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1 **Isoprostanoids quantitative profiling of marine red and brown macroalgae**

2  
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17

18 **Abstract**

19 With the increasing demand for direct human and animal consumption seaweed farming is  
20 rapidly expanding worldwide. Macroalgae have colonized aquatic environments in which  
21 they are submitted to frequent changes in biotic and abiotic factors that can trigger  
22 oxidative stress (OS). Considering that isoprostanoic derivatives may constitute the most  
23 relevant OS biomarkers, we were interested to establish their profile in two red and four  
24 brown macroalgae. Seven phytoprostanes, three phytofuranes, and four isoprostanes  
25 were quantified through a new micro-LC-MS/MS method. The isoprostanoic contents vary  
26 greatly among all the samples, the *ent*-16(*RS*)-9-*epi*-ST- $\Delta^{14}$ -10-PhytoF and the sum of 5-  
27 F<sub>2t</sub>-IsoP and 5-*epi*-5F<sub>2t</sub>-IsoP being the major compounds for most of the macroalgae  
28 studied. We further quantified these isoprostanoic derivatives in macroalgae submitted to heavy  
29 metal (copper) exposure. In most of the cases, their concentrations increased after 24 h of  
30 copper stress corroborating the original hypothesis. One exception is the decrease of *ent*-  
31 9-L1-PhytoP content in *L. digitata*.

32

33 **Keywords**

34 Macroalgae, isoprostanoic derivatives, heavy metal, copper stress, oxylipins, micro-LC-MS/MS

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36

37

## 38 **1-Introduction**

39 Macroalgae, also known as seaweeds, constitute a large group of coastal macro-  
40 organisms playing an important role in marine environment, both as food resource and  
41 engineer species for shaping coastal marine habitats (Hurd, Harrison, Bischof, & Lobban,  
42 2014). Seaweeds have been also part of the human diet for thousands of years, based on  
43 archaeological evidence in Chile (Dillehay, Ramirez, Pino, Collins, Rossen, & Pinot-  
44 Navarro, 2008) and on several other reports (e.g., in China, 300 A. D.; in Ireland, 600 A.D;  
45 Aaronson, 1986; Craigie, 2010; Gantar & Svircev, 2008; Newton, 1951; Tseng, 1981;  
46 Turner, 2003). Nowadays, they are extensively cultivated in the Far East Asia to provide  
47 mainly high-quality food. Indeed, seaweeds are interesting natural sources of functional  
48 ingredients such as carbohydrates, proteins, minerals, vitamins, and present a low content  
49 of lipids with a high level of polyunsaturated  $\omega$ -3 fatty acids (Holdt & Kraan, 2011; Plaza,  
50 Cifuentes, & Ibanez, 2008). In South East Asia and Eastern Africa, the seaweed biomass  
51 is harvested to extract phycocolloids such as carrageenans and agars. With the increasing  
52 demand for direct human and animal consumption, medicines, food additives, fertilizers,  
53 and cosmetics, seaweed farming is rapidly expanding worldwide.

54 Most of the wild populations of macroalgae are thriving in the intertidal and near subtidal  
55 zone, a highly and frequently changing environment, and thus experience repeated biotic  
56 and abiotic (temperature, irradiation, salinity, ...) fluctuations that require physiological  
57 plasticity for stress tolerance (Dittami, Gravot, Renault, Goulitquer, Eggert, Bouchereau, et  
58 al., 2011). Among potential stress factors, exposure to high level of heavy metals is very  
59 common, as illustrated by numerous studies conducted so far on different macroalgae  
60 (Collen, Pinto, Pedersen, & Colepicolo, 2003; Pinto, Sigaud-Kutner, Leitao, Okamoto,  
61 Morse, & Colepicolo, 2003; Roncarati, Sáez, Greco, Gledhill, Bitonti, & Brown, 2015; Saez,  
62 Roncarati, Moenne, Moody, & Brown, 2015).

63 Among heavy metals, copper (Cu(II)) is an essential micronutrient to both land plants and  
64 macroalgae, notably for many electron carriers involved in photosynthetic electron  
65 transport, mitochondrial respiration or oxidative stress (OS) response (Yruela, 2005).  
66 However, above specific threshold concentration, it is considered as a pollutant and thus  
67 toxic. Copper is currently extensively used in antifouling marine paints, and in some  
68 coastal areas copper mining discharges are still very important. Therefore, both natural  
69 and farmed populations of seaweeds can be exposed to copper excess. To understand  
70 the mode of action leading to copper biological function (positive or negative), its chemical  
71 properties have to be considered.

72 Ritter and colleagues demonstrated that copper and H<sub>2</sub>O<sub>2</sub> treatments lead to OS response  
73 in the model brown alga *Ectocarpus siliculosus* (Dillwyn) Lyngbye (A. Ritter, Dittami,  
74 Goulitquer, Correa, Boyen, Potin, et al., 2014). In these studies, the authors underlined a  
75 link between copper stress and fatty acid/lipid metabolism since they observed an increase  
76 of free fatty acid contents and oxylipins after exposure of *E. siliculosus* to this heavy metal.  
77 Similar conclusion were drawn with regards to the brown algal kelp *L. digitate* (A Ritter,  
78 Goulitquer, Salaun, Tonon, Correa, & Potin, 2008). In this context, it was relevant to  
79 complete these previous studies by assessing the potential production of isoprostanoid  
80 derivatives by non-enzymatic oxidation of lipids/fatty acids.

81 Indeed, fatty acids, and more especially polyunsaturated fatty acids (PUFAs), are prone to  
82 oxidation due to their highly reactive bis-allylic hydrogen atoms. This non-enzymatic lipid  
83 peroxidation generates series of lipid mediators such as phytoprostanes (PhytoPs),  
84 isoprostanes (IsoPs), and neuroprostanes (NeuroPs), derived from  $\alpha$ -linolenic acid (ALA  
85 C18:3 n-3), arachidonic acid (AA, C20:4 n-6), and docosahexaenoic acid (DHA, C22:6 n-3)  
86 respectively. It is worth to mention that under high oxygen pressure, further  
87 transformations could occur, leading to the synthesis of furanic forms named phytofurans  
88 (PhytoFs), isofurans (IsoFs), and neurofurans (NeuroFs) (Table 1). All these compounds

89 are robust markers of oxidative stress in biological systems (Milne, Gao, Terry, Zackert, &  
90 Sanchez, 2013). They are also known to have functional roles in living organisms (Galano,  
91 Lee, Gladine, Comte, Le Guennec, Oger, et al., 2015; Galano, Lee, Oger, Vigor,  
92 Vercauteren, Durand, et al., 2017; Jahn, Galano, & Durand, 2008). Previous work by  
93 Barbosa et al. (Barbosa, Collado-Gonzalez, Andrade, Ferreres, Valentao, Galano, et al.,  
94 2015) showed that macroalgae were able to synthesize ALA oxygenated metabolites, and  
95 among them, 9-F<sub>1t</sub>-PhytoP, 9-*epi*-9-F<sub>1t</sub>-PhytoP, 16-B<sub>1</sub>-PhytoP, and 9-L<sub>1</sub>-PhytoP. In view of  
96 these findings, and to go further in the study of potential production of isoprostanoids by  
97 non-enzymatic oxidation of lipids/fatty acids in macroalgae, we were interested we were  
98 interested in identifying and quantifying phytoprostanes potentially produced in  
99 macroalgae from other PUFAs, and also the possible synthesis of furanes in these  
100 organisms. We also forced the stress status with cupric exposure in order to observe an  
101 eventual change in amounts of detected compounds.

102 To better understand the importance of isoprostanoids in seaweeds, we have considered  
103 two distinct groups of seaweeds, the brown (*Phaeophyta*) and the red (*Rhodophyta*)  
104 macroalgae, which belong to two independent eukaryotic lineages and therefore constitute  
105 very interesting biological models (Brodie, Chan, De Clerck, Cock, Coelho, Gachon, et al.,  
106 2017; Cock & Coelho, 2011). Among the brown algae, four species were considered:  
107 *Ectocarpus siliculosus* (Dillwyn) Lyngbye, *Laminaria digitata* (Huds.) Lamouroux, *Fucus*  
108 *spiralis* L., and *Pelvetia canaliculata* (L.) Decaisne & Thuret. Experiments were also  
109 conducted on two *Rhodophyta*, i.e. *Osmundea pinnatifida* (Hudson) Stackhouse, and  
110 *Grateloupia turuturu* Yamada. Isoprostanoïd content was determined for all these species  
111 under normal control growth conditions, and after incubation under copper stress for 24h.  
112 To conduct such experiments, and because oxygenated metabolites were expected to be  
113 present at low concentrations, we first optimized the extraction protocol with *L. digitata*  
114 before applying it to all other algal matrices. In line with this, we developed a process to

115 quantify PhytoPs/PhytoFs, based on micro-LC-MS/MS with increased speed, robustness,  
116 selectivity, and sensitivity of analysis (Medina, Miguel-Elizaga, Oger, Galano, Durand,  
117 Martinez-Villanueva, et al., 2015).

