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## Deeper insight into *Gambierdiscus polynesiensis* toxin production relies on specific optimization of High-Performance Liquid Chromatography High Resolution Mass Spectrometry

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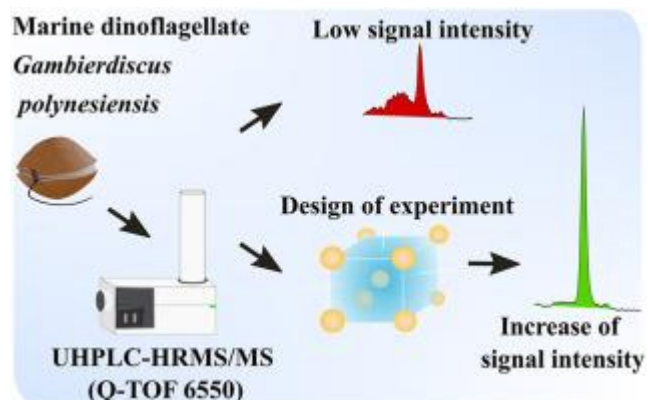
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### Abstract :

Ciguatera food poisoning affects consumer health and fisheries' economies worldwide in tropical zones, and specifically in the Pacific area. The wide variety of ciguatoxins bio-accumulated in fish or shellfish responsible for this neurological illness are produced by marine dinoflagellates of the genus *Gambierdiscus* and bio-transformed through the food web. The evaluation of the contents of ciguatoxins in strains of *Gambierdiscus* relies on the availability of standards and on the development of sensitive and specific tools to detect them. There is a need for sensitive methods for the analysis of Pacific ciguatoxins with high resolution mass spectrometry to ensure unequivocal identification of all congeners. We have applied a fractional factorial design of experiment 2<sup>8</sup>-3 for the screening of the significance of eight parameters potentially influencing ionization and ion transmission and their interactions to evaluate the behavior of sodium adducts, protonated molecules and first water losses of CTX4A/B, CTX3B/C, 2-OH-CTX3C and 44-methylgambierone on a Q-TOF equipment. The four parameters that allowed to significantly increase the peak areas of ciguatoxins and gambierones (up to a factor ten) were the capillary voltage, the sheath gas temperature, the ion funnel low pressure voltage and the ion funnel exit voltage. The optimized method was applied to revisit the toxin profile of *G. polynesiensis* (strain TB92) with a confirmation of the presence of M-seco-CTX4A only putatively reported so far and the detection of an isomer of CTX4A. The improvement in toxin detection also allowed to obtain informative high resolution targeted MS/MS spectra revealing high similarity in fragmentation patterns between putative isomer (4) of CTX3C, 2-OH-CTX3C and CTX3B on one side and between CTX4A, M-seco-CTX4A and the putative isomer on the other side suggesting a relation of constitutional isomerism between them for both isomers.

## Graphical abstract



## Highlights:

► A chemometric method to weigh the contribution of Q-TOF parameters is proposed. ► Ionization and transmission parameter increased sensitivity for ciguatoxin analysis. ► M-seco-P-CTX4A is a major ciguatoxin produced by *G. polynesiensis*. ► Putatively reported "isomer (4) of P-CTX3C" is a constitutional isomer.

**Keywords :** Mass spectrometry, *Gambierdiscus polynesiensis*, ciguatoxins, gambierones, Fractional Factorial Design

## 52 1. Introduction

53 Ciguatera is the largest non-bacterial foodborne illness affecting more than 50 000 people  
54 annually with gastric, cardiac and neurological symptoms [1] . Initially discovered in tropical  
55 and sub-tropical areas, occurrence of the disease appears to increase due to climate and other  
56 environmental changes as well as the expansion of international tourism and trade, see  
57 Chinain et al, [2,3] for a review and references therein. This neurological illness affects  
58 humans within a few hours and can last up to several months after the consumption of  
59 shellfish or fish contaminated *via* feeding either directly on marine ciguatoxin (CTX)-  
60 producing dinoflagellates from the genera *Gambierdiscus* and *Fukuyoa*, or indirectly *via*  
61 accumulation in the food chain of carnivore organisms [1,4].

62 More than 12 different species of *Gambierdiscus* and *Fukuyoa* have been reported with  
63 high variation of ciguatoxin-like toxicity from fg to pg equivalent CTX<sub>3C</sub> cell<sup>-1</sup> (Longo et al.,  
64 and references therein) [5-9]. However the result obtained by bioassays (e.g. mouse-bioassay,  
65 cell-based bioassay, receptor-binding assay) largely depended on the sample preparation, the  
66 complexity of matrix effect and the detection method. To date, only *Gambierdiscus*  
67 *polynesiensis* reported by Chinain et al., [10] has been unequivocally confirmed as a  
68 ciguatoxin producer by analytical tools, i.e. mass spectrometry (MS) and nuclear magnetic  
69 resonance spectroscopy (NMR spectroscopy)[10-13].

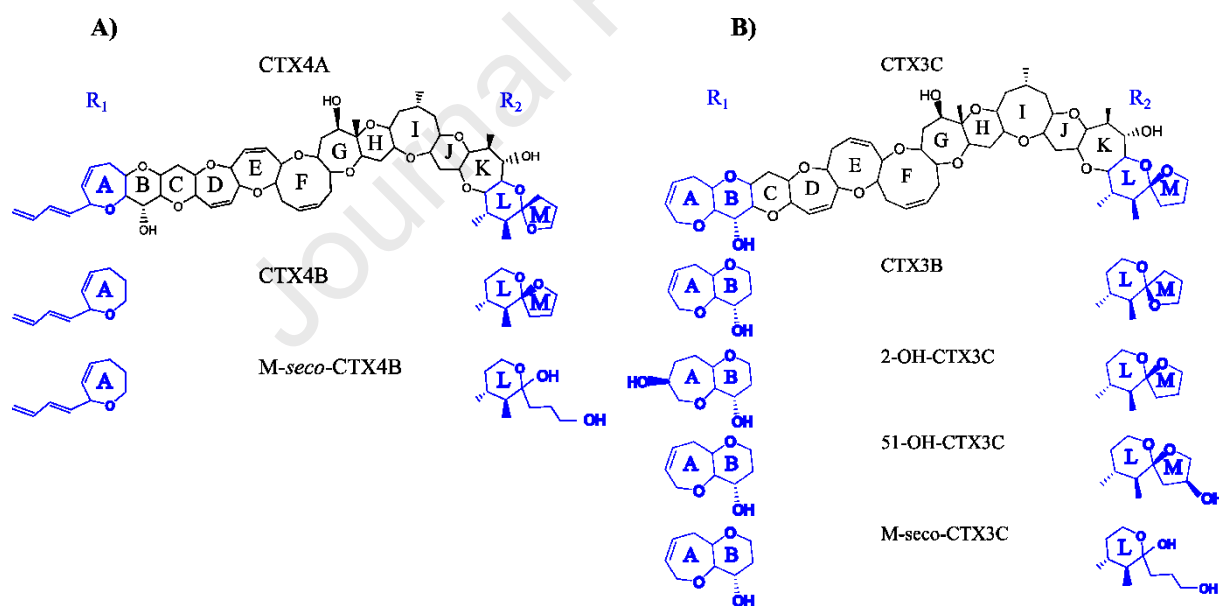
70 Other species such as *G. australes*, *G. belizeanus* and *G. excentricus* were demonstrated as  
71 producers of maitotoxins [6,14-17], *G. toxicus* produced gambieric acids [18,19],  
72 gambieroxide [20] and gambierol [21], and finally gambierones [22-24] were reported in 16

73 species of *Gambierdiscus* and recently also in 2 species of *Fukuyoa* and 2 species of *Coolia*  
 74 [8,25-27].

75 Ciguatoxins are lipophilic polyethers (octanol-water partition coefficients (XlogP3-AA)  
 76 ranging from 2.5 to 4.7) [28] with a mass ranging from 1000 to 1200 Da. Four types of  
 77 structure have been described so far according to their carbon skeleton and origin, i.e. P-CTX  
 78 I (i.e. CTX4A type) and II (i.e. CTX3C type) (Figure 1) both found in the Pacific Ocean,  
 79 Caribbean-CTX (C-CTX) in the Caribbean area and Indian-CTX (I-CTX) in the Indian  
 80 Ocean. [29,30].

81 The complex toxin profile of *G. polynesiensis* has so far mostly been evaluated with low  
 82 resolution mass spectrometry and contains more than 12 analogs of ciguatoxins (Figure 1),  
 83 [8,11,31-35].

84



85

86 **Figure 1.** Reported structure of Pacific Ciguatoxins produced by *G. polynesiensis* according to the type of  
 87 structure: A) CTX4A group and B) CTX3C group [36] (no structure is known for the four isomers of CTX3C)

88

89 In addition to ciguatoxins, *G. polynesiensis* was also reported as producer of gambierone  
 90 and its analog 44-methyl gambierone [8,37-39]. The *G. polynesiensis* strain TB92 was used as

91 a “reference material” in many studies (e.g. [32,34,37,40]) focusing on its toxin profile and  
92 the modifications that may occur throughout the food web.

