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Isoprostanoids quantitative profiling of marine red and brown macroalgae

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Abstract

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

Keywords

Macroalgae, isoprostanoids, heavy metal, copper stress, oxylipins, micro-LC-MS/MS

1-Introduction

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

Most of the wild populations of macroalgae are thriving in the intertidal and near subtidal zone, a highly and frequently changing environment, and thus experience repeated biotic and abiotic (temperature, irradiation, salinity, …) fluctuations that require physiological plasticity for stress tolerance (Dittami, Gravot, Renault, Goulitquer, Eggert, Bouchereau, et al., 2011). Among potential stress factors, exposure to high level of heavy metals is very common, as illustrated by numerous studies conducted so far on different macroalgae (Collen, Pinto, Pedersen, & Colepicolo, 2003; Pinto, Sigaud-Kutner, Leitao, Okamoto, Morse, & Colepicolo, 2003; Roncarati, Sáez, Greco, Gledhill, Bitonti, & Brown, 2015; Saez, 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_2O_2 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 α-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_{1t}-PhytoP, 9-*epi*-9-F_{1t}-PhytoP, 16-B_1-PhytoP, and 9-L_1-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. Isoprostanoid 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

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

2-Material and methods

2.1 Chemicals and reagents

The phytoprostane standard 16-(*RS*)-16-A_1-PhytoP and the deuterated internal standard (IS) d_4-15-F_{2t}-IsoP were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The two IS d_4-10-F_{4t}-NeuroP and C21-15-F_{2t}-IsoP, as well as all the other standards, were synthesized according to previous procedures (Cuyamendous, Leung, Durand, Lee, Oger, & Galano, 2015; Thierry Durand, Cracowski, Guy, & Rossi, 2001; T. Durand, Guy, Vidal, & Rossi, 2002; El Fangour, Guy, Despres, Vidal, Rossi, & Durand, 2004; El Fangour, Guy, Vidal, Rossi, & Durand, 2005; Guy, Flanagan, Durand, Oger, & Galano, 2015; Guy, Oger, Hepekauzen, Signorini, Durand, De Felice, et al., 2014; Oger, Brinkmann, Bouazzaoui, Durand, & Galano, 2008; Oger, Bultel-Poncé, Guy, Balas, Rossi, Durand, et al., 2010). This concerns 9-F_{1t}-PhytoP, 9-*epi*-F_{1t}-PhytoP, *ent*-16-F_{1t}-PhytoP, *ent*-16-*epi*-16-F_{1t}-PhytoP, *ent*-16-B_1-PhytoP, *ent*-9-L_1-PhytoP, as PhytoPs, and *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, and *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF as PhytoFs. Four IsoPs were also evaluated: 15-F_{2t}-IsoP, 15-*epi*-15-F_{2t}-IsoP, 5-F_{2t}-IsoP and 5-*epi*-5-F_{2t}-IsoP. Finally, three NeuroPs were considered: 10-F_{4t}-NeuroP, 10-*epi*-10-F_{4t}-NeuroP, and 4(*RS*)-4-F_{4t}-NeuroP. Stock solutions of standards were prepared in methanol to a concentration of 100 ng.µL^{-1}, and were stored at −20 °C. Appropriate dilutions from the mentioned stock were prepared for calibration purpose. Furthermore, two different solutions of a Standard Mixture (SM) of the 18 compounds mentioned above (SM_{32} = each compound at 32 ng.ml^{-1} or SM_{256} = each compound at 256 ng.ml^{-1}) were made up for validation purpose (extraction yield and matrix effect).

