the input and output values are presented in Tables 4 and 5 in Supplementary Material. As all the
361 constituents of the VF fraction belonged to MechoA 1.1, the predicted values were similar between
362 Method 1 and Method 2 for a given trophic level and exposure (e.g. acute vs. chronic). As all the

363 constituents of the NVF-baf fraction did not belong to MechoA 1.1, predictions for Method 1 (based
364 on MechoA 1.1 ecotoxicity models) were consequently different from those obtained for Method 2
365 (based on MechoA 5.2 and 2.1, depending on the constituent). For a given constituent and trophic
366 level, the values of the predictions obtained with MechoA 1.1 were systematically lower (meaning,
367 more conservative) than the ones obtained with MechoA 5.2 or 2.1.

368

369 Second, using the WAF calculation, a E(r)L10 and E(r)L50 value was predicted for the VF, NVF-baf and
370 WS (Table 3). The E(r)L50 and E(r)L10 were obtained based on different levels of information such as
371 the constituent individual water solubility, ecotoxicity, activity and composition. The *in silico* prediction
372 of VF, NVF-baf and WS toxicity was performed using the concentrations measured by Canton et al. and
373 reasonable worst-case estimations to classify structures. In brief, as not all peaks were attributed to
374 specific structures, some compromise was necessary to process the impacts. In the VF, 68% of the
375 composition was attributed to 18 identified constituents accounting for at least 1%. For the VF *in silico*
376 assessment, it was necessary to conservatively account for the remaining 32% of constituents
377 characterized as unknown substances or representing less than 1% of the composition. Those
378 substances were added to the percentage of the most toxic monoterpene or sesquiterpene derivatives
379 identified in this fraction and being the closest based on the molecular formula and retention time.
380 Thus, three groups of substances were added to the initial list of 18 compounds $\geq 1\%$ as follows: the β -
381 pinene-like substances (2.2%), the guaiol-like substances (13.30%) and the germacrene-B like
382 substances (9.10%). Thus, the vast majority of substance groups were covered by the calculations in a
383 conservative manner, despite the absence of full analytical data. For the NVF-baf, the calculation was
384 performed on the 7 compounds $\geq 1\%$. To account for the inert fraction, which was a large part of the
385 composition from the final experimental result, a correction factor equal to the proportion of the inert
386 compounds in the NVF-baf was applied. For the "inert" fraction, no calculation was performed as no
387 identification of constituents was undertaken, all bioavailable parts of the substance having been
388 leached out by extensive washing (and included in the other fractions). However, as this fraction was
389 found experimentally to be innocuous in OECD 201, 202 and 211 tests, it was included in the calculation
390 for the WS as an inert fraction (i.e., equivalent to a blank space), thereby diluting proportionally, by
391 mass, the toxicity determined in the two other fractions. Finally for the WS, the percentage of each
392 constituent was calculated considering the percentage of the VF (fraction 1) and the NVF-baf (fraction
393 2), respectively. Fraction 1 represented 34.5% of the WS and fraction 2 represented 65.5% as the
394 loading rates where fraction 2 was based on both NVF-baf and inert fraction loading rates. The loading
395 rates obtained were also corrected to account for the inert fraction (fraction 3) at the WS scale
396 ($=51.46\%$ of fraction 2 and 33.7% of the WS). Following the distillation process, NVF (taken as both
397 fractions 2 & 3 ("inert") together) of *Galbanum* resinoid extract represented about $65.5 \pm 1.5\%$ of the
398 WS.

399 The predictions (summarised in Table 3) were performed following 2 methods: *in silico* Method 1:
400 standard WAF calculation taking all substances to be non-polar narcotics (MechoA 1.1) or *in silico*
401 Method 2: WAF calculation between different MechoAs. E(r)L50 and E(r)L10 predictions for VF were
402 the same between the *in silico* Method 1 and 2 as all the constituents belong to MechoA 1.1 and
403 therefore the E(r)C10 and E(r)C50 used for the WAF calculation were the same. For NVF-baf and the
404 WS, the *in silico* Method 1 provided E(r)L50 and E(r)L10 values systematically lower than the *in silico*
405 Method 2. CLP calculation results provided systematically the lowest value (=the most conservative
406 results) of the predictive approaches.