118

## 119 **2-Material and methods**

### 120 **2.1 Chemicals and reagents**

121 The phytostane standard 16-(*RS*)-16-A<sub>1</sub>-PhytoP and the deuterated internal standard  
122 (IS) d<sub>4</sub>-15-F<sub>2t</sub>-IsoP were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The  
123 two IS d<sub>4</sub>-10-F<sub>4t</sub>-NeuroP and C<sub>21</sub>-15-F<sub>2t</sub>-IsoP, as well as all the other standards, were  
124 synthesized according to previous procedures (Cuyamendous, Leung, Durand, Lee, Oger,  
125 & Galano, 2015; Thierry Durand, Cracowski, Guy, & Rossi, 2001; T. Durand, Guy, Vidal, &  
126 Rossi, 2002; El Fangour, Guy, Despres, Vidal, Rossi, & Durand, 2004; El Fangour, Guy,  
127 Vidal, Rossi, & Durand, 2005; Guy, Flanagan, Durand, Oger, & Galano, 2015; Guy, Oger,  
128 Hepekauzen, Signorini, Durand, De Felice, et al., 2014; Oger, Brinkmann, Bouazzaoui,  
129 Durand, & Galano, 2008; Oger, Bultel-Poncé, Guy, Balas, Rossi, Durand, et al., 2010).  
130 This concerns 9-F<sub>1t</sub>-PhytoP, 9-*epi*-F<sub>1t</sub>-PhytoP, *ent*-16-F<sub>1t</sub>-PhytoP, *ent*-16-*epi*-16-F<sub>1t</sub>-  
131 PhytoP, *ent*-16-B<sub>1</sub>-PhytoP, *ent*-9-L<sub>1</sub>-PhytoP, as PhytoPs, and *ent*-9(*RS*)-12-*epi*-ST- $\Delta^{10}$ -13-  
132 PhytoF, *ent*-16(*RS*)-13-*epi*-ST- $\Delta^{14}$ -9-PhytoF, and *ent*-16(*RS*)-9-*epi*-ST- $\Delta^{14}$ -10-PhytoF as  
133 PhytoFs. Four IsoPs were also evaluated: 15-F<sub>2t</sub>-IsoP, 15-*epi*-15-F<sub>2t</sub>-IsoP, 5-F<sub>2t</sub>-IsoP and  
134 5-*epi*-5-F<sub>2t</sub>-IsoP. Finally, three NeuroPs were considered: 10-F<sub>4t</sub>-NeuroP, 10-*epi*-10-F<sub>4t</sub>-  
135 NeuroP, and 4(*RS*)-4-F<sub>4t</sub>-NeuroP. Stock solutions of standards were prepared in methanol  
136 to a concentration of 100 ng. $\mu$ L<sup>-1</sup>, and were stored at -20 °C. Appropriate dilutions from  
137 the mentioned stock were prepared for calibration purpose. Furthermore, two different  
138 solutions of a Standard Mixture (SM) of the 18 compounds mentioned above (SM<sub>32</sub> = each  
139 compound at 32 ng.ml<sup>-1</sup> or SM<sub>256</sub> = each compound at 256 ng.ml<sup>-1</sup>) were made up for  
140 validation purpose (extraction yield and matrix effect).



141 LC-MS methanol, acetonitrile, and HPLC chloroform were obtained from Fisher Scientific  
142 (Loughborough, UK). Hexane (CHROMASOLV, HPLC grade), absolute ethanol, formic  
143 and acetic acids, ammonia and potassium hydroxide (Fluka for mass spectrometry) were  
144 provided by Sigma-Aldrich (Saint Quentin Fallavier, France). Ethyl acetate (HPLC grade)  
145 was purchased from VWR (EC). Water used in this study was purified on a milliQ system  
146 (Millipore).

147 The solid-phase extraction (SPE) cartridges were constituted of a mixed-mode ion-  
148 exchange sorbent (Oasis MAX; 3 mL, 60 mg; from Waters; Milford, MA, USA).

149

## 150 **2.2 Macroalgal samples**

### 151 *2.2.1 Collection*

152 Of the six macroalgae used in this study, one (*E. siliculosus*) was cultivated in laboratory,  
153 and five (*F. spiralis*, *P. canaliculata*, *O. pinnatifida*, *L. digitata*, and *G. turuturu*) were  
154 collected during the summer 2015 at low-tide close to the Station Biologique of Roscoff, a  
155 site with no direct chemical influence from the shore.

156 After collection, four algal species (*F. spiralis*, *P. canaliculata*, *O. pinnatifida*, and *G.*  
157 *turuturu*) were immediately transported to the laboratory where they were cleaned and  
158 then assigned in 40 L tanks with a permanent renewal of both seawater and bubbled air.  
159 After at least 24h of acclimatization, samples were submitted to copper stress (see section  
160 2.2.2).

161 Young sporophytes of *L. digitata* (ca 10-20 cm in length) were also collected in the  
162 intertidal zone close to the Station Biologique of Roscoff, and maintained in 10 L flasks in  
163 autoclaved filtered seawater (FSW), at 13°C, under a photoperiod of 16 h of light (40  $\mu\text{mol}$   
164  $\text{photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) and 8 h of darkness, and well-aerated with filtered (0.22  $\mu\text{m}$ ) compressed  
165 air, up to one week before treatment.

166 *E. siliculosus* (Dilwyn) Lyngbye, unialgal strain 32 (accession CCAP 13104, origin San  
167 Juan de Marcona, Peru, 2002) was cultivated in 10 L flasks as already described (A.  
168 Ritter, Dittami, Goulitquer, Correa, Boyen, Potin, et al., 2014).

169

### 170 *2.2.2 Copper stress for the six species of macroalgae*

171 Each sample corresponded to a mixture of at least three to six individuals in the same  
172 stage of development (depending of the species) to reach a weight of 10 g of fresh algae.

173 Copper stress was triggered by transferring the algal samples to FSW enriched with Cu(II)  
174 as CuCl<sub>2</sub> (Titrisol, Merck) at nominal final concentration of 300 µg.L<sup>-1</sup> (15 µL of a 20 g.L<sup>-1</sup>  
175 stock solution) in 1 L glass flasks washed overnight with 1% HCl to limit Cu adsorption.

176 Control treatment corresponded to another 10 g algal batch incubated without CuCl<sub>2</sub>  
177 addition. After 24 hours in a culture room at 13°C under aeration with filtered (0.22 µm)  
178 compressed air, control and stressed samples were washed with autoclaved FSW, briefly  
179 dried using paper towel, immediately frozen in liquid nitrogen, and then stored at -80 °C  
180 until extraction.

181

### 182 *2.2.3 Algal sample preparation*

183 Approximately 1 g of fresh algal weight was ground with grinding balls (50 mm diameter) in  
184 liquid nitrogen using the Mixer Mill MM400 (Retsch®) bench top unit for 2 min at 20 Hz.

185 After having evaluated the extraction methods suggested by Küpper (Küpper, Gaquerel,  
186 Boneberg, Morath, Salaün, & Potin, 2006), Barbosa (Barbosa, et al., 2015), and Leung  
187 (Leung, Chen, Zhong, Yu, & Lee, 2014) on *L. digitata*, we opted for the latter protocol  
188 since it allowed a better recovery of analytes and showed limited influence of the biological  
189 material (matrix effect) on the extraction procedure. Briefly, 0.10 g of each powdered  
190 sample was weighed in a flask, then added with (i) 25 µL of 1% (w/v) di-*tert*-butyl  
191 hydroxytoluene (BHT) in methanol, (ii) 2 mL of MeOH, and (iii) 1.5 mL of phosphate buffer

192 (pH 2) prepared with saturated sodium chloride solution. At this step, the samples were  
193 spiked with 6 ng of each IS. This mixture was stirred with a vortex mixer for 30 sec, and  
194 then shaken at 100 rpm for 1 hour at room temperature with an IKA KS 4000 control  
195 shaker. Then, extracts were centrifuged at 5,000 rpm for 5 min at room temperature. The  
196 supernatant was separated, and 4.0 mL of cold chloroform were added. This mixture was  
197 stirred with a vortex mixer for 30 s and then centrifuged at 1,500 rpm for 5 min at room  
198 temperature. The lower organic layer was carefully removed, transferred into a Pyrex tube  
199 and then evaporated under N<sub>2</sub> at 40 °C. To perform hydrolysis of samples, the dry extract  
200 was dissolved into 950 µL of KOH (1 M in H<sub>2</sub>O), incubated for 30 min at 40 °C into an IKA  
201 control shaker (100 rpm), and 1 mL of 40 mM formic acid was added. The solution was  
202 then ready for the clean-up step through a SPE process. Oasis mixed polymer phase  
203 cartridges were first conditioned with 2 mL of MeOH and equilibrated with 2 mL of 20 mM  
204 formic acid (pH 4.6). After the SPE column had been loaded with samples, 2 mL of NH<sub>3</sub>  
205 2% (v/v), followed by 2 mL of MeOH/20 mM formic acid (3:7; v/v), 2 mL of hexane, and 2  
206 mL of hexane/ethyl acetate (7/3; v/v) were applied for removing undesired compounds.  
207 Target chemicals were eluted with 2 x 1 mL of a mixture constituted of hexane/EtOH/acetic  
208 acid (70:29.4:0.6; v/v/v), and then dried under nitrogen stream at 40°C. The dried residue  
209 was reconstituted with 100 µL of A/B LC-MS solvents (83:17; v/v). Then 5 µL of sample  
210 were injected and analysed using a micro-LC-MS/MS 5500 QTrap system.

211

### 212 **2.3 Micro-LC-MS/MS analysis**

213 All LC-MS analyses were carried out using an Eksigent® MicroLC 200 plus (Eksigent  
214 Technologies, CA, USA) on a HALO C<sub>18</sub> analytical column (100\*0.5 mm, 2.7 µm; Eksigent  
215 Technologies, CA, USA) kept at 40°C. The mobile phase consisted of a binary gradient of  
216 solvent A (water with 0.1% (v/v) of formic acid) and solvent B (ACN/MeOH; 8:2, v/v; with  
217 0.1% (v/v) of formic acid). The elution was performed at a flow rate of 0.03 mL min<sup>-1</sup> using

218 the following gradient profile: 17% solvent B at 0 min, 22% solvent B at 9.5 min, 30%  
219 solvent B at 11.5 min until 15 min and 95% solvent B at 16 for 2.3 min, and then returned  
220 to the initial conditions. Under these conditions, no sample contamination or sample-to-  
221 sample carry-over was observed.

222 Mass spectrometry analyses were performed on an AB SCIEX QTRAP 5500 (Sciex  
223 Applied Biosystems, ON, Canada). The ionization source was electrospray (ESI), and it  
224 was operated in the negative mode. The source voltage was kept at -4.5 kV, and N<sub>2</sub> was  
225 used as curtain gas. Detection of the fragmentation ion products from each PhytoP,  
226 PhytoF, or IsoP deprotonated molecule [M - H]<sup>-</sup> was performed in the multiple reaction  
227 monitoring mode (MRM). The MS parameters were individually optimized for each  
228 compound.

229

## 230 **2.4 Characterization method**

231 Parameters including extraction yield, matrix effect, sensitivity, linearity, accuracy and  
232 precision were determined to validate the methodology used for PhytoPs, PhytoFs and  
233 IsoPs quantification in *L. digitata* from an extractive and LC-MS analytical point of view.