93 The low number of strong acidic or basic functional groups in algal ciguatoxins appears to  
94 result in low ionization yield in electrospray type source, thus raising the detection limits  
95 compared to many other algal-derived polyethers [41]. However, the use of MRM mode  
96 operated in tandem mass spectrometers allows to focus on a limited number of compounds  
97 and thus, achieves relatively low limits of detection for ciguatoxins in fish or algal extracts (1-  
98 2 ng mL<sup>-1</sup>) [34,42]. A major drawback of the MRM mode is that it requires knowledge on the  
99 mass spectral behavior of compounds to define the transitions monitored (precursor/product  
100 ion pairs) and their ion ratio. So far, only two standards of CTX are commercially available  
101 and thus, quantitative estimation of concentrations were typically carried out by assuming  
102 equal response factors with CTX1B or CTX3C and results were commonly expressed as  
103 CTX1B or CTX3C equivalents [41].

104 An alternative to confirm the presence of known compounds or to detect related,  
105 unknown compounds in complex extracts is high-resolution mass spectrometry (HRMS)  
106 [34,41]. With hybrid high resolution systems such as quadrupole-time-of-flight (Q-TOF)  
107 technology, both targeted and untargeted analyses can be performed allowing for  
108 retrospective data analysis [43,44]. Compared to MRM mode performed in tandem mass  
109 spectrometry (i.e. triple quadrupole or Q-Trap), the full scan acquisition carried out on a Q-  
110 TOF mass spectrometer suffers from a 10 to 12 times lower sensitivity [34]. To overcome the  
111 sensitivity issue, a rigorous optimization of the ionization and transmission parameters is  
112 required to detect trace amounts of CTXs. So far, such optimization of ciguatoxin detection  
113 was only performed with low resolution mass spectrometry, using either one parameter at the  
114 time or multiple parameters with an experimental design [34,42,45]. Chemometric  
115 optimization of ion funnel parameters has already been proven to efficiently increase peak

116 area of urine metabolites [46]. In this study, a fractional factorial design of experiment (FFD)  
117 was used on a Q-TOF, equipped with the dual ion funnel technology to evaluate the  
118 significance of 8 electrospray ionization and ion transmission parameters on the  
119 chromatographic peak area obtained for ciguatoxins and gambierones present in methanolic  
120 extracts of *G. polynesiensis* strain TB92. Improving the detection of polyethers while  
121 enhancing the formation of protonated ions are key objectives to obtain intense and  
122 informative MS/MS spectra for ciguatoxins. Optimal values for significant parameters were  
123 implemented and allowed us to describe the toxin profile of *G. polynesiensis* with HRMS and  
124 notably the presence of M-*seco*-CTX4A that was only putatively reported so far. The increase  
125 of peak area of CTX3B/C by a factor five resulting from the optimization enabled the  
126 acquisition of targeted HRMS/MS spectra of CTX3B and isomer (4) of CTX3C confirming  
127 the constitutional isomerism between the two compounds.

## 128 **2. Materials and Methods**

### 129 **2.1. Chemicals**

130 LC-MS grade methanol, formic acid (98% purity) and ammonium formate were  
131 purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Water was deionized and  
132 purified at 18 M $\Omega$  cm thanks to a Milli-Q integral 3 system (Millipore, France).

133 For HRMS, acetonitrile, methanol and high purity water were purchased from Optima  
134 Fisher chemical (Illkirch, France).

135 A mix of ciguatoxin standards (M-*seco*-CTX3C, 2-OH-CTX3C, 51-OH-CTX3C, CTX2,  
136 CTX3B, CTX3C, CTX4A and CTX4B) was provided by Dr. Mireille Chinain, Institut Louis  
137 Malardé (ILM), Tahiti, French Polynesia.

### 138 **2.2. Sample preparation**

139 Dried methanolic extracts corresponding to one million cells of *Gambierdiscus*  
140 *polynesiensis* TB92 (Tubuai, Australes archipelago, French Polynesia) were prepared at  
141 Institut Louis Malardé (ILM), Tahiti, French Polynesia using the same procedure as published  
142 in Chinain et al., [11]. Briefly, fresh cell pellet was extracted twice with 20 mL of methanol  
143 100% and twice with 20 mL of aqueous methanol 50% under sonication for 10 min each.  
144 Supernatants were obtained after centrifugation at 2800g for 10 min, pooled and evaporated.

145 Two dried extracts corresponding to  $1.7 \times 10^6$  and  $1 \times 10^6$  cells equivalent/mL were  
146 resuspended each in 1 mL of methanol, filtered through 0.25  $\mu\text{m}$  GF/F filters (Whatman,  
147 France) and were used for the design of experiment and the toxin profile assessment,  
148 respectively.

### 149 **2.3. Instrumental conditions for the design of experiment**

150 Analyses were carried out using a system composed of ultra-high performance liquid  
151 chromatography UHPLC (1290 Infinity II, Agilent Technologies, CA, USA) coupled to a  
152 6550 ion funnel Q-TOF (Agilent Technologies, CA, USA) equipped with a Dual Jet Stream<sup>®</sup>  
153 ESI source. The nebulizer was set at 35 Psi in agreement with Agilent recommendation for a  
154 flow rate of  $0.4 \text{ mL min}^{-1}$  and the sheath gas flow (SGF) varied according to the sheath gas  
155 temperature (SGT) (5, 6 and  $11 \text{ L min}^{-1}$  for 250, 325 and  $400 \text{ }^\circ\text{C}$ , respectively). All other  
156 source and transmission parameters varied as described in the fractional factorial design (see  
157 section 2.5 and Figure 2).

158 The instrument was operated in full scan positive mode, at a scan rate of  $2 \text{ spectra s}^{-1}$  over  
159 a mass-to-charge ratio ( $m/z$ ) from 100 to 1700. Reference mass  $m/z$  922.0099 (hexakis  
160 phosphazene) was injected continuously over the entire run to ensure no deviation in mass  
161 measurement.

162 Instrument control and data treatment were carried out using the MassHunter software  
163 version B.08 (Agilent Technologies, CA, USA) and peaks were integrated using the algorithm



164 Agile2 of the Mass Hunter Qualitative Analysis software. The chromatographic method was  
 165 adapted from Sibat et al., [34]. Briefly, the stationary phase was a Kinetex C18 column (50 x  
 166 2.1 mm, 1.7  $\mu\text{m}$  100  $\text{\AA}$ , Phenomenex, CA, USA) maintained at 40  $^{\circ}\text{C}$ . The mobile phases  
 167 were 100% water (eluent A) and acetonitrile/water (95:5, v/v) (eluent B), both contained 2  
 168 mM ammonium formate and 50 mM formic acid. The flow rate was 0.4  $\text{mL min}^{-1}$  and the  
 169 elution gradient was as follows: 10% to 95% of B from 0 to 12 min, held at 95 % B for 2 min  
 170 and then return to initial condition (10 %B) at 14.1 min and held for 6 min.

171 The chromatographic conditions used in this study allowed the detection of a wide variety of  
 172 compounds including ciguatoxins, maitotoxins and gambierones. To reduce the number of  
 173 experiments, the detection of ciguatoxins and gambierones was achieved simultaneously,  
 174 resulting in the coelution of CTX3B with 3C and CTX4A with 4B.

175 Conditions were randomized in the run sequence and experimental blanks were added  
 176 between two different conditions to avoid any cross-contamination and ensure the stability  
 177 (i.e. temperature) between each condition. Triplicate injections under the same conditions  
 178 were, however, performed successively to reduce the equilibration time.

179 Finally, the average of the peak area of the extracted ion chromatogram (EIC) was  
 180 selected as the response for each toxin and ion species (Table 1).

181 **Table 1.** Toxin, retention time and selected ion species (accurate mono isotopic  $m/z$ ) for the fractional factorial  
 182 design of experiment (RT = retention time)

Toxin	Molecular Formula	RT (min)	$[\text{M}-\text{H}_2\text{O}+\text{H}]^+$ ( $m/z$ )	$[\text{M}+\text{H}]^+$ ( $m/z$ )	$[\text{M}+\text{Na}]^+$ ( $m/z$ )
44-methylgambierone	$\text{C}_{51}\text{H}_{76}\text{O}_{19}\text{S}$	7.5	1021.4825	1039.4931	1061.4750*
2-OH-CTX3C	$\text{C}_{57}\text{H}_{84}\text{O}_{17}$	9.1	1023.5676	1041.5781*	1063.5601
CTX3C and 3B	$\text{C}_{57}\text{H}_{82}\text{O}_{16}$	11.8	1005.5570	1023.5676	1045.5495
CTX4A and 4B	$\text{C}_{60}\text{H}_{84}\text{O}_{16}$	12.4	1043.5726	1061.5832	1083.5652

183 \*Ions excluded from the FFD as they were not detected in the majority of the runs

#### 184 **2.4. Fractional Factorial Design (FFD) of experiment**

185 A fractional factorial design (FFD) ( $2_{IV}^{8-3}$ ) with a resolution of 4 was selected for the  
186 investigation of the direct effects of 8 factors and their interactions (order 2). An FFD can  
187 only evaluate the significance of the effects (i.e. contribution) of factors and the level giving  
188 the best response among the experimental domain considered (i.e. either -1 or +1 level for  
189 each factor).