407

408

409 Along with the calculation, a Pareto graph was edited to highlight the constituents leading the toxicity
410 of the WS. An example is provided in Fig. 3 for the WS toxicity to daphnids. Under acute toxicity, β -

411 pinene, galbanic acid and guaiol-like compounds explained ca. 50% of the toxicity. Then, 24 other
412 compounds of the VF and the NVF-baf were identified to increase the toxicity of the WS. Under chronic
413 exposure, a restricted number of compounds were identified to drive the toxicity of the WS, with
414 Germacrene and Germacrene B-like compounds leading ca. 60% of the toxicity. Then, 12 other
415 compounds were identified as contributing to the toxicity of the WS.

416

417

418 **4 Discussion**

419 The assessment of NCS2, such as *Galbanum* resinoid, remains a significant challenge in regulatory
420 ecotoxicology due to the variable and often poorly characterized nature of these UVCB substances. This
421 study demonstrates the utility of a combined experimental and *in silico* approach for evaluating the
422 acute and chronic aquatic toxicity of NCS2 substances and provides mechanistic insight into observed
423 toxic effects.

424 For all fractions tested, Method 1, using standard WAF calculations (MechoA 1.1 whatever the actual
425 MechoA), were close to the measured values of EL50 and EL10 (within a factor of 3) for both acute and
426 chronic studies with the *in silico* results the same as or slightly more conservative than the experimental
427 values. For Method 2, constituents within the NCS were treated as having different MechoAs (1.1, 2.1
428 & 5.2) using an additivity approach for non-narcotics, while the toxicity of non-polar narcotics present
429 were calculated following the WAF calculation. This method underestimated toxicity significantly in
430 nearly all fractions.

431 Specifically for the WS, the standard WAF calculation (method 1) was considered to perform extremely
432 well for the daphnid acute and algae acute and chronic. The chronic daphnid prediction appeared
433 overly conservative at first sight compared to the experimental results overpredicting toxicity by a
434 factor of 6. One explanation may be due to losses of germacrene B observed during the daphnid chronic
435 study resulting in a slight reduction of toxicity in the experimental study (SI table 3). On the other hand,
436 Method 2 significantly underestimated the toxicity of both acute and chronic studies throughout. As a
437 general result, the *in silico* Method 1 provided E(r)L50 and 10 values lower than the values obtained
438 with the *in silico* Method 2. Values deviate from a factor of ca. 2 to 8 for acute toxicity, and from a factor
439 of 25 to 63 for chronic toxicity for daphnids. Such a difference between both methods can be explained
440 by the fact that NVF-baf and WS contains constituents belonging to another MechoA than 1.1. Thus,
441 the E(r)L50 and 10 were calculated in the Method 1 from E(r)C50 and 10 values obtained from
442 ecotoxicological model for MechoA 1.1 in Method 1 and from models for MechoA 5.2 and 2.1 in
443 Method 2. The regressions for MechoA 1.1 being steeper than the regressions for MechoA 5.2 and 2.1
444 (for acute and chronic algae and daphnids toxicity) and the constituents being poorly soluble, the
445 E(r)C50 and 10 values were derived from a part of the regression line where MechoA 1.1 exhibit a
446 higher toxicity than the two other MechoAs. Thus, the resulting E(r)L50 and 10 values obtained from
447 Method 1 were more conservative than for Method2. Nevertheless, Method 1 predicted experimental
448 toxicity exceedingly well in all cases, contrarily to Method 2. A precise explanation for this finding
449 remains to be elucidated as one might expect that toxicity estimations for mixtures would be improved,
450 not worsened by taking into account the specific mechanisms of toxic action of all the constituents.

451 The CLP approach significantly overestimates toxicity in virtually all cases (with the exception of the
452 NVF in the daphnid chronic study) with values at least 5 times and up to 80 times more conservative
453 than observed results. It is recommended to consider use of this method with extreme care to calculate
454 ecotoxicity of natural complex substances.

455 A key finding is that the volatile fraction of the NCS2 was the principal contributor to the acute and
456 chronic aquatic toxicity, while the residual non-volatile or presumed "inert" fraction exhibited limited
457 and negligible toxic impacts, respectively. This supports the hypothesis that fractionation can help
458 isolate the bioavailable and toxicologically relevant components of complex mixtures. Importantly, the

459 fraction-specific results allowed for the elucidation of relative toxicity contributions, much of the *in*
460 *vivo* data could be directly compared to the *in silico* values.