234

### 235 **2.4.1 Validation of sample preparation**

236 In order to determine the extraction yield and the matrix effect, experiments described  
237 below were performed in triplicate using the same algal powder. Briefly, three sets were  
238 prepared: 1) 100 mg of algal samples spiked with two different concentrations of a  
239 standard mixture (SM<sub>32</sub> or SM<sub>256</sub>) before following the extraction procedure described in  
240 part 2.2.3; 2) 100 mg of algal samples treated according to the method described in 2.2.3,  
241 and then spiked with SM<sub>32</sub> or SM<sub>256</sub>; 3) SM<sub>32</sub> or SM<sub>256</sub> directly prepared into the mobile  
242 phase.

243 SPE extraction yield (EY) was evaluated for each compound (standards and internal

standards) by comparing peak area of set 1 (spike before SPE) *versus* set 2 (spike after SPE). Result is expressed in percentage by the following calculation:  $EY = A_{\text{set1}}/A_{\text{set2}}*100$ . The matrix effect (ME), also expressed in percentage and evaluated for each compound, was determined as the difference between peak areas obtained for the standards added to the extracted samples (set 2) and pure standard (set 3). The calculation is:  $ME = A_{\text{set2}}/A_{\text{set3}}*100$ .

250

#### 2.4.2 Sensitivity linearity and quantification

In order to determine the sensitivity of the analytical method we evaluated the limit of detection (LOD) and the limit of quantification (LOQ) for each compound. These values corresponded respectively to 3 and 10 times the signal-to-noise ratio. The linearity of the response was evaluated using 15 concentrations of compounds (in triplicate). Calibration curves were calculated by the least-squares linear regression method, and linearity was determined to range between  $3.125 \times 10^{-3}$  and  $512 \text{ pg} \cdot \mu\text{L}^{-1}$  for compounds injected in column. Analytes quantification was based on the analyte to IS ratio using the obtained calibration curves. Data processing was achieved using the MultiQuant 3.0 software (Sciex Applied Biosystems).

261

#### 2.4.3 Accuracy and precision

Trueness, precision and accuracy were determined by validation standard analysis performed in triplicate at defined concentrations, and on two different days. Intra-batch reflects intra-day precision or repeatability, and inter-batch the inter-day precision or reproducibility. These parameters express the error of the analytical measurement.

267

### 2.5 Statistical Analyses

Standard deviation (SD) and relative standard deviation (RSD) were used to determine

270 significant differences of data. As mentioned above, algal samples corresponded to a  
271 mixture of individuals placed together in a same glass flask. Each flask thus represented  
272 one experimental condition, *i.e.* control or copper stress. Despite numerous individual  
273 algae being present in flasks, each individual cannot be considered as an independent  
274 biological replicate because all algae within a flask were harvested as a pool before  
275 preparation of powder for subsequent treatment. Three independent extractions were  
276 performed on each algal sample, thus the SD calculated reflected a technical triplicate and  
277 not a biological triplicate. Two-way ANOVA were calculated with GraphPad Prism 7. The  
278 level of significance was set at  $P < 0.05$ .

279

### 280 **3-Results**

281 Before dealing with the analysis of the acquired data on various biological samples, an  
282 important part of this work consisted in the validation of the methodology presented below  
283 (section 3.1).

284

#### 285 **3.1 Sample processing validation on *L. digitata***

286 Sample validation was a pre-condition for extract preparation and analysis of the six  
287 macroalgae selected for this analysis. The aim was to verify that the work was done under  
288 favourable conditions for the detection and quantification of PhytoPs, PhytoFs, IsoPs, and  
289 eventually NeuroPs.

290 First, the extraction yield and matrix effect determination assessed the efficiency of the  
291 sample processing (Table 2). The extraction yield, a parameter specific of each compound  
292 (standards and IS), allowed the evaluation of product losses that could happen by  
293 retention on the SPE cartridge and/or by partial elution during the washing steps. For the  
294 majority of analytes, the apparent loss of compounds during SPE was low (<10%), yielding  
295 recovery rates similar to as if they were added after SPE. Regarding the type of

296 compounds (PhytoPs, PhytoFs, IsoPs or NeuroPs), no specific trend could be noticed, *i.e.*  
297 some PhytoPs exhibited good recovery ratio (*e.g.* *ent*-16-*epi*-16-F<sub>1t</sub>-PhytoP; EY<sub>32</sub>= 99.6%),  
298 while it was lower for other ones (*e.g.* 16(*RS*)-16-A<sub>1</sub>-PhytoP; EY<sub>32</sub>= 68.6%). The extraction  
299 yield calculated was more than 100% for some analytes, corresponding probably to the co-  
300 elution of a compound that presents the same MRM transition. It is the case for instance  
301 for the mixture of 5-F<sub>2t</sub>-IsoP and 5-*epi*-F<sub>2t</sub>-IsoP with an EY of 144.4% at 32 pg.μL<sup>-1</sup>. We  
302 could also notice that EYs were better at low than at high concentrations. To complete this  
303 validation, the matrix effect, corresponding to an ion-suppression/enhancement of co-  
304 eluted matrix compounds, was evaluated. As for EY, ME is specific to each isoprostanoids,  
305 and there was no similar behaviour across the same class of compounds. We observed a  
306 clearly marked effect at low concentration, for instance for 9-F<sub>1t</sub>-PhytoP and 9-*epi*-F<sub>1t</sub>-  
307 PhytoP between SM<sub>32</sub> and SM<sub>256</sub> conditions. Indeed, ME values were 169% (SM<sub>32</sub>) and  
308 123% (SM<sub>256</sub>) for the first compound, and 204% (SM<sub>32</sub>) and 125% (SM<sub>256</sub>) for the second  
309 one.

310 In order to determine the linear range in the quantification process, 15 concentrations  
311 ranging from 3.125x10<sup>-3</sup> to 512 pg.μL<sup>-1</sup> and prepared in triplicate were injected. This  
312 allowed establishing calibration curves and calculating the linear regression equation. The  
313 detector response was linear across the range tested. LOD and LOQ were also  
314 determined and ranged from 0.16 to 0.63 pg injected for LOD and between 0.16 and 1.25  
315 pg injected for LOQ. These values depended on the type of isoprostanoids but are quite  
316 homogenous.

317 Finally, for testing the repeatability and precision of the method, the intra- and inter-day  
318 analysis of two selected concentrations (SM<sub>32</sub> and SM<sub>256</sub>) was performed. Among the  
319 twenty compounds tested, the majority presented an intra-day variation lower than 2%,  
320 exceptions being 16(*RS*)-16-A<sub>1</sub>-PhytoP (2.2%), 5(*RS*)-5-F<sub>2t</sub>-IsoP + 5-*epi*-5-F<sub>2t</sub>-IsoP (2.7%),  
321 and 4(*RS*)-4-F<sub>4t</sub>-NeuroP (5.6%). Mean inter-day variation was 9.9%, with 7.8% and 13.7%

322 as minimum and maximum values, respectively. In light of these results, we concluded on  
323 the robustness of the developed method that was reproducible and usable for the purpose  
324 of isoprostanoids quantification in macroalgae.

325

### 326 **3.2. Profiling of isopropanoid in six species of brown and red algae**

327 It is well established that in seaweeds, the total fatty acid content and composition, as well  
328 as the proportions of the different lipid fractions, may vary during the algal cycle life and  
329 also according to the physiological state of the algae or growth conditions, and of the  
330 genetic status or taxonomic entity. Mean total lipid content for brown seaweeds (phylum  
331 *Phaeophyta*) is 3% of dry weight, and is comprised within a range of 0.1% to 20%. Based  
332 on published data reporting lipid composition in this phylum, no specific trend depending of  
333 the phylogenetic order could be inferred, and a similar conclusion was drawn for red algae  
334 (phylum *Rhodophyta*) (Wielgosz-Collin, Kendel, & Couzinet-Mossion, 2016). Analysis of  
335 fatty acid distribution allowed a better discrimination of phylogenetic order or sub-order.  
336 Indeed, many studies about the proportion of saturated fatty acids (SFAs),  
337 monounsaturated fatty acids (MUFAs) and PUFAs seemed to be in favour of a significant  
338 link between fatty acid signature and phylogenetic lineage (Galloway, Britton-Simmons,  
339 Duggins, Gabrielson, & Brett, 2012; P Kumari, M Kumar, C R K Reddy, & Bk Jha, 2013).  
340 In any cases, seaweeds are mainly characterized by a high content of PUFAs, such as  $\alpha$ -  
341 linolenic acid (18:3, n-3, ALA), stearidonic (18:4, n-3, STA), arachidonic (20:4, n-6, AA)  
342 and eicosapentaenoic acids (20:5, n-3, EPA), but each phylum presented a characteristic  
343 PUFA signature as evidenced by clustering studies of algae belonging to the same phylum  
344 (Pereira, Barreira, Figueiredo, Custodio, Vizetto-Duarte, Polo, et al., 2012). In our work, we  
345 studied profiles of PUFA oxidized derivatives across two distinct phyla and tried to  
346 determine if such clustering could be observed. In other words, it was of interest to  
347 investigate selected species of two taxonomic groups to know if difference in PUFA



348 composition subsequently resulted in occurrence of different oxidized products. From the  
349 study of Ritter *et al.* on *E. siliculosus*, we already knew that a macroalga could produce  
350 PhytoPs (A. Ritter, et al., 2014). More recently, Barbosa *et al.* (Barbosa, et al., 2015)  
351 tested 24 different species belonging to *Chlorophyta*, *Phaeophyta* and *Rhodophyta*, and  
352 highlighted the presence of four oxygenated metabolites derived from ALA among ten  
353 available PhytoPs standards. These compounds were 9-F<sub>1t</sub>-PhytoP and 9-*epi*-9-F<sub>1t</sub>-PhytoP  
354 found in 13 species, 16-B<sub>1</sub>-PhytoP quantified in seven species among the 13 previous  
355 ones, and 9-L<sub>1</sub>-PhytoP detected only in two species. Considering that macroalgae do not  
356 contain exclusively ALA but also STA, AA, or EPA, it appeared interesting not to focus only  
357 on PhytoPs but to investigate also other oxygenated metabolites potentially originating  
358 from such PUFAs (P. Kumari, M. Kumar, C. R. K. Reddy, & B. Jha, 2013). Therefore, we  
359 were more interested in investigating the diversity of oxidised derivatives of PUFA  
360 containing 18 to 22 carbons rather than in conducting a study on a wide range of algal  
361 species among the *Phaeophyta* and *Rhodophyta*.