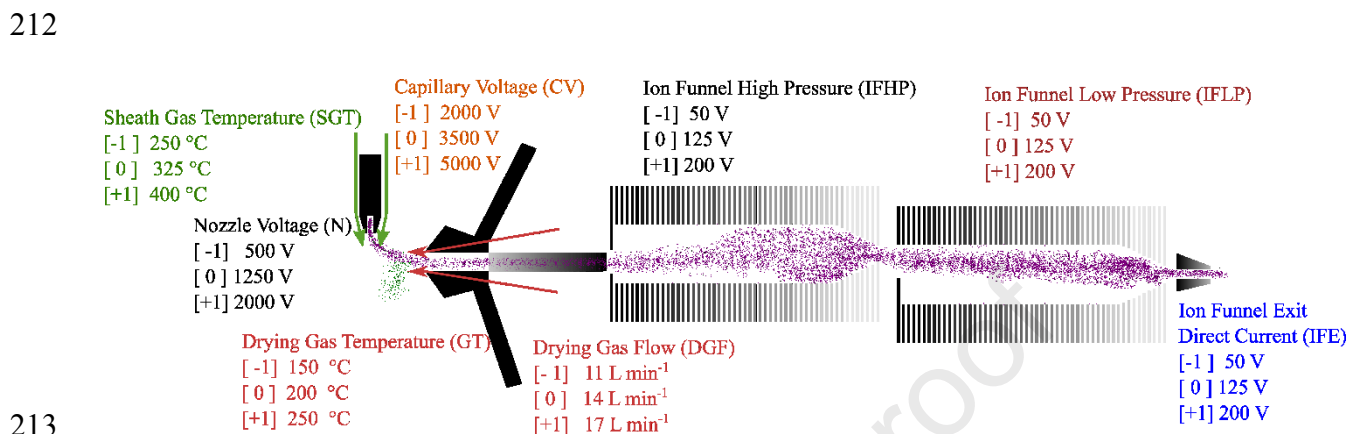
190 The selection of significant factors and their interactions was performed assuming that  
191 interactions between three or more factors were supposed as negligible and the interaction  
192 between non-significant factors were considered as non-significant and thus removed from the  
193 results.

194 Statgraphics Centurion 18 (Version 18.1.02) was used for the experimental design and  
195 the statistical analysis.

196 For the ion funnel Q-TOF (Figure 2), the ionization and transmission parameters are  
197 separated into two categories: flow-dependent (nebulizer pressure (NP), drying gas  
198 temperature (DGT) and flow (DGF), sheath gas flow (SGF)) and compound-dependent  
199 (capillary voltage (CV), nozzle voltage (N), ion funnel voltages (high pressure (IFHP), low  
200 pressure (IFLP) and exit (IFE) direct current), sheath gas temperature (SGT) and fragmentor  
201 voltage (FV)) [47]. The parameters which can be set in the system (the IFHP and IFLP) are  
202 time-varying potentials used to compress the ion beam and improve the transmission. The dual  
203 ion funnel technology first removes the gas and neutral noise with radio frequency applied in  
204 the IFHP, then focuses the ion beam to the center in the IFLP and finally the direct current  
205 (DC) voltage applied in the IFE accelerates the ions (Figure 2) [48].

206 The 8 factors used in the FFD were selected using Agilent recommendations [47] for  
207 compound-dependent parameters. The levels were defined either by the limit of the  
208 instrument for the high level, or by selecting the lowest value allowing to detect the reference

209 mass  $m/z$  922.0099 (hexakis phosphazene). Levels of the 8 factors (low [-1], center [0] and  
 210 high [+1] values) are summarized in Figure 2 and the matrix corresponding to the 32  
 211 experiments is provided in Table S1.



213  
 214 **Figure 2.** Scheme of Agilent Q-TOF 6550 electrospray source and dual ion funnel with the parameters (factors)  
 215 and their levels (low [-1], center [0] and high [+1]) used in the fractional factorial design

216  
 217 LC-HRMS systems can provide accurate masses but suffer from low sensitivity. As the  
 218 only commercial standard of ciguatoxin (CTX3C, Wako, Tokyo, Japan) is limited by cost and  
 219 availability, it was not possible to optimize MS parameters using this standard. Alternatively,  
 220 and to account for matrix effect and the possible divergence of responses between ciguatoxin  
 221 analogs, we used an extract of *G. polynesiensis* (strain TB92 estimated as  $5.8 \pm 0.85$  pg  
 222 CTX3C equivalent cell<sup>-1</sup> [32]) corresponding to 1.7 million cells (*i.e.* 10  $\mu\text{g mL}^{-1}$  of  
 223 ciguatoxins (sum of CTX3B/C and 4A/B)), making this sample highly suitable for this HRMS  
 224 optimization.

225 The peak area corresponding to the ion  $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ ,  $[\text{M}+\text{H}]^+$  and  $[\text{M}+\text{Na}]^+$  of each  
 226 compound was log(10) normalized before statistical analyses. The protonated molecule  
 227  $[\text{M}+\text{H}]^+$  of 2-OH-CTX3C and the sodium adduct  $[\text{M}+\text{Na}]^+$  of 44-methylgambierone were  
 228 excluded from the FFD as they were detected in only few conditions. When no peak was  
 229 detected, the value was set as half of the average noise (*i.e.* 100).

230 For all responses, an ANOVA was performed to test the statistical significance of each  
231 effect at a level of confidence of 95% comparing the least square against the estimation of the  
232 experimental error. A Durbin-Watson test assessing the correlation between the residuals  
233 values and the order of experiment was performed to ensure at a level of confidence of 95%  
234 that the residuals were not correlated to the order of injections.

## 235 **2.5. Instrumental conditions for the toxin profile of *G. polynesiensis***

236 For the revisit of the toxin profile of *G. polynesiensis*, two chromatographic methods  
237 were used. For gambierones, the chromatographic method was the same as used for the FFD  
238 as it allowed to separate gambierone from 44-methylgambierone. For ciguatoxins, the  
239 chromatographic method used in the FFD was not able to separate related compounds (i.e.  
240 CTX3B from CTX3C), hence, and to facilitate comparison with the literature, we used a  
241 chromatographic method already published by Sibat et al., [34], the “LC method n°2”.  
242 Shortly, a linear gradient using water 100% as eluent A and methanol 100% as eluent B both  
243 containing 2 mM of ammonium formate and 50 mM of formic acid was used on a Zorbax  
244 Eclipse Plus C18 column (50 × 2.1 mm, 1.8 μm, 95 Å, Agilent Technologies, Santa Clara,  
245 CA, USA). The elution gradient started at 78% of B then rose up to 88% of B in 10 min and  
246 held for 4 min before the reconditioning for 5 min. The flow rate was 0.4 mL min<sup>-1</sup>, the  
247 column was maintained at 40 °C and the injection volume was 5 μL.

248 Peak area and signal-to-noise ratio (S/N) were obtained using MassHunter algorithm for  
249 Extracted Ion Chromatogram (EIC) of the ions presented in Table 2 with a delta ppm  
250 tolerance of 10.

251 For structural comparison between CTX3B, the isomer (4) of CTX3C and 2-OH-CTX3C  
252 on one side and CTX4A, M-*seco*-CTX4A and the isomer of CTX4A on the other side, mass  
253 spectra were acquired by targeted MS/MS over a *m/z* range of 50-1300 at 10 spectra s<sup>-1</sup> for  
254 MS and 3 spectra s<sup>-1</sup> for MS/MS levels. Three collision energies were used (10, 20 and 40

255 eV). Instrument control and data treatment were carried out using the MassHunter software  
 256 version B.08 and B.07 (Agilent Technologies, CA, USA).

257

258 **Table 2.** Toxin, retention time and selected ion species (accurate mono isotopic  $m/z$ ) for toxin profile

	<b>Toxin</b>	<b>Retention Time (min)</b>	<b>[M-H<sub>2</sub>O+H]<sup>+</sup> (<math>m/z</math>)</b>	<b>[M+H]<sup>+</sup> (<math>m/z</math>)</b>	<b>[M+NH<sub>4</sub>]<sup>+</sup> (<math>m/z</math>)</b>
Method	gambierone	7.4	1007.4669	1025.4774	1042.5040
gambierones	44-methylgambierone	7.6	1021.4825	1039.4931	1056.5196
Method	2-OH-CTX3C	3.9	1023.5676	1041.5781	1058.6047
CTXs	M- <i>seco</i> -CTX4A	5.2	1061.5832	1079.5938	1096.6203
	Isomer (4) of CTX3C	8.7	1005.5570	1023.5676	1040.5941
	CTX3B	9.6	1005.5570	1023.5676	1040.5941
	CTX3C	9.8	1005.5570	1023.5676	1040.5941
	Isomer of CTX4A	9.8	1043.5726	1061.5832	1078.6098
	CTX4A	10.7	1043.5726	1061.5832	1078.6098
	CTX4B	11.2	1043.5726	1061.5832	1078.6098