461 Among the predictive approaches evaluated, WAF calculation method, as implemented in the *in silico*
462 model of Bichere and Thomas (2021), performed best. Despite being originally developed for NCS1
463 substances composed primarily of constituents known to be non-polar narcotics, the method showed
464 high predictive accuracy even for the more complex and partially unknown NCS2. Particularly for acute
465 endpoints, the predicted loading rates were closely aligned with the experimental data. Chronic
466 predictions were also accurate, with the exception that the model overestimated toxicity by a factor of
467 approximately six in the chronic daphnid endpoint for the WS, where the observed toxicity was 0.8
468 mg/L experimentally but predicted as 0.14 mg/L *in silico*. This overestimation of toxicity can be
469 plausibly explained by the observed (and unavoidable) experimental loss of key C15 compounds
470 (notably germacrene B and thus, presumably, structurally similar substances) during the chronic
471 daphnid study compared to the smaller constituents during chronic testing, likely due to volatility or
472 sorptive losses where such losses were not observed in the algae or short term daphnid study. C15
473 constituents were identified in the *in silico* model as major drivers of chronic (but less so in acute)
474 toxicity. This finding underscores the importance of ensuring test material integrity over time in chronic
475 studies and supports the role of *in silico* methods in identifying specific chemical and mechanistic
476 drivers of observed effects.

477 The comparative underperformance of the standard CLP additivity method highlights a broader
478 limitation in its applicability to UVCBs and complex mixtures. The CLP method was found to be
479 excessively conservative in all cases except for the daphnid NVFs, overpredicting toxicity by factors of
480 5 to 80 depending on the test. This was due to assumptions of total bioavailability and toxic
481 contribution from all components. Use of a modified hybrid method combining both CLP (additivity)
482 with WAF principles also failed to provide consistent or conservative predictions suggesting that the
483 exclusion of the non-bioavailable fraction throughout may be a significant contributor to accurate
484 estimation of the toxicity.

485 This study employed a two-tiered chemical analysis strategy. First, a detailed characterization was
486 conducted on the two fractions of the substance expected to contain the bioavailable and
487 toxicologically relevant constituents. The values from this in-depth analysis were used as a basis for
488 determining the nominal loading rates used in the *in silico* predictions. Second, a more targeted
489 analytical approach was used, employing a limited set of marker compounds (sentinels) to monitor the
490 behaviour of representative structures throughout the course of the ecotoxicity studies. It was not
491 possible to analyse more than a handful of constituents in this step and therefore *in silico* analysis
492 based on measured values was not appropriate. Furthermore, the WAF calculations have been
493 developed for use with the constituent concentrations from fully characterised multi-constituent
494 substances. Only the comprehensive compositional data previously provided (of which an overview
495 was published by Canton *et al.*) therefore proved adequate for generating reliable toxicity estimates.
496 Nonetheless, the targeted analytical data collected during the toxicity tests provided valuable insight
497 into key factors such as chemical stability, particularly under test conditions. For example, the observed
498 loss of germacrene B between fresh and aged test solutions during the chronic daphnid study provided
499 a plausible explanation for discrepancies between predicted and observed EC₁₀ values in the daphnid
500 chronic study. Germacrene B and other C15 components were lesser contributors to short-term toxicity
501 due to their lower bioavailability and thus their lower potential to reach equilibrium with the test
502 organisms over the course of the study. and therefore have a lower contribution to toxicity (figure 3).

503

504 When comparing experimental outcomes across different species, exposure durations, and
505 fractionation strategies, the most accurate predictions were achieved using the standard WAF
506 calculation method despite the presence of three characterised mechanisms of toxic action amongst

507 the constituents (MechoA 1.1 in the volatile fraction and MechoA 3.1 and 5.2 in the non-volatile
508 fraction). This was an unexpected result, suggesting that non-polar narcotic mechanisms (MechoA 1.1),
509 at least when they are abundantly present, overturn the other mechanisms of action and dominate the
510 overall toxic mechanism of *Galbanum* resinoid. Moreover, approaches involving full or partial additivity
511 (such as those prescribed by CLP, or hybrid models that apply WAF calculations only to narcotic
512 constituents), generally performed poorly. One possible explanation is that the presence of
513 hydrophobic narcotic substances may facilitate removal of non-narcotic substances in the WAF, thereby
514 reducing their bioavailability. This would explain why the CLP calculations were generally far too low.
515 Alternatively, the toxicity may simply be driven predominantly by the narcotic fraction, with non-
516 narcotic substances exerting relatively minor toxic impact in this case.