362

### 363 3.2.1 *Laminaria digitata* (Huds.) Lamouroux (Fig. 1; Table 3)

364 It was worth considering this alga because its phytoprostane production had not been  
365 investigated yet. We showed for the first time that it produces F<sub>1</sub>-, B<sub>1</sub>-, L<sub>1</sub>- and A<sub>1</sub>-PhytoPs.  
366 Identification of PhytoPs relied on retention times observed during spiked experiments,  
367 determination of molecular masses, and determination of specific MS/MS transitions.  
368 Among our results, F<sub>1</sub>-PhytoPs represented an interesting case. These metabolites are  
369 characterized by the SRM transition (m/z) 327.2 → 283.2, which is common to 9-F<sub>1t</sub>-  
370 PhytoP (+ 9-*epi*-9-F<sub>1t</sub>-PhytoP) and to *ent*-16-F<sub>1t</sub>-PhytoP (+ *ent*-16-*epi*-16-F<sub>1t</sub>-PhytoP). In  
371 order to distinguish between these compounds, the second SRM transition was  
372 considered, which is usually more specific but less sensitive. The 9-F<sub>1t</sub>-PhytoP and 9-*epi*-  
373 9-F<sub>1t</sub>-PhytoP presented the second SRM transition (m/z) 327.2 → 171.2, whereas *ent*-16-

374 F<sub>1t</sub>-PhytoP and *ent*-16-*epi*-16-F<sub>1t</sub>-PhytoP were observable by following the SRM transition  
375 (m/z) 327.2 → 151.2. Based on the combination of transitions 1 and 2, we were able to  
376 quantify 9-F<sub>1t</sub>-PhytoP and *ent*-16-*epi*-16-F<sub>1t</sub>-PhytoP. Unfortunately, due to matrix effect, the  
377 peaks separation corresponding to 9-*epi*-9-F<sub>1t</sub>-PhytoP and *ent*-16-F<sub>1t</sub>-PhytoP was  
378 ineffective, and allowed only integration of the two compounds together by following  
379 transition 1, while transition 2 value was below the quantification threshold. For B<sub>1</sub>-, L<sub>1</sub>-  
380 and A<sub>1</sub>-PhytoPs, the main transition of each series was already specific. A precursor ion at  
381 m/z 307 and a product ion at m/z 235 were observed for B<sub>1</sub>-PhytoP; a precursor ion at m/z  
382 307 and a product ion at m/z 185 for L<sub>1</sub>-PhytoP; a precursor ion at m/z 307 and a product  
383 ion at m/z 249 for A<sub>1</sub>-PhytoP. The content of these compounds reached values of 28.2  
384 ng/g and 69.2 ng/g of fresh algae for *ent*-16-B<sub>1</sub>-PhytoP and 16(*RS*)-16-A<sub>1</sub>-PhytoP,  
385 respectively.

386 PhytoPs were not the only compounds identified in *L. digitata*. Indeed, PhytoFs were  
387 detected for the first time in macroalgae. These compounds have recently been  
388 discovered in nuts, seeds, or melon leaves (Cuyamendous, Leung, Durand, Lee, Oger, &  
389 Galano, 2015; Yonny, Rodriguez Torresi, Cuyamendous, Reversat, Oger, Galano, *et al.*,  
390 2016). *Ent*-9(*RS*)-12-*epi*-ST-Δ<sup>10</sup>-13-PhytoF, *ent*-16(*RS*)-13-*epi*-ST-Δ<sup>14</sup>-9-PhytoF, and *ent*-  
391 16(*RS*)-9-*epi*-ST-Δ<sup>14</sup>-10-PhytoF were three compounds found in quantities similar to those  
392 observed for PhytoPs by following their specific SMR transitions: m/z 343.2 → m/z 237.1  
393 for the first transition, m/z 343.2 → m/z 201 for the second transition and m/z 343.2 → m/z  
394 209 for the last transition.

395 Finally, we also succeeded in quantifying the AA derivatives 15-F<sub>2t</sub>-IsoP, 15-*epi*-15-F<sub>2t</sub>-  
396 IsoP, and the mixture of the two diastereoisomers of 5-F<sub>2t</sub>-IsoP. These compounds were  
397 the most abundant in term of amounts measured, ranging from 79.1 to 342.6 ng/g of fresh  
398 algae.

399 Considering these last results, it is possible to suggest that 5-F<sub>2t</sub>-IsoP and/or 5-*epi*-5-F<sub>2t</sub>-  
400 IsoP are particularly relevant as lipid OS biomarker. Indeed, a compound present in high  
401 quantity can be more easily and reliably quantified, any concentration change being more  
402 detectable. However, before making this simple observation in *L. digitata* a rule, it is  
403 necessary to carefully analyse data gathered for many other species of macroalgae.

404

### 405 3.2.2 *Ectocarpus siliculosus* (Dillwyn) Lyngbye (Fig. 1; Table 3)

406 Analysis of *E. siliculosus* revealed the presence of PhytoPs different from those previously  
407 described (A. Ritter, et al., 2014a). Indeed, this macroalga contained F<sub>1</sub>-, L<sub>1</sub>-PhytoPs, but  
408 also PhytoFs and IsoPs. It is worth to mention the high amount of *ent*-16(*RS*)-9-*epi*-ST-  
409  $\Delta^{14}$ -10-PhytoF, with a concentration of 332.9 ng/g of fresh algae. As for *L. digitata*, the  
410 content in 5-F<sub>2t</sub>-IsoP + 5-*epi*-5-F<sub>2t</sub>-IsoP was higher than for 15-F<sub>2t</sub>-IsoP and its epimer  
411 (106.3 versus 19.1 or 21.4 ng/g of fresh algae, respectively). Based on this observation, it  
412 is possible to suggest that the two diastereoisomers of 5-F<sub>2t</sub>-IsoP are better potential OS  
413 biomarkers than 15-F<sub>2t</sub>-IsoP for *E. siliculosus*, as already described for *L. digitata*. In *E.*  
414 *siliculosus*, after taking into account each class of compounds, PhytoFs seemed to be  
415 more relevant due to higher amounts with a total of 486 ng/g of fresh algae for three  
416 metabolites, against 310 ng/g of fresh algae for six PhytoPs, and 146 ng/g of fresh algae  
417 for three IsoPs.

418

### 419 3.2.3 *Fucus spiralis* L., *Pelvetia canaliculata* (L.) Decaisne & Thuret, *Grateloupia turuturu* 420 Yamada and *Osmundea pinnatifida* (Hudson) Stackhouse (Table 3)

421 To complete the analysis on brown algae, isoprostanoids composition was also assessed  
422 in *F. spiralis* and *P. canaliculata*. These algae contained the same three PhytoFs, PhytoPs  
423 (except for series A<sub>1</sub>), and IsoPs as observed in *L. digitata* and *E. siliculosus*, but at lower  
424 concentrations.

425 Fewer compounds were observed across the two investigated red algae, *i.e.* *G. turuturu*  
426 and *O. pinnatifida*. Indeed, only 8 or 4 compounds were respectively identified in these two  
427 organisms among the 16 compounds available. PhytoPs and PhytoFs were detected in  
428 low concentration, in contrast to IsoPs in *G. turuturu*, an alga known to be rich in AA. By  
429 looking at the heterogeneity of the results, a first suggestion to explain them could be  
430 related to the efficiency of the extraction method considering important differences  
431 between the algae investigated, *e.g.* in their cell wall composition, and the fact that the  
432 optimization process was carried out only on *L. digitata* (Jimenez-Escrig, Gomez-Ordonez,  
433 & P, 2012). However, if we place these results in a different context, *i.e.* by making  
434 correlation between isoprostanoids profile and phylogenetic classification, an additional  
435 hypothesis can be put forward. Indeed, a heatmap representing the levels of  
436 isoprostanoids identified across the six seaweeds considered in our study underlined the  
437 emergence of a phylogenetic clustering, in particular with all brown algae showing similar  
438 patterns of isoprostanoid composition, but with quantitative differences (Fig. 2). We  
439 observed that the closest evolutionary relationship between *E. siliculosus* and *L. digitata*  
440 versus *P. canaliculata* and *F. spiralis* was corroborated with the heatmap clustering. Thus,  
441 this part of the study suggests that isoprostanoids signature may be used as a  
442 chemotaxonomic tool to differentiate macroalgae at the taxonomic level. Obviously, this is  
443 an assumption that will need to be tested on a larger diversity of macroalgae.

444

### 445 **3.3 Accumulation of isoprostanoids under copper stress condition**

446 The rationale supporting these experiments was based on previous reports describing that  
447 copper treatment induced oxidative stress in macroalgae, and was then expected to alter  
448 the profile and content of isoprostanoids (A. Ritter, et al., 2014a).