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

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

2.2 Macroalgal samples

2.2.1 Collection

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

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

Young sporophytes of *L. digitata* (*ca* 10-20 cm in length) were also collected in the intertidal zone close to the Station Biologique of Roscoff, and maintained in 10 L flasks in autoclaved filtered seawater (FSW), at 13°C, under a photoperiod of 16 h of light (40 µmol photons.m^{-2}.s^{-1}) and 8 h of darkness, and well-aerated with filtered (0.22 µm) compressed 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 $CuCl_2$ (Titrisol, Merck) at nominal final concentration of 300 $\mu g.L^{-1}$ (15 µL of a 20 $g.L^{-1}$
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 $CuCl_2$
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 shaked 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_2 at 40 °C. To perform hydrolysis of samples, the dry extract
200 was dissolved into 950 µL of KOH (1 M in H_2O), 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_3
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_{18} 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^{-1} using

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

Mass spectrometry analyses were performed on an AB SCIEX QTRAP 5500 (Sciex Applied Biosystems, ON, Canada). The ionization source was electrospray (ESI), and it was operated in the negative mode. The source voltage was kept at −4.5 kV, and N_2 was used as curtain gas. Detection of the fragmentation ion products from each PhytoP, PhytoF, or IsoP deprotonated molecule $[M - H]^-$ was performed in the multiple reaction monitoring mode (MRM). The MS parameters were individually optimized for each compound.

2.4 Characterization method

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

2.4.1 Validation of sample preparation

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

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

244 standards) by comparing peak area of set 1 (spike before SPE) *versus* set 2 (spike after
245 SPE). Result is expressed in percentage by the following calculation: EY= A_{set1}/A_{set2}*100.
246 The matrix effect (ME), also expressed in percentage and evaluated for each compound,
247 was determined as the difference between peak areas obtained for the standards added to
248 the extracted samples (set 2) and pure standard (set 3). The calculation is: ME =
249 A_{set2}/A_{set3}*100.
250
251 *2.4.2 Sensitivity linearity and quantification*
252 In order to determine the sensitivity of the analytical method we evaluated the limit of
253 detection (LOD) and the limit of quantification (LOQ) for each compound. These values
254 corresponded respectively to 3 and 10 times the signal-to-noise ratio. The linearity of the
255 response was evaluated using 15 concentrations of compounds (in triplicate). Calibration
256 curves were calculated by the least-squares linear regression method, and linearity was
257 determined to range between $3.125x10^{-3}$ and 512 $pg.\mu L^{-1}$ for compounds injected in
258 column. Analytes quantification was based on the analyte to IS ratio using the obtained
259 calibration curves. Data processing was achieved using the MultiQuant 3.0 software (Sciex
260 Applied Biosystems).
261
262 *2.4.3 Accuracy and precision*
263 Trueness, precision and accuracy were determined by validation standard analysis
264 performed in triplicate at defined concentrations, and on two different days. Intra-batch
265 reflects intra-day precision or repeatability, and inter-batch the inter-day precision or
266 reproducibility. These parameters express the error of the analytical measurement.
267
268 ***2.5 Statistical Analyses***
269 Standard deviation (SD) and relative standard deviation (RSD) were used to determine

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

3-Results

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

3.1 Sample processing validation on L. digitata

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

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

compounds (PhytoPs, PhytoFs, IsoPs or NeuroPs), no specific trend could be noticed, *i.e.* some PhytoPs exhibited good recovery ratio (*e.g. ent*-16-*epi*-16-F_{1t}-PhytoP; EY_{32}= 99.6%), while it was lower for other ones (*e.g.* 16(*RS*)-16-A_1-PhytoP; EY_{32}= 68.6%). The extraction yield calculated was more than 100% for some analytes, corresponding probably to the co-elution of a compound that presents the same MRM transition. It is the case for instance for the mixture of 5-F_{2t}-IsoP and 5-*epi*-F_{2t}-IsoP with an EY of 144.4% at 32 pg.µL^{-1}. We could also notice that EYs were better at low than at high concentrations. To complete this validation, the matrix effect, corresponding to an ion-suppression/enhancement of co-eluted matrix compounds, was evaluated. As for EY, ME is specific to each isoprostanoids, and there was no similar behaviour across the same class of compounds. We observed a clearly marked effect at low concentration, for instance for 9-F_{1t}-PhytoP and 9-*epi*-F_{1t}-PhytoP between SM_{32} and SM_{256} conditions. Indeed, ME values were 169% (SM_{32}) and 123% (SM_{256}) for the first compound, and 204% (SM_{32}) and 125% (SM_{256}) for the second one.