259

## 260 3. Results and discussion

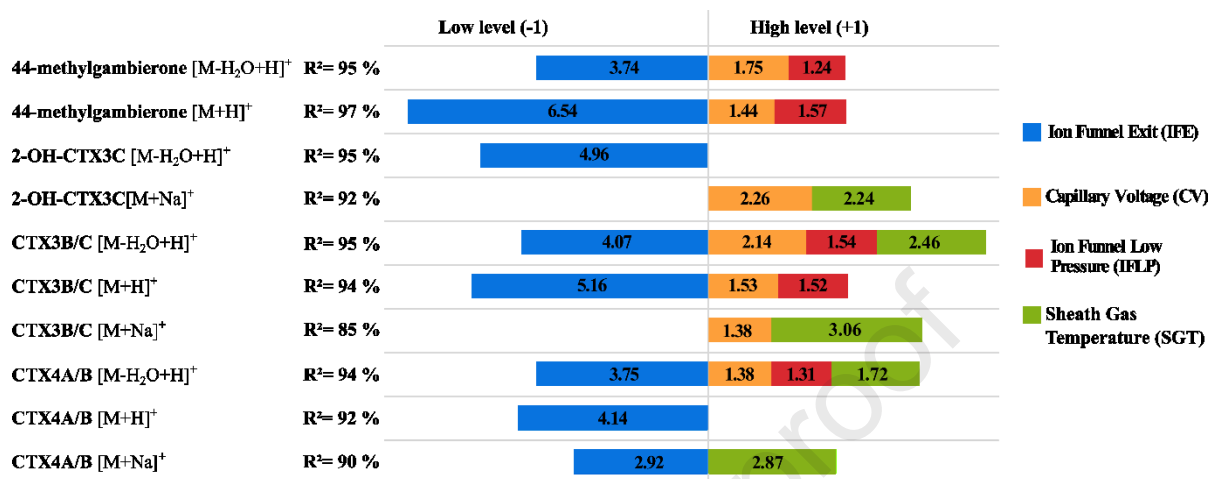
### 261 3.1. Significance of the factors

262 The FFD allowed to assess the contribution (or weight) of the eight factors on the peak  
 263 area of the ten selected response providing information about in-source fragmentation, adduct  
 264 formation as well as protonated molecule ionization and transmission.

265 The peak areas of all ion species (Table S2) showed high variations and ranged from 100  
 266 (undetected) to  $4 \times 10^6$  counts depending on the conditions. The significant factors at a  
 267 confidence level of 95 % ( $p$ -values from 0.05 to 0.0001) and their contribution on the

268 responses are presented in Figure 3. The variability explained by the model was excellent ( $R^2$   
 269 ranged from 85% to 97%).

270



271

272 **Figure 3.** Combined Pareto chart with the contribution (bar size) and levels (-1 or +1) of all significant (at a confidence level  
 273 of 95 %) factors on the responses, and percentage of variability explained by the model ( $R^2$ )

274

275 The most influent parameters (*i.e.* with the highest contribution) were the CV, the SGT,  
 276 the IFLP and the IFE (Figure 3).

277 Therefore, among the ionization parameters, the increase of the CV significantly  
 278 enhanced the areas of CTXs and 44-methylgambierone. The SGT when set at high level (*i.e.*  
 279 400°C) appeared to increase the first water loss and sodium adduct formation of CTXs while  
 280 all other parameters (N, DGT and DGF) were not significant ( $p$ -value > 0.05).

281 The CV increases the number of charges available for the ionization and the SGT  
 282 improves the desolvation of compound and removes noise. The results are in agreement with  
 283 Yogi et al., [45] and Mead et al., [49] suggesting that the electrospray ionization of such  
 284 polyethers with very low number of ionizable groups (*i.e.* acidic or basic functional groups)  
 285 can be challenging and requires stronger conditions. Our results also demonstrated that none  
 286 of the ionization parameters could selectively increase the protonated molecular ion without

287 increasing the first water loss or the sodium adduct and thus, it is recommended to set a high  
288 value of CV and SGT to increase the ionization yield.

289 The dual ion funnel technology was implemented in the Q-TOF 6550 to overcome the  
290 difficulties of controlling the ion motion in presence of strong gas dynamics and thus to  
291 increase the ion transmission [47,50]. Among ion transmission parameters, the IFLP and IFE  
292 voltages were significant at a confidence level of 95% whereas the IFHP voltage was not  
293 significant. Consistent results were also reported by Peris-Diaz et al., [46] on the same  
294 analytical system. A lower value of voltage applied in the IFE resulted in an increase of the  
295 transmission of the protonated molecule and first water loss ions of all polyethers (Figure 3).  
296 This observation suggests that ions may be fragmented or poorly transmitted in the ion funnel  
297 if they are exceedingly accelerated by the IFE voltage [48]. Noteworthy, the first water loss  
298 ion did not increase when IFE parameter was set at mid or high level (*i.e.* 125 or 200 V,  
299 respectively) revealing that this water loss is more likely to appear in the electrospray source  
300 rather than into the ion funnel.

301 However, when we observed a significant effect of the IFLP factor (mostly for [M-  
302 H<sub>2</sub>O+H]<sup>+</sup> and [M+H]<sup>+</sup> ions) the best response was obtained with the higher level (*i.e.* 200 V)  
303 suggesting that these ions still needed high voltage to be efficiently focused.

304 The sodium adduct formation was increased by either a high value of CV and SGT or a  
305 low value of IFE for all responses, as those parameters increased also the protonated molecule  
306 and the first water loss, none of the conditions studied could reduce significantly the adduct  
307 formation.

308 Hence, we recommend to use the IFLP at high level (*i.e.* 200 V) and the IFE at low level  
309 (*i.e.* 50V) to enhance the overall sensitivity for the Q-TOF detection of polyethers from  
310 *Gambierdiscus*.

311 The FFD provided the significance and the contribution of the ionization and  
312 transmission parameters on the responses with a limited number of experiments compared to a  
313 full factorial design. Here, with only 133 injections corresponding to 44 h of chromatographic  
314 run time (one blank and a triplicate of injection for each condition) instead of 1024 injections  
315 (*i.e.* 14 d and 5 h of chromatographic run time) for a full factorial design, four parameters  
316 were clearly demonstrated as significant to increase the peak area of CTXs and 44-  
317 methylgambierone. This approach could have been strengthened using a second design of  
318 experiment focusing on the four key parameters with more than 2 levels. However, these  
319 results already highlighted the behavior of sodium adducts, protonated molecules and first  
320 water loss ions according to the different factors tested. It should be noted that the significant  
321 parameters were all compound-dependent and thus, should improve the ionization and  
322 transmission of ions independently of the mobile phase used.

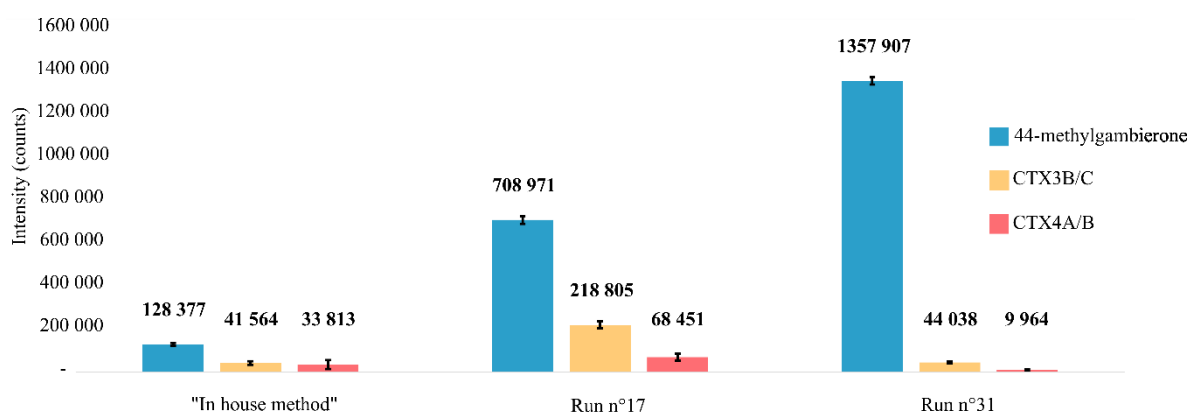
### 323 **3.2. Signal enhancement**

324 Q-TOF type systems can perform targeted MS/MS that relies on the fragmentation of  
325 selected precursor ions in the collision chamber to obtain the fragments (*i.e.* product ions) and  
326 gain insight into structural information. As demonstrated for other types of polyethers [51],  
327 the presence of sodium adducts can dramatically reduce the degree of fragmentation in the  
328 CID chamber compared to the protonated molecule. It is therefore critical to use parameters  
329 that favor the protonated molecules without increasing the in-source fragmentation or sodium  
330 adduct formation to achieve a higher sensitivity.

331 Accordingly, the parameters of the run n°17 and n°31 used in the FFD (Table S2 and S3)  
332 were chosen for subsequent analyses of CTXs and gambierones respectively, and compared to  
333 the previous “in house” method used in the laboratory (derived from “LC method 3” in Sibat  
334 et al., [34]). The peak area of each protonated molecule is presented in Figure 4 and details of  
335 the ionization and transmission parameters for the three methods can be found in Table S3.



336



337

338 **Figure 4.** Peak area of Extract Ion Chromatograms (tolerance  $\pm 10$  ppm) of protonated molecules of 44-methylgambierone,  
 339 CTX3B/C and CTX4A/B from full scans acquired with ESI+ LC-HRMS using the parameters of the "In house method", run  
 340 n°17 and run n°31 (Table S3).