449 For *L. digitata*, we observed higher content for most of the detected compounds (excepted  
450 for *ent*-9-L<sub>1t</sub>-phytoP and *ent*-16-B<sub>1t</sub>-phytoP) under stress compared to control condition

451 (Fig. 3, Table 3). Quantities of PhytoFs increased by +91%, +114% and +155% for *ent-*  
452 *16(RS)-9-epi-ST- $\Delta^{14}$ -10-PhytoF*, *ent-9(RS)-12-epi-ST- $\Delta^{10}$ -13-PhytoF*, and *ent-16(RS)-13-*  
453 *epi-ST- $\Delta^{14}$ -9-PhytoF* respectively, while this trend was less marked for other categories of  
454 compounds. Conversely, the content of *ent-9-L<sub>1</sub>-PhytoP* decreased under the stress  
455 condition, and this could be explained by physiological considerations. Production of  
456 PhytoPs has been suggested to occur in the same way as for IsoPs, through hydrogen  
457 abstraction from ALA to give G<sub>1</sub>-PhytoPs isomers, which are subsequently metabolized to  
458 the detectable A-type to J-type PhytoPs. Under alkaline conditions, A- and J-types  
459 undergo isomerization of the double bond to form the thermodynamically more stable final  
460 metabolites (Jahn, Galano, & Durand, 2008) .Thus, the A-type isomerized into B-type, and  
461 the J-type led to the L-type. To explain the decrease of *ent-9-L<sub>1</sub>-PhytoP* under copper  
462 stress, a probable assumption would be the absence of alkaline conditions, making  
463 isomerization impossible, so stopping the PhytoPs pathway at the step of A- and J-types.  
464 The accumulation of *16(RS)-16-A<sub>1t</sub>-PhytoP* could corroborate this hypothesis. Another way  
465 to explain this pattern is to consider that, due to the consumption of ALA for synthesis of  
466 all PhytoPs accumulated under copper stress, there is not enough of it left to support  
467 production of the B-type and L-type PhytoPs isomer precursors, which thus decrease. To  
468 be more precise in the mechanism, we can consider the biosynthetic pathways of PhytoPs  
469 as proposed by Galano and co-workers (GALANO 2017). If we consider that PhytoPs  
470 follow the same routes as IsoPs, the G<sub>2</sub>-IsoP intermediate (endoperoxide-hydroperoxide  
471 intermediate) could represent a key metabolite for PhytoPs production that may be  
472 modulated depending on physiological conditions. Indeed, G<sub>2</sub>-IsoP could be partially (i) or  
473 completely (ii) reduced. To date, no information about the prevalence of one way  
474 compared to the other is known. In the first case (i), 15-D<sub>2</sub>-hydroperoxide is formed,  
475 leading by dehydration to the compounds 14,15-epoxyde-15-D<sub>2</sub>-IsoP (single dehydration)  
476 or 14,15-epoxyde-15-J<sub>2</sub>-IsoP (double dehydration). If these compounds are produced

477 (they were not quantified by our method), they limit the bioavailability of G2-IsoP for the  
478 second pathway. When the level of G2-IsoP decrease, less H<sub>2</sub>-IsoP can be formed and  
479 consequently less final products of this pathway accumulate. In the second case (ii), the  
480 G2-IsoP is completely reduced to produce H<sub>2</sub>-IsoP. This latter intermediate represents  
481 also a key regulation point. Indeed, as for G2-IsoP, reduction of H<sub>2</sub>-IsoP could be partial or  
482 complete. A partial reduction leads to the formation of E2-IsoP or D2-IsoP which are  
483 precursors of A2-IsoP and J2-IsoP respectively. These latter compounds are susceptible  
484 to be changed into B2-IsoP and L2-IsoP respectively under basic conditions. A complete  
485 reduction of H<sub>2</sub>-IsoP leads to the F2-IsoP family. We can suggest that copper stress  
486 induces the complete reduction of the H<sub>2</sub>-IsoP intermediate, leading to an accumulation of  
487 the F2 derivatives at the expense of B2 and L2 derivatives. So far, nothing is known about  
488 the conditions leading to a partial or total reduction of intermediates. We can only propose  
489 that high concentration of copper supports the way of a partial reduction of the G2-IsoP  
490 intermediate, and/or a complete reduction of H<sub>2</sub>-IsoP intermediate, which could explain the  
491 decrease in the formation of the derivatives B2 and L2. To finish on this, we can also  
492 suggest that copper stress, by altering the physiological status of the alga, may lead to  
493 changes in fatty acids and/or lipid metabolism that will affect the amount ALA available in  
494 algal cells, and thus influence the enzymatically and non-enzymatically production of its  
495 subsequent oxidized derivatives. In line with this, comparison between intact and wounded  
496 rice tissues has shown that ALA concentration, and not wounding by itself, was the key  
497 regulator of the octadecanoid pathway activity under stress condition(Christeller & Galis,  
498 2014).

499 Changes in isoprostanoïds content between control and stress condition were more  
500 obvious in *E. siliculosus* than in *L. digitata*. All the isoprostanoïds identified in *Ectocarpus*  
501 accumulated after copper treatment, fold changes ranging from 3 to 6 (Fig. 3, Table 3). For  
502 the two other brown algae and the two *Rhodophyta* investigated, similar observations were

503 made, with variable increase depending on the alga considered and on the PUFAs  
504 oxygenated derivatives (Table 3).

505 Interestingly, no alteration in the diversity of the molecules identified could be noticed  
506 between control and stress condition. Almost all the compounds observed under control  
507 condition were still identified after copper stress. The only exception was 16(*RS*)-16-A<sub>1</sub>-  
508 PhytoP in *E. siliculosus* that could not be quantified after heavy metal exposure due to the  
509 peak overlapping with overexpressed matrix compounds. Finally, no compound not  
510 detected under control condition was detected after copper stress. Thus, this treatment did  
511 not trigger the production of new isoprostanooids, but modified their relative quantities. It is  
512 important to draw attention to the fact that, in our targeted lipidomic approach, we have  
513 limited our analysis to non-enzymatically produced oxidized fatty acids that we were able  
514 to identify. Therefore, it cannot be ruled out that new non-targeted enzymatic and/or non-  
515 enzymatic oxylipids were produced during this treatment, and that our analysis has  
516 probably overlooked changes for some metabolites. Additional studies are necessary to  
517 deal with these aspects.

518 In light of these results, *E. siliculosus* is, among those tested, the macroalgal species for  
519 which we observed the strongest response in term of isoprostanooid profile under copper  
520 stress. Based on previous observations in land plants, this may indicate that *E. siliculosus*  
521 is well suited to support heavy metals pollution. Indeed, several PhytoPs have been shown  
522 to activate plant defence and detoxification responses. In 2003, Thoma and co-workers  
523 demonstrated the ability of cyclopentenone PhytoPs, induced by Reactive Oxygen  
524 Species, to trigger expression of genes involved in defence mechanisms and the  
525 accumulation of phytoalexin in plant cells (Thoma, Loeffler, Sinha, Gupta, Krischke,  
526 Steffan, et al., 2003). Further research of the same group strongly suggest that PhytoPs  
527 may be an endogenous mediator capable of counteracting cell damages caused by  
528 various toxicants, especially those causing severe oxidative stress (Loeffler, Berger, Guy,

529 Durand, Bringmann, Dreyer, et al., 2005; Mueller, Hilbert, Dueckershoff, Roitsch, Krischke,  
530 Mueller, et al., 2008). The ability to produce high amount of PhytoPs, compounds  
531 potentially involved in response against environmental stressors, may confer to *E.*  
532 *siliculosus* a protective role to alleviate copper-induced toxicity and thus a survival  
533 advantage.

534 Interestingly, the high accumulation of isoprostanoids observed in this alga may also  
535 account for a higher sensitivity of *E. siliculosus* to OS in this species to the other ones  
536 investigated. Such potential lethality or phytotoxicity response suggests that isoprostanoid  
537 profiling in *E. siliculosus* may serve as a possible diagnostic tool for assessing potential  
538 heavy metal pollution in the marine environment. In a more comprehensive way, we  
539 observed that most of the isoprostanoids detected under control accumulated in this alga  
540 after exposure to copper. This supports a direct correlation between this stress and the  
541 non-enzymatic production of oxidized PUFA derivatives. Our observation corroborates  
542 results previously published by Ritter *et al.* demonstrating that copper stress induced OS in  
543 the model brown alga *E. siliculosus*, as illustrated by the overlapping of transcriptomic  
544 response observed after copper and H<sub>2</sub>O<sub>2</sub> treatments (A. Ritter, et al., 2014b). The proof  
545 that we bring today is not directly related to signalling pathway, but is quite relevant  
546 because it concerns end-products of oxidation also considered as “gold” OS biomarkers.  
547 Furthermore, in recent years, it has become accepted that these molecules not only serve  
548 as biomarkers but also exhibit a wide range of bioactivities (Galano, et al., 2017). Our  
549 observations lay the ground to determine the physiological role(s) of these lipid mediators  
550 in macroalgae, for instance in signalling and/or as effectors altering gene expression.

551

## 552 **Conclusion**

553 To our knowledge, this is the first report of PhytoF production in macroalgae. Our analysis  
554 also allowed the detection of PhytoPs or IsoPs never reported so far. The *ent-16(RS)-9-*



555 *epi*-ST- $\Delta^{14}$ -10-PhytoF and the 5-F<sub>2t</sub>-IsoP epimers are ubiquitous and the most abundant of  
556 the isoprostanooids identified and quantified. This study suggests the possible use of  
557 isoprostanooid signature as a potential chemotaxonomic tool to discriminate macroalgae.  
558 Our study is also the first to establish a link between significant changes in the  
559 isoprostanooid profiles of macroalgae and heavy metal stress. For instance, the total  
560 isoprostanooid concentration in *E. siliculosus* was in the range of 945.8 ng/g and 3957.8  
561 ng/g of fresh algae before and after cupric treatment, respectively. Furthermore, these  
562 data could open prospects for the use of *E. siliculosus* as a model in the case of marine  
563 pollution and environmental emergencies. In addition, based on recent studies showing  
564 promising biological activities for PhytoPs, IsoPs and NeuroPs, (Minghetti, Salvi, Lavinia  
565 Salvatori, Ajmone-Cat, De Nuccio, Visentin, *et al.*, 2014; Noschka, Moore, Peroni, Lewis,  
566 Morrow, & Robertson, 2009; Roy, Fauconnier, Oger, Farah, Angebault-Prouteau, Thireau,  
567 *et al.*, 2017), and due to high amounts quantified in some of the tested macroalgae, it may  
568 be worth exploring these organisms as a potential natural bio-resource for extraction of  
569 these molecules, including as an alternative to their current production by complex  
570 chemical syntheses. In this context, further work should focus on assessing how  
571 manipulating culture conditions could enhance the production of isoprostanooids in  
572 macroalgae, notably by targeting the ALA biosynthetic pathway. Conditions to be tested  
573 may include nitrate depletion in the culture medium during acclimation before stress  
574 treatment, and/or alternative oxidative stress (*e.g.* H<sub>2</sub>O<sub>2</sub>). Besides nutritional interests,  
575 additional studies will be necessary to unravel the biological effects of algal isoprostanooids  
576 in humans, since they show very similar structures to the relevant bioactive IsoPs and  
577 PGs.