517 This finding has significant implications for both experimental design and regulatory assessment. It
518 suggests that exhaustive chemical characterization of all fractions may not be necessary to obtain a
519 reliable mechanistic and quantitative ecotoxicological profile for complex substances. Rather, detailed
520 analysis of the volatile fraction, along with a basic understanding of the carbon chain lengths and
521 structural classes present in the non-volatile fraction, may be sufficient to enable effective *in silico*
522 modelling. Experimentally, testing the relative toxicity of individual fractions also aids in interpreting
523 WS toxicity. If the so-called "inert" fraction is found to be devoid of bioavailable toxicants, further
524 analytical investigation of that fraction may be superfluous.

525 Another important observation relates to the unexpectedly high acute-to-chronic toxicity ratio for the
526 WS (observed as 30 experimentally; predicted as 177 *in silico*), which significantly exceeds typical
527 expectations of a factor of 10 for MechoA 1.1 mono-constituent substances. This discrepancy was
528 mechanistically explained by the differential contribution of constituents to acute versus chronic
529 toxicity. Smaller, more soluble (= less toxic) compounds (e.g., β -pinene) dominated acute effects,
530 whereas larger, more hydrophobic (= more toxic) molecules in the WAF (e.g., germacrene B) became
531 toxicologically relevant only under prolonged exposure. This, despite the fact that they are both
532 MechoA 1.1, was likely due to the increased bioaccumulation potential, lower solubility and therefore
533 bioavailability and thus slower partitioning kinetics of the larger molecules. The losses of Germacrene
534 B in the chronic study may help to explain discrepancies between the experimental and predicted acute
535 to chronic ratios. This phenomenon highlights the critical role of hydrophobicity in modulating
536 bioavailability and toxic action over time, even within a single MechoA class.

537

538 **5 Conclusion**

539 This study tested a novel approach using an NCS2 substance (*Galbanum* resinoid) where only a small
540 fraction had been previously characterized. The substance was fractionated based on physicochemical
541 properties into three components: volatile, non-volatile bioavailable, and "inert" fractions. Our
542 hypothesis proposed that only bioavailable fractions (volatile and non-volatile) contribute to acute and
543 chronic aquatic toxicity, allowing *in silico* modeling to be performed exclusively on these known
544 constituents while excluding the non-bioavailable fraction. The *in silico* Method 1, considering that all
545 the constituents belongs to MechoA 1.1, provided the closest match to the *in vivo* data while the
546 additivity approach recommended by CLP appeared as over-conservative in most cases.

547 Acute-to-chronic ratios for the daphnid studies were substantially higher than those typically observed
548 for mono-constituent substances. Our analysis revealed different substances driving toxicity in acute
549 versus chronic tests. With smaller, more soluble molecules (terpenes) mainly responsible for toxicity
550 during short-term studies and larger molecules (e.g., C15) in longer-term studies, resulting in relatively
551 higher toxicity. This phenomenon produces substantially larger acute-to-chronic ratios than would be
552 observed with mono-constituent substances, a prediction that was experimentally confirmed. These
553 findings demonstrate that for complex substances like the NCS2 tested, chronic toxicity cannot be
554 reliably predicted from acute experimental data alone. Nor can it be readily calculated by the simplistic
555 GHS/CLP method and this highlights the important role of including higher level competent predictive
556 methodologies in testing regimes for complex substances. Finally, this study highlights that *in silico*

557 approaches combined with in depth analytics of the test material and carefully chosen experimental
558 testing, constitute a winning combination for such complex substances. The analytics provide a useful
559 characterisation of the NCS2 that can be exploited in the *in silico* tests. The results from the
560 experiments substantially validated the *in silico* predictions which accurately predicted the toxicity of
561 the fractions and the WS (even if the prediction was slightly conservative for chronic daphnid toxicity).
562 Furthermore, the *in silico* method provide mechanistic understanding of the WAF test results and
563 attributed the observed toxicity to specific constituents. However, the CLP/GHS method overpredicted
564 toxicity in all cases and failed to explain observed toxicity. This work represents the first of its kind in
565 this field and a second phase is currently ongoing to validate the approach and the results obtained
566 with *Galbanum* resinoid using another NCS2.