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

Finally, for testing the repeatability and precision of the method, the intra- and inter-day analysis of two selected concentrations (SM_{32} and SM_{256}) was performed. Among the twenty compounds tested, the majority presented an intra-day variation lower than 2%, exceptions being 16(*RS*)-16-A_1-PhytoP (2.2%), 5(*RS*)-5-F_{2t}-I*s*oP + 5-*epi*-5-F_{2t}-I*s*oP (2.7%), and 4(*RS*)-4-F_{4t}-NeuroP (5.6%). Mean inter-day variation was 9.9%, with 7.8% and 13.7%

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

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

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

348 composition subsequently resulted in occurence 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_{1t}-PhytoP and 9-*epi*-9-F_{1t}-PhytoP
354 found in 13 species, 16-B_1-PhytoP quantified in seven species among the 13 previous
355 ones, and 9-L_1-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_1-, B_1-, L_1- and A_1-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_1-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_{1t}-
370 PhytoP (+ 9-*epi*-9-F_{1t}-PhytoP) and to *ent*-16-F_{1t}-PhytoP (+ *ent*-16-*epi*-16-F_{1t}-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_{1t}-PhytoP and 9-*epi*-
373 9-F_{1t}-PhytoP presented the second SRM transition (m/z) 327.2 → 171.2, whereas *ent*-16-

374 F_{1t}-PhytoP and *ent*-16-*epi*-16-F_{1t}-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_{1t}-PhytoP and *ent*-16-*epi*-16-F_{1t}-PhytoP. Unfortunately, due to matrix effect, the
377 peaks separation corresponding to 9-*epi*-9-F_{1t}-PhytoP and *ent*-16-F_{1t}-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_1-, L_1-
380 and A_1-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_1-PhytoP; a precursor ion at m/z
382 307 and a product ion at m/z 185 for L_1-PhytoP; a precursor ion at m/z 307 and a product
383 ion at m/z 249 for A_1-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_1-PhytoP and 16(*RS*)-16-A_1-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^{10}-13-PhytoF, *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF, and *ent*-
391 16(*RS*)-9-*epi*-ST-Δ^{14}-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_{2t}-IsoP, 15-*epi*-15-F_{2t}-
396 IsoP, and the mixture of the two diastereoisomers of 5-F_{2t}-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_{2t}-IsoP and/or 5-*epi*-5-F_{2t}-
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_1-, L_1-PhytoPs, but
408 also PhytoFs and IsoPs. It is worth to mention the high amount of *ent*-16(*RS*)-9-*epi*-ST-
409 Δ^{14}-10-PhytoF, with a concentration of 332.9 ng/g of fresh algae. As for *L. digitata*, the
410 content in 5-F_{2t}-IsoP + 5-*epi*-5-F_{2t}-IsoP was higher than for 15-F_{2t}-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_{2t}-IsoP are better potential OS
413 biomarkers than 15-F_{2t}-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_1), 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 macrolagae 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_{1t}-phytoP and *ent*-16-B_{1t}-phytoP) under stress compared to control condition