578

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586

### 587 **List of abbreviations**

588 AA: arachidonic acid

589 ALA: linolenic acid

590 BHT: butylated hydroxytoluene

591 DHA: docosahexaenoic acid

592 DW: dry weight

593 EI: electron ionization

594 EPA: eicosapentaenoic acid

595 ESI: electrospray ionization

596 EY: extraction yield

597 FSW: filtered sea water

598 FW: fresh weight

599 IS: internal standard

600 IsoFs: isofurans

601 IsoPs: isoprostanes

602 LC: liquid chromatography

603 LOD: limit of detection

604 LOQ: limit of quantification

605 ME: matrix effect

606 MRM: multiple reaction monitoring  
607 MS: mass spectrometry  
608 MUFA: monounsaturated fatty acid  
609 m/z: mass to charge ratios  
610 NeuroFs: neurofurans  
611 NeuroPs: neuroprostanes  
612 OS: oxidative stress  
613 PhytoFs: phytofurans  
614 PhytoPs: phytoprostanes  
615 PUFAs: polyunsaturated fatty acids  
616 ROS: reactive oxygen species  
617 SFA: saturated fatty acid  
618 SPE: solid phase extraction  
619 SM: standard mixture  
620 MRM: multiple reaction monitoring  
621 UV: ultraviolet  
622  
623

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788

789 **Figure captions:**

790

791 Fig. 1: Isoprostane, phytoprostane and metabolite content in *Laminaria digitata* (Huds.)  
792 Lamouroux and *Ectocarpus siliculosus* (Dillwyn) Lyngbye. Results are expressed as  
793 means  $\pm$  S.D. from three technical replicates per algal sample.

794

795 Fig. 2: Hierarchical cluster analysis on isoprostanoid derivatives of the six seaweeds  
796 investigated. Results are expressed as means from three technical replicates per algal  
797 sample.

798 Fig. 3: Qualitative and quantitative isoprostanoid profiles of *Laminaria digitata* (Huds.)  
799 Lamouroux and *Ectocarpus siliculosus* (Dillwyn) Lyngbye under control condition and  
800 copper stress. Results presented as box plot were obtained from three technical replicates  
801 per algal sample.

802

803

804 **Table captions:**

805

806 Table 1: Structures of some isoprostanoid isomers derived from  $\alpha$ -linolenic acid (ALA) and  
807 arachidonic acid (AA), as well as of some of the internal standards considered in our  
808 study.

809

810 Table 2: Determination of matrix effect and extraction efficiency for isoprostanoid  
811 extraction from *Laminaria digitata* (Huds.) Lamouroux. Results are expressed as means  $\pm$   
812 S.D. from three technical replicates per algal sample.

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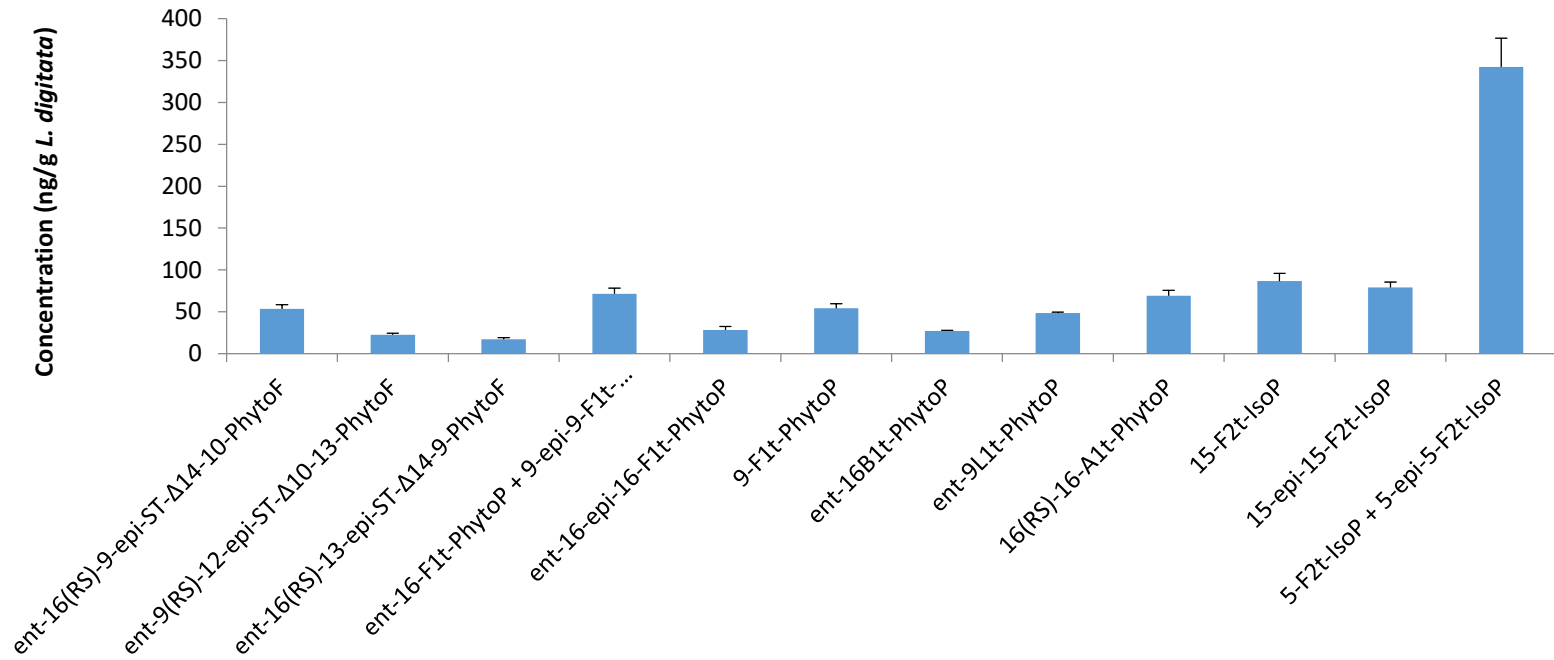
814



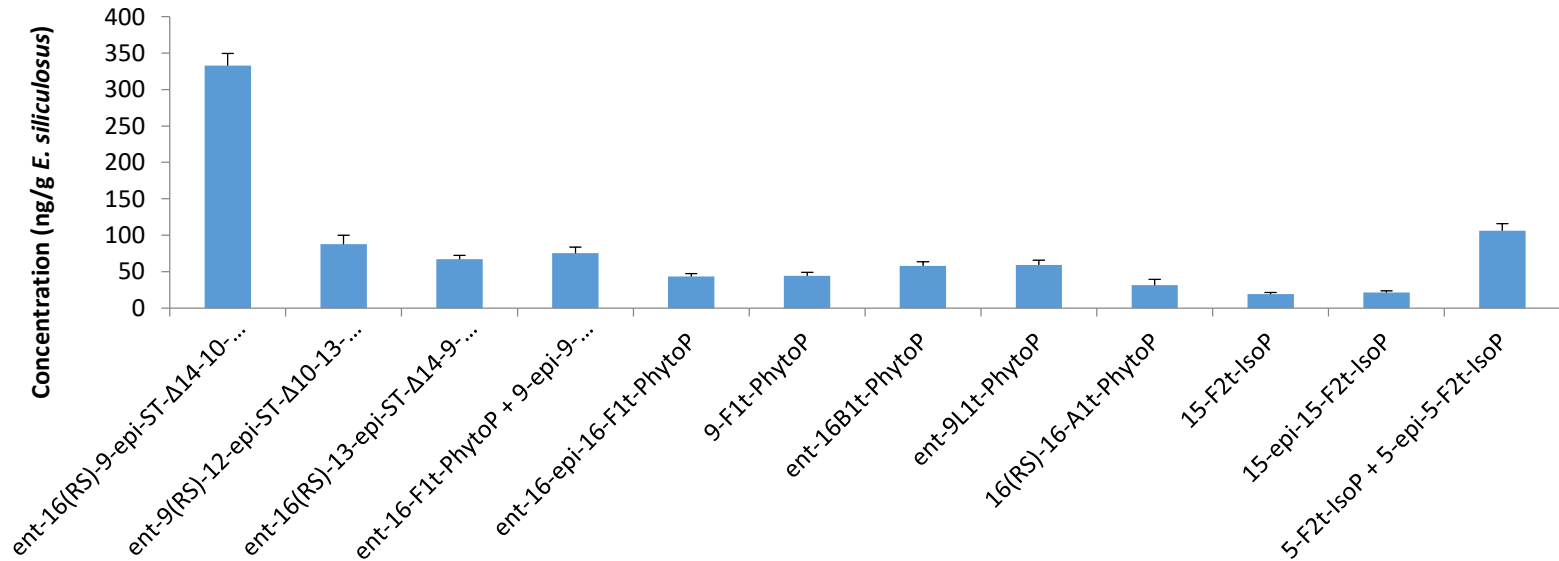
815 Table 3: Quantification of isoprostanes, phytoprostanes and metabolites in six algae  
816 incubated under control and copper stress condition based on the method described in  
817 section 2. Results are expressed as means  $\pm$  S.D. from three technical replicates per algal  
818 sample. Statistical differences between control and stress condition were tested by two-  
819 way ANOVA. The limit of statistical significance was set at  $p < 0.05$ .