341

342 When using optimized parameters, the average peak area (triplicate of injection) of the  
 343  $[M+H]^+$  ion of 44-methylgambierone was increased by a factor 10 compared to the method  
 344 previously used in the laboratory (Figure 4). Similarly, for ciguatoxins, peak areas were  
 345 increased by a factor 5 and 2 for CTX3B/C and CTX4A/B, respectively.

346 As reflected by Figure 4 and Figure S1, ionization and transmission parameters can  
 347 significantly change the relative toxin profile observed here on a sample of *G. polynesiensis*  
 348 strain TB92, highlighting the necessity to optimize HRMS parameters to lower the limits of  
 349 detection of gambierones and ciguatoxins on a Q-TOF system. When focusing only on  
 350 gambierones, the parameters used for the run n°31 are thus recommended. However, as the  
 351 peak area for 44-methylgambierone was always more than 3 times higher than the one of  
 352 CTX3B/C and CTX4A/B, for the subsequent analyses the parameters increasing CTX areas  
 353 were considered as more important than those increasing only the area of gambierones.

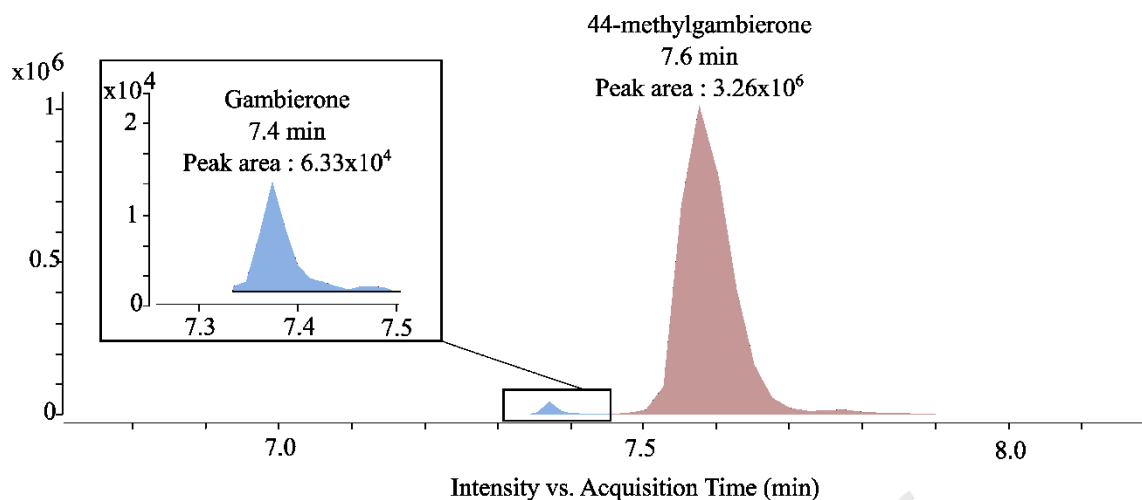
### 354 3.3. Toxin profile of *G. polynesiensis* TB92

355 So far, the toxin profile of TB92 had only been explored with low resolution mass  
356 spectrometry, using the MRM mode of acquisition with two or three transitions per toxin  
357 [11,34,38,40,52]. The identity was confirmed by the comparison of transitions, ion ratio and  
358 retention time with purified CTX standards. Further identifications were achieved based on  
359 literature information using identical chromatographic methods as reported and comparing the  
360 retention order, in addition to retention time and tandem-MS spectrum. A major issue with  
361 this approach was the misidentification of unknown ciguatoxins, when the retention time was  
362 unknown and the transitions were unspecific. For example, the signal observed for the  
363 transition  $[M+H]^+/[M-H_2O+H]^+$  of CTX3B/C could be attributed to either a new CTX3B/C  
364 analog or to the transition  $[M-H_2O+H]^+/[M-2H_2O+H]^+$  of a hydroxylated analog that  
365 underwent an in-source water loss. In this study, the use of HRMS in Full Scan mode  
366 followed by a targeted MS/MS on unknown compound was performed in order to provide a  
367 complete picture of the different form of ions corresponding to gambierones and CTXs in *G.*  
368 *polynesiensis* TB92 including adduct formation protonated molecule or even in-source water  
369 loss.

### 370 3.3.1. Gambierones

371 The peak area of 44-methylgambierone was 50 times higher than gambierone (Figure 5)  
372 and the detection of gambierone here was only possible thanks to the significant enhancement  
373 of signal resulting from the previous optimization.

374



375  
376 **Figure 5.** Extract Ion Chromatograms (tolerance  $\pm 10$  ppm) corresponding to the sum of areas of first water loss,  
377 protonated molecule and ammonium adduct of gambierone and 44-methylgambierone from full scan acquired with ESI+ LC-  
378 HRMS.

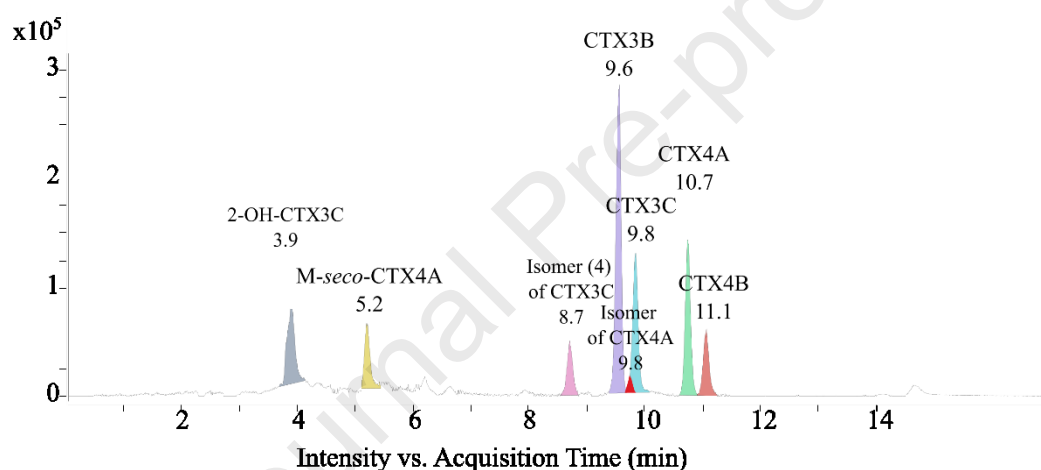
379  
380 The ubiquitous presence of gambierone and 44-methylgambierone in four  
381 *G. polynesiensis* clones has recently been reported [8,33]. Initially found in *G. belizeanus*  
382 [22,23], gambierone and 44-methylgambierone now seem to be common to 16 species of  
383 *Gambierdiscus*, 2 species of *Fukuyoa* and 2 species of *Coolia* [16,25,26]. To date, only *G.*  
384 *excentricus* was not demonstrated as a gambierone or 44-methylgambierone producer but  
385 interestingly, this species produced a compound responding to the transition  $m/z$  1037/97 but  
386 with a different retention time [16]. In addition, *G. jejuensis* has not been tested for the  
387 presence of gambierone analogs yet [53].

388 The quantitative profile of gambierones in *G. polynesiensis* has been described for four  
389 mono-clonal strains, by comparing the exponential and stationary growth phases and the  
390 intracellular/extracellular proportions [8]. The cell quotas were strain-dependent but  
391 gambierone was always produced in higher amount than 44-methylgambierone with a  
392 difference ranging from 2 to 7.5-fold. For this sample (*G. polynesiensis* strains TB92), the 44-  
393 methylgambierone is clearly the major product with an abundance 50 times higher than

394 gambierone. This difference can be attributed to either strain variability or method for  
 395 extraction and storage.

### 396 3.3.2. Ciguatoxins

397  
 398 LC-HRMS analysis of the methanolic extract of the *G. polynesiensis* strain TB 92  
 399 demonstrated the presence of eight CTX analogs (Figure 6), namely 2-OH-CTX3C, M-*seco*-  
 400 CTX4A, isomer (4) of CTX3C, CTX3B, CTX3C, an isomer of CTX4A, CTX4A and CTX4B.  
 401



402  
 403 **Figure 6.** Extract Ion Chromatograms (tolerance  $\pm 10$  ppm) corresponding to the sum of areas of first water loss, protonated  
 404 molecule and ammonium adduct ions of CTXs from full scan acquired with ESI+ LC-HRMS.

405 Reliable identification of the toxin congeners was carried out on the full scan analysis  
 406 based on mass and spectral accuracy for the first water loss, the protonated molecule and the  
 407 ammonium or sodium adduct (details provided in Figures S3-to-S10). In addition, the toxin  
 408 profile obtained by low resolution mass spectrometry in MRM mode acquired with the  
 409 method published in Sibat et al., [34] was compared to the mix of CTXs standard available  
 410 from ILM (detail provided in Figure S2). The toxin profile obtained was rather consistent with  
 411 previous studies on *G. polynesiensis* strains [8,11,32-34,38,40]. In recent studies, four  
 412 CTX3C-like compounds (*i.e.* peak responding to at least two transitions of CTX3C in MRM

413 mode but with a different retention time) that were identified as potential isomers according to  
414 their elution order in reversed phase chromatography, have been reported in *G. polynesiensis*  
415 [8,34,38,40] with retention times between *M-seco*-CTX3C and CTX3B. No structural  
416 information is available for these isomers as they never had been isolated and analyzed either  
417 by HRMS or NMR spectroscopy.