567

568

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570

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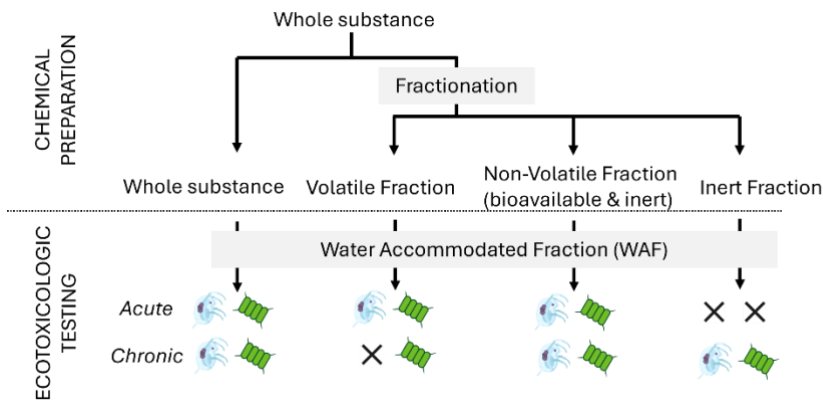
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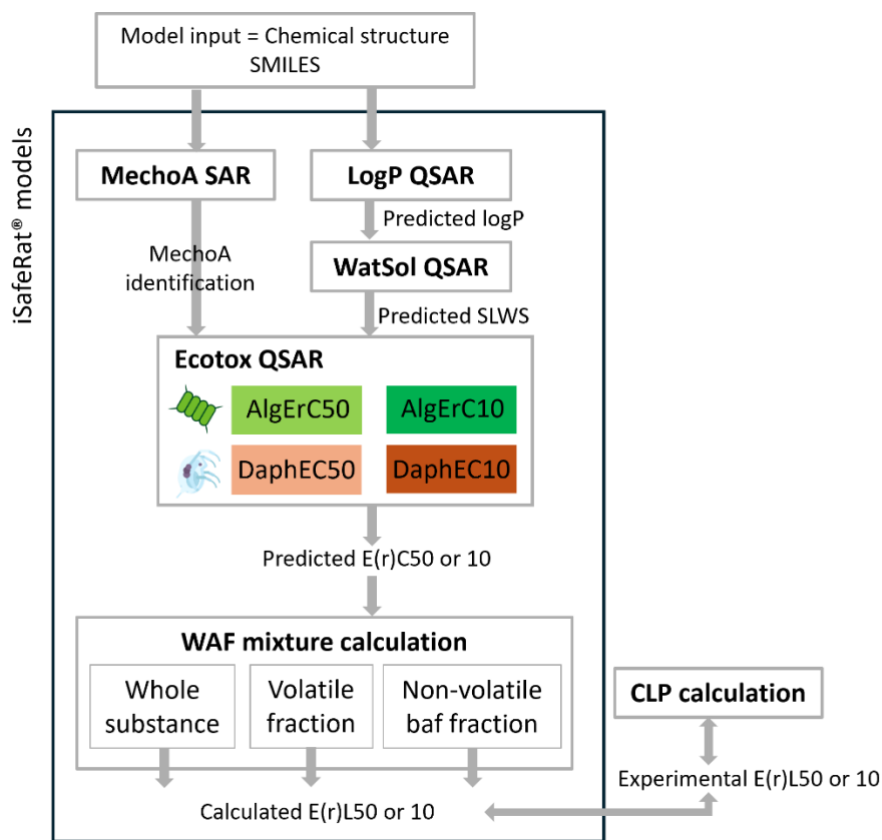
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655 **FIGURES**



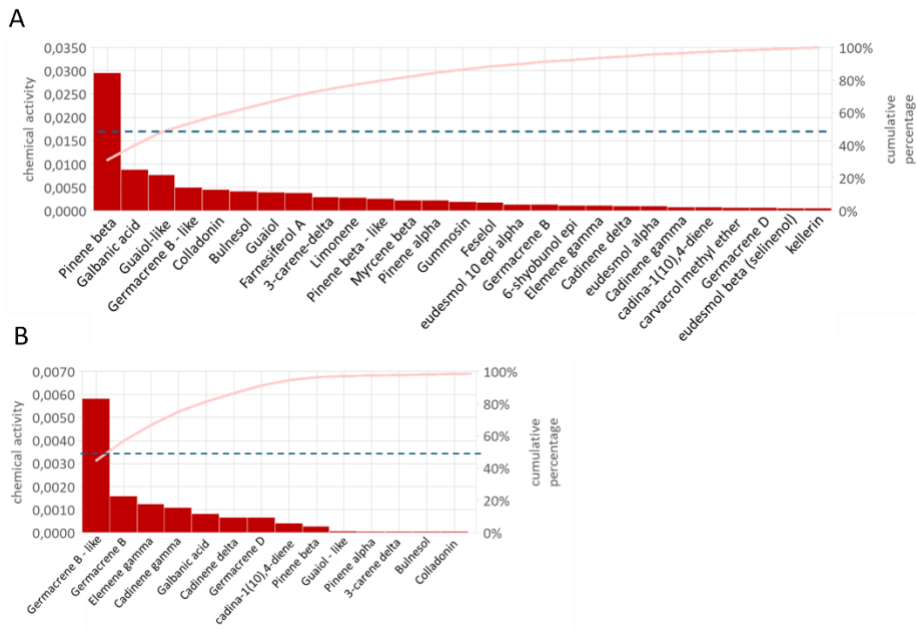
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Figure 1: Experimental workflow for chemical samples preparation and ecotoxicological testing. A cross means that no test was performed.