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## *L. digitata*

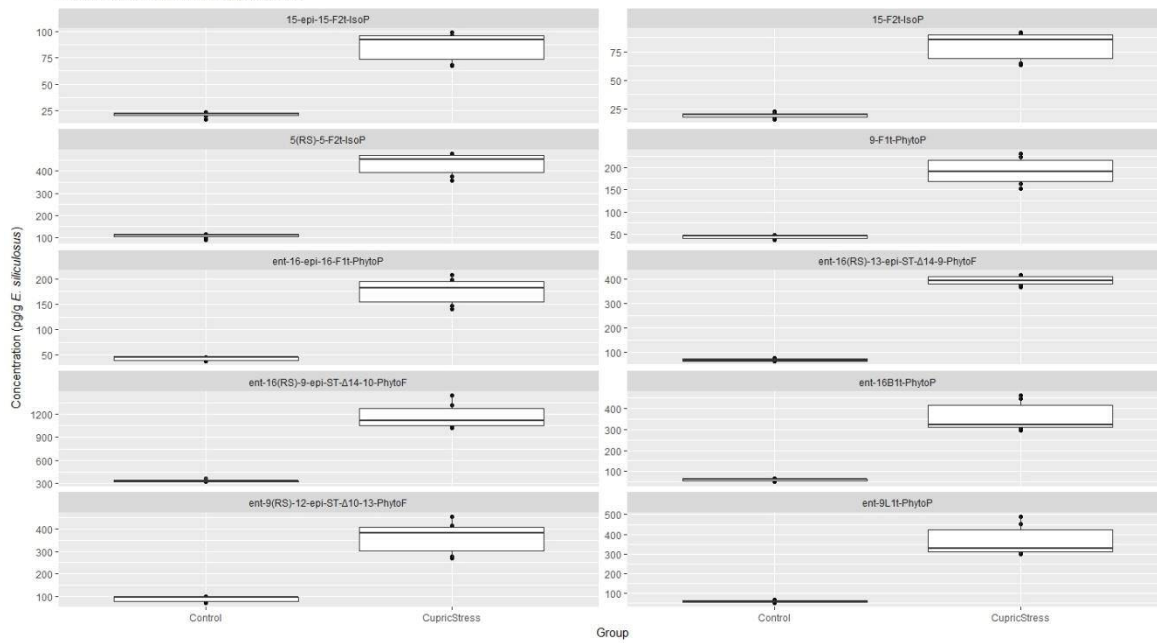


## *E. siliculosus*

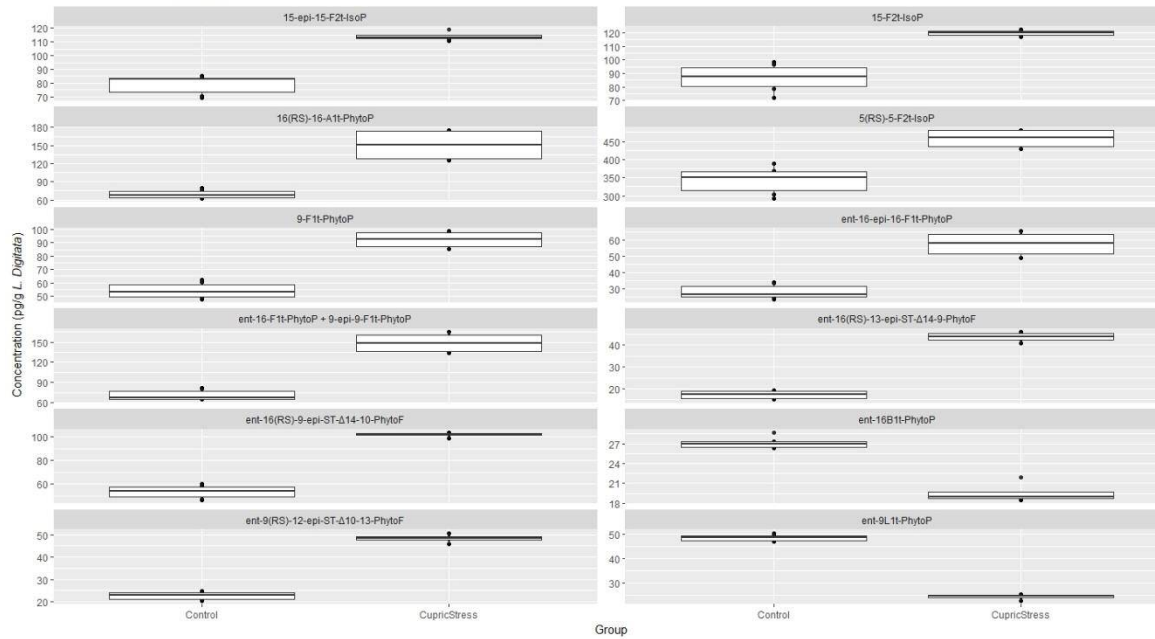


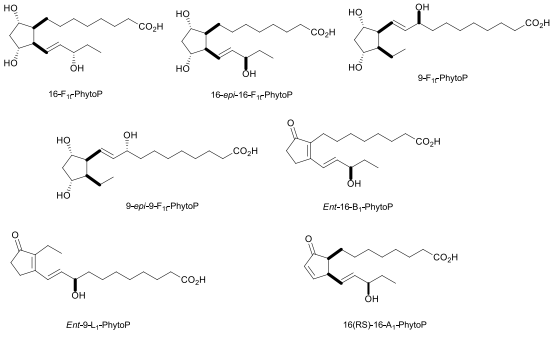
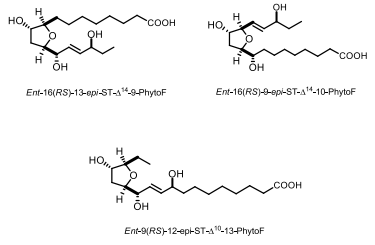
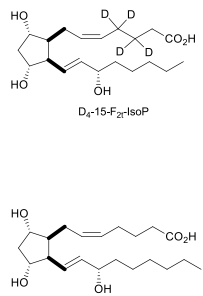
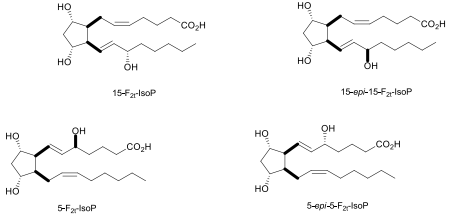
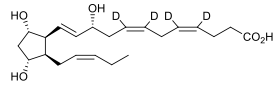
		Compounds (ng/g)											
		PhytoFs			PhytoPs						IsoPs		
		<i>ent</i> -16(RS)-9- <i>epi</i> -ST- $\Delta^{14}$ -10-PhytoF	<i>ent</i> -9(RS)-12- <i>epi</i> -ST- $\Delta^{10}$ -13-PhytoF	<i>ent</i> -16(RS)-13- <i>epi</i> -ST- $\Delta^{14}$ -9-PhytoF	<i>ent</i> -16-F <sub>1t</sub> -PhytoP + 9- <i>epi</i> -9-F <sub>1t</sub> -PhytoP	<i>ent</i> -16- <i>epi</i> -16-F <sub>1t</sub> -PhytoP	9-F <sub>1t</sub> -PhytoP	<i>ent</i> -16-B <sub>1t</sub> -PhytoP	<i>ent</i> -9-L <sub>1t</sub> -PhytoP	16(RS)-16-A <sub>1t</sub> -PhytoP	15-F <sub>2t</sub> -IsoP	15- <i>epi</i> -15-F <sub>2t</sub> -IsoP	5-F <sub>2t</sub> -IsoP + 5- <i>epi</i> -5-F <sub>2t</sub> -IsoP
Brown algae	<i>Ectocarpus siliculosus</i>	332.88	87.80	67.25	75.28	43.47	44.29	57.60	59.10	31.23	19.10	21.42	106.30
	<i>Laminaria digitata</i>	53.35	22.63	17.16	71.23	28.18	54.26	27.08	48.47	69.24	86.66	79.09	342.60
	<i>Pelvetia canaliculata</i>	42.40	17.86	9.77	20.65	6.34	14.74	13.44	23.34	0.00	37.49	31.13	99.55
	<i>Fucus spiralis</i>	25.32	7.18	8.64	8.66	4.03	5.24	5.07	5.91	0.00	5.79	5.15	27.41
Red algae	<i>Osmundea pinnatifida</i>	11.13	0.00	0.00	6.58	2.89	3.30	0.00	0.00	0.00	0.00	0.00	0.00
	<i>Grateloupia turuturu</i>	2.48	0.00	3.49	18.42	8.96	9.59	0.00	0.00	0.00	112.78	93.35	689.56

*E. siliculosus* : Control Vs. Cupric Stress



*L. Digitata* : Control Vs. Cupric Stress



Parent PUFAs	Isoprostanoid compounds	Isofuranoid compounds	Internal Standard (IS)
<p><b><math>\alpha</math>-linolenic Acid (ALA)</b></p>	 <p>16-F<sub>1r</sub>-PhytoP 16-epi-16-F<sub>1r</sub>-PhytoP 9-F<sub>1r</sub>-PhytoP 9-epi-9-F<sub>1r</sub>-PhytoP Ent-16-B<sub>1</sub>-PhytoP Ent-9-L<sub>1</sub>-PhytoP 16(RS)-16-A<sub>1</sub>-PhytoP</p>	 <p>Ent-16(RS)-13-epi-ST-1<sup>14</sup>-9-PhytoF Ent-16(RS)-9-epi-ST-1<sup>14</sup>-10-PhytoF Ent-9(RS)-12-epi-ST-1<sup>13</sup>-13-PhytoF</p>	 <p>D<sub>4</sub>-15-F<sub>2r</sub>-IsoP C<sub>21</sub>-15-F<sub>2r</sub>-IsoP</p>
<p><b>Arachidonic Acid (AA)</b></p>	 <p>15-F<sub>2r</sub>-IsoP 15-epi-15-F<sub>2r</sub>-IsoP 5-F<sub>2r</sub>-IsoP 5-epi-5-F<sub>2r</sub>-IsoP</p>		 <p>D<sub>4</sub>-10-epi-10-F<sub>4r</sub>-NeuroP</p>