418 In this study, due to a still lower sensitivity of the Q-TOF compared to a hybrid  
419 quadrupole ion-trap mass spectrometer operating in MRM mode [34,38], only the isomer (4)  
420 of CTX3C (*i.e.* the most intense) was detected at a retention time of 8.7 min while *M-seco*-  
421 CTX3C and 3-OH-CTX3C were not detected.

422 In contrast to gambierone and 44-methylgambierone, the variation of ciguatoxin profile of  
423 *G. polynesiensis* strain TB92 since the first description in 2010 by Chinain et al., [11] was  
424 low. The major toxin produced is still CTX3B, followed by CTX3C and CTX4A and B and  
425 then 2-OH-CTX3C, *M-seco*-CTX4A and the presence of CTX3C isomers is consistent with  
426 the intensities reported previously [33,34,40].

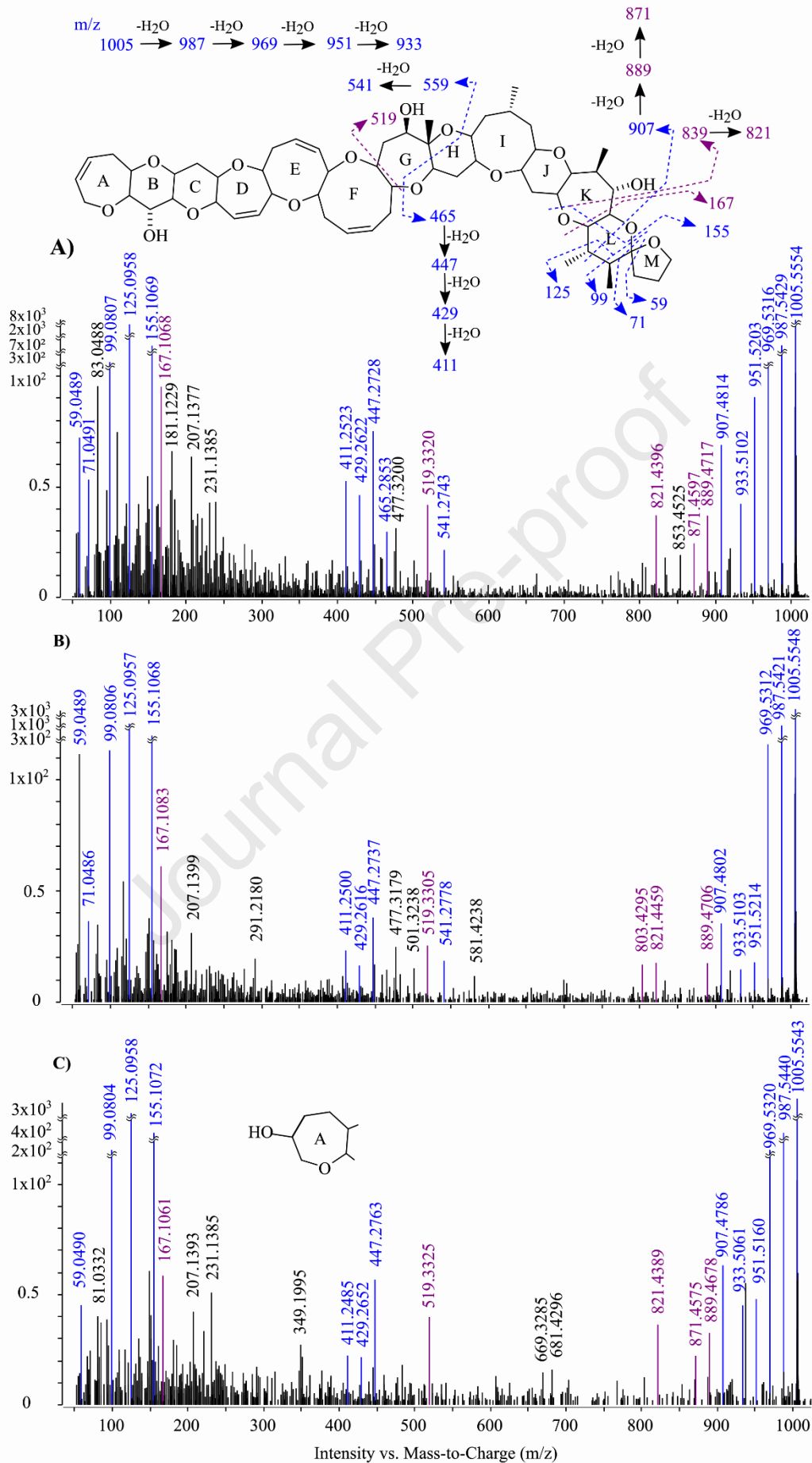
427 The low difference in retention time between CTX3B and the isomer (4) of CTX3C (0.9  
428 min) suggested a high structural similarity. In contrast, the hydroxylated form of CTX3C (2-  
429 OH-CTX3C), also produced by *G. polynesiensis*, eluted at 3.9 min (*i.e.* 5.7 min earlier than  
430 CTX3B), this high difference in retention time suggested that the isomer (4) was more closely  
431 related to CTX3B or C than to a hydroxylated form of CTX3B/C. To obtain more insights  
432 into the structure of the isomer (4) of CTX3C, targeted HRMS/MS spectrum was obtained on  
433 the  $m/z$  1005.5570 as it was the most abundant ion found in the full scan spectra (Figures  
434 S3,5,6).

### 435 **3.3.3. Isomer (4) of CTX3C**

436 Some parts of the fragmentation pattern of CTX3C were described in previous studies  
437 [11,34,41] (Figure 7A in blue). In this study, the fragmentation pattern of the epimer of

438 CTX3C (CTX3B) was acquired as the chromatographic peak was more intense (Figure 6) but  
439 both compounds share the same spectrum (as expected since the only structural difference is  
440 in the stereochemistry of the L- and M- rings). The reported fragmentation pathway was  
441 characterized by a succession of water losses from  $m/z$  1023.5541 ( $[M+H]^+$ ) to  $m/z$  933.5084  
442 ( $[M-5H_2O+H]^+$ ) corresponding to the successive dehydration on B-, G- and K- ring followed  
443 by the opening and water losses occurring on L- and M- ring. The opening of the L-ring  
444 resulted also in the formation of  $m/z$  907.4838. The opening of the G- to H-ring followed by  
445 several water losses was observed with the cluster from  $m/z$  465.2847 to 411.2530 for the H-  
446 to M-side of the molecule and with the  $m/z$  541.2796 (water loss originated from the  $m/z$  559)  
447 for the A- to G-side of the molecule. The opening of K-, L- and M-rings resulted in five  
448 characteristic small ions:  $m/z$  155.1071, 125.0960, 99.0807, 71.0491 and 59.0491.

449



451 **Figure 7.** Fragmentation pathways based on HRMS/MS spectra obtained on Q-TOF by targeted MS/MS (average of  
452 three collision energies 10, 20 and 40 eV) on the precursor ions  $m/z$  1005.5570 for chromatographic peak  
453 corresponding to A) CTX3B, B) Isomer (4) of CTX3C and C) 2-OH-CTX3C. Described in literature (in blue) and  
454 proposed (in purple).

455  
456 The high concentration of CTX3B present in the sample associated with the improvement  
457 of ionization and transmission from the source to the analyzer of both protonated molecule  
458 and first water loss contributed to the observation of hitherto unreported fragments in the  
459 spectrum (Figure 7A in purple). The molecular formula of these ions and the associated errors  
460 are presented in Table 3. The attribution of the fragments was carried out using the  
461 “molecular formula analysis” provided by Chemcalc (<https://www.chemcalc.org/>) [54] with  
462 an error of less than 5 ppm in mass accuracy associated with the number of unsaturations.  
463 Hence, we report that the opening of the L-ring in our condition was followed by two water  
464 losses  $m/z$  889.4733 (-1.8 ppm) and  $m/z$  871.4627 (-3.4 ppm). Additionally, the opening of the  
465 K-ring ( $m/z$  839.4576 not observed) was followed by one water loss  $m/z$  821.4471 (-4.3 ppm).  
466 Fragmentation of the G-ring resulted in the formation of  $m/z$  519.3316 (+0.8 ppm). For the  
467 small fragments, the opening of the K-ring produced  $m/z$  167.1067 (+0.6 ppm).  
468



469 **Table 3.** Ion species corresponding to the accurate mono-isotopic  $m/z$  of CTX3B, isomer (4) of CTX3C and 2-OH-  
 470 CTX3C. Mass differences ( $\Delta$ ppm) were compared between theoretical exact mass and measured  $m/z$ .