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Figure 2. Workflow for ecotoxicity value prediction (constituent level) and loading rate calculation (VF, NVF-baf, WS) used for algal and daphnids acute and chronic toxicity assessment.



666
667 Figure 3. Pareto graphs of the WS acute (A) and chronic (B) toxicity to daphnids based on method 1.
668

669 **TABLES**

670 Table 1. Specific constituents monitored throughout the ecotoxicological studies for the VF, NVF-baf
671 and WS samples.

Constituent	CAS number	Acute ecotoxicity testing		Chronic ecotoxicity testing	
		Algae (OECD 201)	Daphnids (OECD 202)	Algae (OECD 201)	Daphnids (OECD 211)
β -pinene	127-91-3	VF, WS	VF, WS	VF, WS	WS
Guaiol	489-86-1	VF, WS	VF, WS	VF, WS	
Bulnesol	22451-73-6	VF, WS	VF, WS	VF, WS	
Germacrene B	15423-57-1				WS*
Galbanic acid	3566-55-0	NVF-baf, WS	NVF-baf, WS	NVF-baf, WS	NVF-baf, WS
Farnesiferol A	511-33-1	NVF-baf, WS	NVF-baf, WS	NVF-baf, WS	NVF-baf

672 *Germacrene B was measured additionally in this study.

673
674 Table 2: hypotheses tested for VF (Volatile fraction), NVF-baf (Non-volatile, potentially bioavailable
675 fraction) and WS.

Tested entity	Features	Method 1	Method 2
		All the constituents act as non-polar narcotics	Each constituent acts according to its specific MechoA
VF fraction	Predicted MechoA	Non-polar narcosis (1.1)	
	Ecotoxicity calculation approach	WAF	
NVF-baf fraction	Predicted MechoA	Non-polar narcosis (1.1)	Acidification of cells (5.2), Combination of narcosis of the parent molecule and enzymatic hydrolysis (2.1)

	Ecotoxicity calculation approach	WAF	Additivity (CLP)
Whole substance	Predicted MechoA	Non-polar narcosis (1.1)	Non-polar narcosis (1.1), acidification of cells (5.2), Combination of narcosis of the parent molecule and enzymatic hydrolysis (2.1)
	Ecotoxicity calculation approach	WAF	Additivity between VF& NVF-baf

676

677 Table 3: Summary of the experimental E(r)L50 and E(r)L10 ecotoxicity results for daphnids and algae
678 for each fraction and the WS versus predictions using 2 methods: Method 1 WAF based calculation
679 (from iSafeRat®) and Method 2 a modified additivity method (mixture of WAF and additivity
680 approaches) and the CLP (standard additivity) approach.

681

Endpoint	Fraction	<i>In silico</i> Method1	<i>In silico</i> Method2	CLP	Experimental
Daphnids EL50 (mg/L)	VF	7.73		1.04	6.9
	NVF	46.3	93.2	14.8	>50
	WS	23.5	60	3.21	23.5
Daphnids EL10 (mg/L)	VF	0.04		0.01	-
	NVF	0.72	18	2.07	2.01
	WS	0.14	8.87	0.02	0.8
Algal ErL50 (mg/L)	VF	8		0.88	4.8
	NVF	52.7	131	8.41	>50
	WS	24.4	198	2.62	34.5
Algal ErL10 (mg/L)	VF	5.03		0.08	1.5
	NVF	29.7	NC*	NC*	50
	WS	14.8	NC*	NC*	13.4

682

683 *Not calculable as no QSAR model for galbanic acid available for algae EL10 using MechoA 5.2

684 **No data for algae EC10 located for the critical constituents

685