IS	Compounds	Concentration	Extraction Yield		Matrix Effect		Total Extraction Yield	
			±	SD	±	SD	±	SD
	D4-10(R)-10F <sub>4t</sub> NeuroP	300 µg	93,54	± 3,52	63,92	± 6,56	56,97	± 2,75
	D4-15-F <sub>2t</sub> -IsoP	300 µg	89,81	± 4,41	68,71	± 5,47	53,19	± 2,55
	C21 15-F <sub>2t</sub> -IsoP	300 µg	77,10	± 8,43	43,83	± 6,34	42,12	± 4,03
	<i>ent</i> -16(RS)-9- <i>epi</i> -ST-Δ <sup>14</sup> -10-PhytoF	SM <sub>32</sub>	99,17%	± 2,43%	159,11	± 5,16%	67,54%	± 2,44%
		SM <sub>256</sub>	88,48%	± 4,08%	124,76	± 7,44%	54,25%	± 2,93%
	<i>ent</i> -9(RS)-12- <i>epi</i> -ST-Δ <sup>10</sup> -13-PhytoF	SM <sub>32</sub>	100,60%	± 3,63%	119,06	± 4,77%	56,40%	± 1,97%
		SM <sub>256</sub>	88,27%	± 4,03%	119,06	± 7,55%	46,83%	± 3,13%
	<i>ent</i> -16(RS)-13- <i>epi</i> -ST-Δ <sup>14</sup> -9-PhytoF	SM <sub>32</sub>	103,83%	± 2,21%	144,98	± 4,37%	66,44%	± 2,53%
		SM <sub>256</sub>	90,15%	± 4,22%	122,76	± 8,15%	49,21%	± 2,91%
	<i>ent</i> -16-F <sub>1t</sub> -PhytoP	SM <sub>32</sub>	108,03%	± 3,29%	92,36	± 3,30%	55,67%	± 3,02%
		SM <sub>256</sub>	87,30%	± 9,02%	97,03	± 11,57%	46,48%	± 5,00%
	<i>ent</i> -16- <i>epi</i> -16-F <sub>1t</sub> -PhytoP	SM <sub>32</sub>	99,62%	± 3,79%	135,16	± 4,36%	52,89%	± 4,61%
		SM <sub>256</sub>	86,92%	± 3,79%	123,57	± 7,24%	44,82%	± 3,48%
	9-F <sub>1t</sub> -PhytoP	SM <sub>32</sub>	93,36%	± 2,46%	169,63	± 2,52%	61,21%	± 3,79%
		SM <sub>256</sub>	87,10%	± 3,45%	123,73	± 7,33%	46,02%	± 3,31%
	9- <i>epi</i> -9-F <sub>1t</sub> -PhytoP	SM <sub>32</sub>	94,25%	± 1,85%	204,81	± 4,93%	61,73%	± 7,77%
		SM <sub>256</sub>	86,10%	± 3,89%	125,18	± 7,71%	46,93%	± 3,06%
	<i>ent</i> -16B <sub>1t</sub> -PhytoP	SM <sub>32</sub>	115,63%	± 3,01%	98,46	± 5,53%	90,44%	± 5,06%
		SM <sub>256</sub>	87,61%	± 3,77%	91,42	± 7,91%	81,16%	± 2,96%
	<i>ent</i> -9L <sub>1t</sub> -PhytoP	SM <sub>32</sub>	128,95%	± 3,74%	119,84	± 5,12%	99,50%	± 6,44%
		SM <sub>256</sub>	96,08%	± 4,43%	94,17	± 6,99%	92,82%	± 3,38%
	16(RS)-16-A <sub>1t</sub> -PhytoP	SM <sub>32</sub>	68,63%	± 6,98%	160,94	± 8,37%	50,74%	± 7,95%
		SM <sub>256</sub>	15,02%	± 3,30%	104,48	± 8,37%	9,48%	± 6,57%
	15-F <sub>2t</sub> -IsoP	SM <sub>32</sub>	116,17%	± 2,97%	155,19	± 4,52%	78,84%	± 7,22%
		SM <sub>256</sub>	109,46%	± 2,76%	97,12	± 7,75%	68,27%	± 2,52%
	15- <i>epi</i> -15-F <sub>2t</sub> -IsoP	SM <sub>32</sub>	97,23%	± 2,42%	142,96	± 2,57%	67,01%	± 6,64%
		SM <sub>256</sub>	79,82%	± 2,66%	98,44	± 7,52%	52,30%	± 2,38%
	5-F <sub>2t</sub> -IsoP + 5- <i>epi</i> -5-F <sub>2t</sub> -IsoP	SM <sub>32</sub>	144,44%	± 3,08%	224,53	± 4,88%	99,55%	± 8,46%
		SM <sub>256</sub>	161,69%	± 1,03%	100,87	± 6,70%	108,83%	± 1,15%

Compounds	Group	<i>L. digitata</i> ng/g	±	SD	p-value	<i>E. siliculosus</i> ng/g	±	SD	p-value	<i>F. spiralis</i> ng/g	±	SD	p-value	<i>P. canaliculata</i> ng/g	±	SD	p-value	<i>O. pinnatifida</i> ng/g	±	SD	p-value	<i>G. turururu</i> ng/g	±	SD	p-value
ent-16(RS)-9-epi-ST-Δ <sup>14</sup> -10-PhytoF	Control	53,35	±	5,19	<0,0001	332,88	±	16,86	<0,0001	25,32	±	2,49	<0,0001	42,40	±	7,10	<0,0001	11,13	±	1,38	<0,0001	2,48	±	0,61	>0,9999
	Cupric Stress	101,82	±	1,75		1176,45	±	152,82		51,08	±	11,24		86,28	±	14,77		36,84	±	4,87		2,43	±	0,58	
ent-9(RS)-12-epi-ST-Δ <sup>16</sup> -13-PhytoF	Control	22,63	±	1,65	0,0182	87,80	±	12,09	<0,0001	7,18	±	1,09	0,0764	17,86	±	2,95	<0,0001	NQ	±	NQ	<0,0001	NQ	±	NQ	>0,9999
	Cupric Stress	48,39	±	1,80		363,69	±	68,37		13,71	±	1,73		43,83	±	9,31		11,23	±	2,70		NQ	±	NQ	
ent-16(RS)-13-epi-ST-Δ <sup>14</sup> -9-PhytoF	Control	17,16	±	1,67	0,0127	67,25	±	5,01	<0,0001	8,64	±	1,18	0,0098	9,77	±	2,27	0,004	NQ	±	NQ	<0,0001	3,49	±	0,92	>0,9999
	Cupric Stress	43,81	±	2,01		395,71	±	18,57		16,76	±	2,55		27,55	±	7,88		13,47	±	1,98		4,50	±	1,44	
ent-16-F <sub>11</sub> -PhytoP + 9-epi-9-F <sub>11</sub> -PhytoP	Control	71,23	±	7,10	<0,0001	75,28	±	8,66	<0,0001	8,66	±	2,34	0,6635	20,65	±	2,64	0,1466	6,58	±	0,80	<0,0001	18,42	±	2,68	0,5355
	Cupric Stress	149,03	±	13,48		326,04	±	47,63		12,73	±	2,68		32,76	±	5,41		13,87	±	2,40		56,90	±	13,82	
ent-16-epi-16-F <sub>11</sub> -PhytoP	Control	28,18	±	4,14	0,0036	43,47	±	3,62	<0,0001	4,03	±	1,08	0,9987	6,34	±	0,85	0,9933	2,89	±	0,36	0,0062	8,96	±	1,75	0,9931
	Cupric Stress	57,78	±	6,92		176,14	±	24,78		5,90	±	1,19		10,94	±	1,61		6,01	±	1,10		28,54	±	6,46	
9-F <sub>11</sub> -PhytoP	Control	54,26	±	5,38	<0,0001	44,29	±	4,82	<0,0001	5,24	±	1,37	0,9987	14,74	±	1,39	0,2473	3,30	±	0,40	0,0003	9,59	±	1,68	0,9933
	Cupric Stress	92,33	±	5,78		190,89	±	28,98		7,12	±	1,44		25,79	±	2,96		7,13	±	1,03		29,10	±	6,14	
ent-16B <sub>11</sub> -PhytoP	Control	27,08	±	0,82	0,9932	57,60	±	5,86	<0,0001	5,07	±	0,50	0,0055	13,44	±	1,77	0,0004	NQ	±	NQ	>0,9999	NQ	±	NQ	>0,9999
	Cupric Stress	19,52	±	1,38		359,78	±	68,16		13,60	±	2,46		34,30	±	7,29		NQ	±	NQ		NQ	±	NQ	
ent-9L <sub>11</sub> -PhytoP	Control	48,47	±	1,18	0,0355	59,10	±	6,46	<0,0001	5,91	±	0,71	0,0102	23,34	±	2,49	<0,0001	NQ	±	NQ	>0,9999	NQ	±	NQ	>0,9999
	Cupric Stress	24,43	±	1,05		367,26	±	75,45		14,01	±	2,47		62,16	±	11,90		NQ	±	NQ		NQ	±	NQ	
16(RS)-16-A <sub>11</sub> -PhytoP	Control	69,24	±	6,33	<0,0001	31,23	±	7,91	0,9734	NQ	±	NQ	>0,9999	NQ	±	NQ	>0,9999	NQ	±	NQ	>0,9999	NQ	±	NQ	>0,9999
	Cupric Stress	150,42	±	23,46		NQ	±	NQ		NQ	±	NQ		NQ	±	NQ		NQ	±	NQ		NQ	±	NQ	
15-F <sub>22</sub> -IsoP	Control	86,66	±	9,32	0,0007	19,10	±	2,29	0,2968	5,79	±	1,39	0,4331	37,49	±	3,68	<0,0001	NQ	±	NQ	>0,9999	112,78	±	18,82	0,0011
	Cupric Stress	119,77	±	2,08		80,23	±	11,72		10,54	±	1,62		69,02	±	6,97		NQ	±	NQ		195,80	±	32,68	
15-epi-15-F <sub>22</sub> -IsoP	Control	79,09	±	6,43	0,0003	21,42	±	2,29	0,2319	5,15	±	0,92	0,6394	31,13	±	2,55	<0,0001	NQ	±	NQ	>0,9999	93,35	±	11,54	0,8049
	Cupric Stress	113,83	±	2,94		85,70	±	12,89		9,29	±	2,07		59,42	±	6,31		NQ	±	NQ		124,69	±	19,10	
5-F <sub>22</sub> -IsoP + 5-epi-5-F <sub>22</sub> -IsoP	Control	342,60	±	34,25	<0,0001	106,30	±	9,61	<0,0001	27,41	±	4,91	<0,0001	99,55	±	9,86	<0,0001	NQ	±	NQ	>0,9999	689,56	±	40,74	0,0104
	Cupric Stress	456,87	±	23,30		432,68	±	48,11		57,49	±	9,23		156,84	±	22,28		NQ	±	NQ		619,76	±	139,46	