Ion Formula	Theoretical mono isotopic mass (Da)	NI*	CTX3B	$\Delta$ ppm	Isomer (4) of CTX3C	$\Delta$ ppm	2 OH-CTX3C	$\Delta$ ppm
$[C_{57}H_{81}O_{15}]^+$	1005.5570	18	1005.5554	-1.6	1005.5548	-2.2	1005.5543	-2.7
$[C_{57}H_{79}O_{14}]^+$	987.5464	19	987.5429	-3.5	987.5421	-4.4	987.544	-2.4
$[C_{57}H_{77}O_{13}]^+$	969.5359	20	969.5316	-4.4	969.5312	-4.8	969.532	-4.0
$[C_{57}H_{75}O_{12}]^+$	951.5253	21	951.5203	-5.3	951.5214	-4.1	951.516	-9.8
$[C_{57}H_{73}O_{11}]^+$	933.5147	22	933.5102	-4.8	933.5103	-4.7	933.5061	-9.2
$[C_{51}H_{71}O_{14}]^+$	907.4838	17	907.4817	-2.3	907.4802	-4.0	907.4786	-5.7
$[C_{51}H_{69}O_{13}]^+$	889.4733	18	889.4717	-1.8	889.4706	-3.0	889.4678	-6.2
$[C_{51}H_{67}O_{12}]^+$	871.4627	19	871.4597	-3.4			871.4575	-6.0
$[C_{47}H_{65}O_{13}]^+$	839.4576	15						
$[C_{47}H_{63}O_{12}]^+$	821.4471	16	821.4436	-4.3	821.4459	-1.5	821.4389	-10
$[C_{47}H_{61}O_{11}]^+$	803.4365	17			803.4295	-8.7		
$[C_{31}H_{41}O_8]^+$	541.2796	12	541.2743	-9.8	541.2778	-3.3		
$[C_{30}H_{47}O_7]^+$	519.3316	7.5	519.332	0.8	519.3305	-2.1	519.3325	1.7
$[C_{30}H_{45}O_6]^+$	501.3211	8.5			501.3238	5.4		
$[C_{26}H_{41}O_7]^+$	465.2847	7	465.2853	1.3	465.284	-1.5		
$[C_{26}H_{39}O_6]^+$	447.2741	8	447.2728	-2.9	447.2737	-0.9	447.2763	4.9
$[C_{26}H_{37}O_5]^+$	429.2636	9	429.2622	-3.3	429.2616	-4.7	429.2652	3.7
$[C_{26}H_{35}O_4]^+$	411.2530	10	411.2523		411.25	-7.3	411.2485	-11
$[C_{10}H_{15}O_2]^+$	167.1067	4	167.1068	0.6	167.1083	9.6	167.1061	-3.6
$[C_9H_{15}O_2]^+$	155.1067	2.5	155.1069	1.3	155.1068	0.6	155.1072	3.2
$[C_8H_{13}O]^+$	125.0961	2	125.0958	-2.4	125.0957	-3.2	125.0958	-2.4
$[C_6H_{11}O]^+$	99.0804	2	99.0807	3.0	99.0806	2.0	99.0804	0.0
$[C_4H_7O]^+$	71.0491	2	71.0491	0.0	71.0486	-7.0		
$[C_3H_7O]^+$	59.0491	1	59.0489	-3.4	59.0489	-3.4	59.049	-1.7

471 \*NI: number of unsaturation

472 The fragmentation observed in this study as well as the pathways reported in the literature  
 473 corresponded mainly to fragments resulting from opening of the cycles from the G- to M- side  
 474 suggesting that A- to F- rings required higher collision energy to open.

475 The intensity of ions observed in the spectrum of CTX3B was two times higher than for  
 476 isomer (4) or for 2-OH-CTX3C. Despite the variation in intensity, only small differences were  
 477 observed in the fragmentation spectra when comparing CTX3B and 2-OH-CTX3C (Figure 7A  
 478 and C). The precursor ion  $m/z$  1005 used in this study corresponded to either  $[M-H_2O+H]^+$  for  
 479 isomer (4) and CTX3B or to  $[M-2H_2O+H]^+$  for 2-OH-CTX3C, as one of these two water  
 480 losses corresponded to the loss of the hydroxyl group on the A- ring, both the structure of 2-  
 481 OH-CTX3C and CTX3B became identical after the rearrangement explaining the similarity in

482 fragmentation pathways. The opening of the G- and H-rings was not observed ( $m/z$  541.2796)  
483 and other ions were observed with no clear correlation with the fragmentation pathway.

484 Isomer (4) of CTX3C previously reported by low resolution mass spectrometry  
485 [8,11,34,38,40] was only putatively characterized by SIM mode with the  $m/z$  1023.8 and  
486 1045.8 and by MRM mode using the transitions  $m/z$  1040.6/1005.6, 1023.6/1005.6 and  
487 1045.6/1045.6. The same compound was probably detected by Roué et al., [37]. In our study,  
488 we used HRMS and HRMS/MS to improve the mass accuracy of the ion measured compared  
489 to low resolution mass spectrometry. Firstly, the masses of the first water losses and the  
490 protonated molecule were highly accurate (error < 0.5 ppm) (Figure S6) showing that the  
491 molecular formula of this compound was  $C_{57}H_{82}O_{16}$ . Secondly, this compound showed a  
492 fragmentation pattern closely related to the one of CTX3B. The presence of  $m/z$  541.2778 (-  
493 3.3 ppm) suggested that the A- to G- side of the molecule of this analog was common with  
494 CTX3B. The opening and water losses of the G- to M-ring of the molecule were the same as  
495 found for CTX3B too, hence no clear difference in structure was found. However, the  
496 similarity in terms of retention time with CTX3B compared to 2-OH-CTX3C (Figure 6)  
497 suggests that the difference between the isomer (4) of CTX3C and CTX3B was due to the  
498 change in the position of a methyl or a hydroxyl group along the carbon skeleton of the  
499 compound. Furthermore, as the stereochemical differences between CTXs congeners  
500 described in previous studies [31,36,55] were restricted to the orientation or configuration of  
501 the spiroketal L-, M- ring, the stereochemistry of other chiral centers could not be determined  
502 so a difference based in the position of the methyl or hydroxyl group remain possible.

503

#### 504 **3.3.4. M-*seco*-CTX4A and isomer of CTX4A**

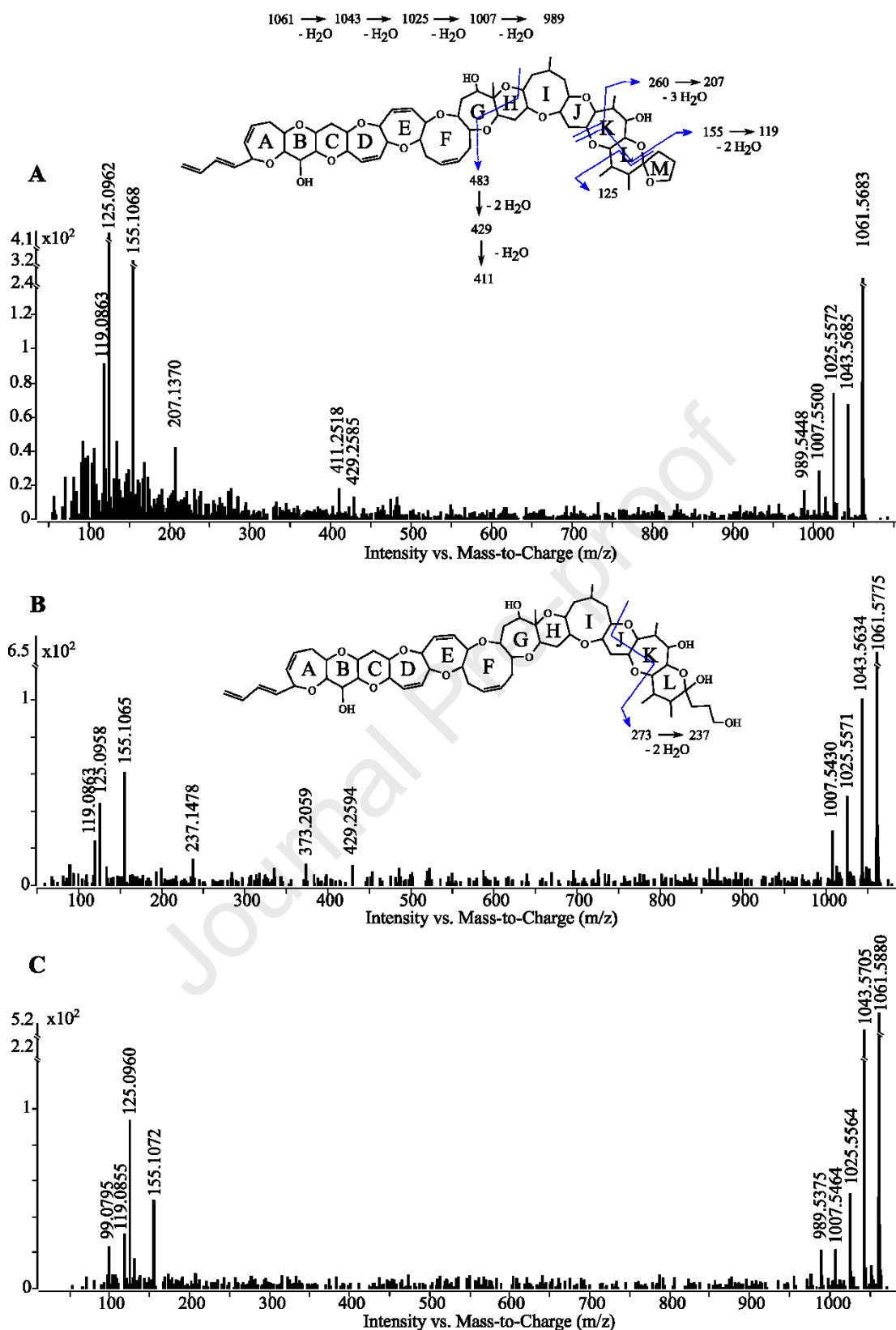
505

506 So far, the presence of M-*seco*-CTX4A at a significant amount (*i.e.* peak area similar to 2-  
507 OH-CTX3C) was only putatively reported with low-resolution mass spectrometry in  
508 *G. polynesiensis* TB92 (*i.e.* without standard confirmation) by Chinain et al., [11] and Roué et  
509 al., [32]. A compound eluting earlier than CTX4A sharing the same MRM transitions as  
510 CTX4A was also reported by Murray et al., [33] in *G. polynesiensis* CAWD212. In this study,  
511 the presence of M-*seco*-CTX4A in *G. polynesiensis* TB92 was ascertained by mass and  
512 spectral accuracy (Figure S4) with an error less than 5 ppm for monoisotopic  $m/z$   
513 corresponding to  $[M-H_2O+H]^+$  (-0.4 ppm),  $[M-H_2O+NH_4]^+$  (-4.7 ppm) and  $[M+Na]^+$  (+0.3  
514 ppm).

515 Furthermore, on the sum of EIC corresponding to the different form of ions (water loss,  
516 protonated molecule or adduct) observed for CTX4A and CTX4B, a small peak eluting at 9.8  
517 min was also observed.

518 To obtain more information about M-*seco*-CTX4A and the putative isomer of CTX4A,  
519 targeted HRMS/MS spectrum was obtained on the  $m/z$  1061.5833 (Figure 8) as it was the  
520 most abundant and common ion found in the full scan spectra of CTX4A, M-*seco*-CTX4A  
521 and the isomer of CTX4A (Figures S4, 8 and 9).

522



523

524

525

526

527

**Figure 8.** Proposed fragmentation pathways based on HRMS/MS spectra obtained by targeted MS/MS on the precursor ions  $m/z$  1061.5833 for chromatographic peak corresponding to A) CTX4A at eluting at 10.8 min (with a collision energy of 40 eV), B) *M-seco*-CTX4A at 5.2 min (collision energy 20 eV) and C) a potential isomer of CTX4A at 9.8 min (collision energy 20 eV)

528 The fragmentation pattern of CTX4A type share similarity with the CTX3C type such as  
529 the succession of four water losses from  $m/z$  1061.5833 to  $m/z$  989.541 corresponding to the  
530 successive dehydration on B-, G- and K- ring followed by opening and a water loss of L- ring  
531 (Figure 8) [11,34,41]

532 Both structure type share the same opening of the G- to H-ring followed by several water  
533 losses as demonstrated by the  $m/z$  429.2636 and 411.2530 for the H- to M-side of the  
534 molecule. In accordance with Sibat et al, [34] and Suzuki et al., [41] the two characteristic  
535 ions of the CTXs:  $m/z$  155.1067 and 125.0960 resulting from the opening of K-, L- and M-  
536 rings were detected in all three fragmentation spectra. Such fragmentation was not observed  
537 in CTX1B due to presence of the hydroxyl group on the M-ring.

538 The molecular formula of observed ions and the associated errors compared to theoretical  
539 monoisotopic mass are presented in Table 4.

540 The fragmentation spectra of CTX4A (Figure 8A and Table 4) presented two previously  
541 unreported ions,  $m/z$  207.1370 (-4.8 ppm) corresponding to the opening of the K- ring and  $m/z$   
542 119.0863 (+6.7 ppm) originating from the opening of K- and L- ring followed by one water  
543 loss also present in two others spectra (Figure 8B and C).

544 For M-*seco*-CTX4A (Figure 8B), the opening of the J- and K- ring yielded in the  
545 formation of the  $m/z$  237.1418 (-3.0 ppm).

546 As for the isomer (4) of CTX3C, the small difference in terms of retention time between  
547 the putative isomer of CTX4A (9.8 min, Figure S8) compared to the one of CTX4A (10.7  
548 min, Figure S9) and the one of M-*seco*-CTX4A (5.2 min, Figure S4) suggest only a small  
549 difference on the structure as the compound eluted only 0.9 min earlier than CTX4A. No  
550 difference was found between targeted HRMS/MS spectra of CTX4A and the isomer of  
551 CTX4A (Figure 8A and C) pointing out that the difference between the two compound is  
552 potentially only related to a difference of a position of functional group within the structure.

553 Unfortunately, as the compound was only present at low amount in the sample, a potential in-  
 554 source water loss could be identified on the full scan spectra (Figure S8). More analysis on a  
 555 purified (or a more concentrated) sample will be required to determine the isomerism  
 556 relationship between the isomer of CTX4A and CTX4A.

557

558 **Table 4.** Ion species corresponding to the accurate mono-isotopic  $m/z$  of CTX4A, M-*seco*-CTX4A and isomer of  
 559 CTX4A. Mass differences ( $\Delta$ ppm) were compared between theoretical exact mass and measured  $m/z$ .

Ion Formula	Theoretical mono isotopic mass (Da)	CTX4A	$\Delta$ ppm	M- <i>seco</i> -CTX4A	$\Delta$ ppm	Isomer of CTX4A	$\Delta$ ppm
[C <sub>60</sub> H <sub>85</sub> O <sub>16</sub> ] <sup>+</sup>	1061,5832	1061,5683	-14,0	1061,5775	-5,4	1061,588	4,5
[C <sub>60</sub> H <sub>83</sub> O <sub>15</sub> ] <sup>+</sup>	1043,5726	1043,5685	-3,9	1043,5634	-8,8	1043,5705	-2,0
[C <sub>60</sub> H <sub>81</sub> O <sub>14</sub> ] <sup>+</sup>	1025,5621	1025,5572	-4,8	1025,5571	-4,9	1025,5564	-5,6
[C <sub>60</sub> H <sub>79</sub> O <sub>13</sub> ] <sup>+</sup>	1007,5515	1007,55	-1,5	1007,543	-8,4	1007,5464	-5,1
[C <sub>60</sub> H <sub>77</sub> O <sub>12</sub> ] <sup>+</sup>	989,541	989,5448	3,8	989,5348	-6,3	989,5375	-3,5
[C <sub>26</sub> H <sub>37</sub> O <sub>5</sub> ] <sup>+</sup>	429,2636	429,2594	-9,8	429,2594	-9,8	429,2594	-9,8
[C <sub>26</sub> H <sub>35</sub> O <sub>4</sub> ] <sup>+</sup>	411,253	411,2518	-2,9			411,2518	-2,9
[C <sub>14</sub> H <sub>21</sub> O <sub>3</sub> ] <sup>+</sup>	237,1485			237,1478	-3,0		
[C <sub>13</sub> H <sub>18</sub> O <sub>2</sub> ] <sup>+</sup>	207,138	207,137	-4,8			207,137	
[C <sub>9</sub> H <sub>15</sub> O <sub>2</sub> ] <sup>+</sup>	155,1067	155,1068	0,6	155,1065	-1,3	155,1072	3,2
[C <sub>8</sub> H <sub>13</sub> O] <sup>+</sup>	125,0961	125,0962	0,8	125,0958	-2,4	125,096	-0,8
[C <sub>8</sub> H <sub>11</sub> ] <sup>+</sup>	119,0855	119,0863	6,7	119,0863	6,7	119,0855	0,0
[C <sub>6</sub> H <sub>11</sub> O] <sup>+</sup>	99,0804					99,0795	-9,1

560

561

## 562 4. Conclusions

563 This study demonstrated the benefits of using a time- and cost-saving fractional factorial  
 564 design of experiment to assess the significance of HRMS ionization and ion transmission  
 565 parameters in order to improve the sensitivity for the detection of algal polyethers. It was a  
 566 first step towards a better understanding of their behavior in an electrospray source and in the  
 567 dual ion funnel that led to lowering their limits of detection. The increase of sensitivity  
 568 contributed to obtain higher-quality MS/MS fragmentation, which ultimately provided more  
 569 relevant structural information on ciguatoxin-like compounds. The toxin profile of  
 570 *G. polynesiensis* strain TB92 was re-described using high resolution mass spectrometry

571 resulting in the report of 44-methylgambierone produced at 50 times higher level than  
572 gambierone, the confirmation based on Full Scan and targeted MS/MS analysis of the  
573 presence of M-*seco*-CTX4A and the report of a putative isomer of CTX4A. For the first time,  
574 the signal enhancement in HRMS allowed for the acquisition of MS/MS spectra for CTX3B,  
575 2-OH-CTX3C, the isomer (4) of CTX3C, CTX4A, M-*seco*-CTX4A and an isomer of  
576 CTX4A. Their structural comparison resulted in the report of constitutional isomerism for  
577 both type of isomers with a difference in the position of likely a methyl- or hydroxyl-group  
578 with no clear difference in the fragmentation pattern using MS<sup>2</sup>. The perspective for the  
579 structural elucidation of the two isomers relies on the use of MS<sup>n</sup> mass spectrometry or NMR  
580 spectroscopy on the purified compound.

581

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- 745

**Highlights :**

- A chemometric method to weigh the contribution of Q-TOF parameters is proposed
- Ionization and transmission parameter increased sensitivity for ciguatoxin analysis
- M-seco-P-CTX4A is a major ciguatoxin produced by *G. polynesiensis*
- Putatively reported “isomer (4) of P-CTX3C” is a constitutional isomer

Journal Pre-proof

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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