(Fig. 3, Table 3). Quantities of PhytoFs increased by +91%, +114% and +155% for *ent*-16(*RS*)-9-*epi*-ST-Δ^{14}-10-PhytoF, *ent*-9(*RS*)-12-*epi*-ST-Δ^{10}-13-PhytoF, and *ent*-16(*RS*)-13-*epi*-ST-Δ^{14}-9-PhytoF respectively, while this trend was less marked for other categories of compounds. Conversely, the content of *ent*-9-L_1-PhytoP decreased under the stress condition, and this could be explained by physiological considerations. Production of PhytoPs has been suggested to occur in the same way as for IsoPs, through hydrogen abstraction from ALA to give G_1-PhytoPs isomers, which are subsequently metabolized to the detectable A-type to J-type PhytoPs. Under alkaline conditions, A- and J-types undergo isomerization of the double bond to form the thermodynamically more stable final metabolites (Jahn, Galano, & Durand, 2008) .Thus, the A-type isomerized into B-type, and the J-type led to the L-type. To explain the decrease of *ent*-9-L_1-PhytoP under copper stress, a probable assumption would be the absence of alkaline conditions, making isomerization impossible, so stopping the PhytoPs pathway at the step of A- and J-types. The accumulation of 16(*RS*)-16-A_{1t}-PhytoP could corroborate this hypothesis. Another way to explain this pattern is to consider that, due to the consumption of ALA for synthesis of all PhytoPs accumulated under copper stress, there is not enough of it left to support production of the B-type and L-type PhytoPs isomer precursors, which thus decrease. To be more precise in the mechanism, we can consider the biosynthetic pathways of PhytoPs as proposed by Galano and co-workers (GALANO 2017). If we consider that PhytoPs follow the same routes as IsoPs, the G2-IsoP intermediate (endoperoxide-hydroperoxide intermediate) could represent a key metabolite for PhytoPs production that may be modulated depending on physiological conditions. Indeed, G2-IsoP could be partially (i) or completely (ii) reduced. To date, no information about the prevalence of one way compared to the other is known. In the first case (i), 15-D_2-hydroperoxide is formed, leading by dehydration to the compounds 14,15-epoxyde-15-D_2-IsoP (single dehydration) or 14,15-epoxyde-15-J_2-IsoP (double dehydration). If these compounds are produced

(they were not quantified by our method), they limit the bioavailability of G2-IsoP for the second pathway. When the level of G2-IsoP decrease, less H_2-IsoP can be formed and consequently less final products of this pathway accumulate. In the second case (ii), the G2-IsoP is completely reduced to produce H_2-IsoP. This latter intermediate represents also a key regulation point. Indeed, as for G2-IsoP, reduction of H_2-IsoP could be partial or complete. A partial reduction leads to the formation of E2-IsoP or D2-IsoP which are precursors of A2-IsoP and J2-IsoP respectively. These latter compounds are susceptible to be changed into B2-IsoP and L2-IsoP respectively under basic conditions. A complete reduction of H_2-IsoP leads to the F2-IsoP family. We can suggest that copper stress induces the complete reduction of the H2-IsoP intermediate, leading to an accumulation of the F2 derivatives at the expense of B2 and L2 derivatives. So far, nothing is known about the conditions leading to a partial or total reduction of intermediates. We can only propose that high concentration of copper supports the way of a partial reduction of the G2-IsoP intermediate, and/or a complete reduction of H2-IsoP intermediate, which could explain the decrease in the formation of the derivatives B2 and L2. To finish on this, we can also suggest that copper stress, by altering the physiological status of the alga, may lead to changes in fatty acids and/or lipid metabolism that will affect the amount ALA available in algal cells, and thus influence the enzymatically and non-enzymatically production of its subsequent oxidized derivatives. In line with this, comparison between intact and wounded rice tissues has shown that ALA concentration, and not wounding by itself, was the key regulator of the octadecanoid pathway activity under stress condition(Christeller & Galis, 2014).

Changes in isoprostanoids content between control and stress condition were more obvious in *E. siliculosus* than in *L. digitata*. All the isoprostanoids identified in *Ectocarpus* accumulated after copper treatment, fold changes ranging from 3 to 6 (Fig. 3, Table 3). For 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_1-
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 isoprostanoids, 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 isoprostanoid 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_2O_2 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-Δ^{14}-10-PhytoF and the 5-F_{2t}-IsoP epimers are ubiquitous and the most abundant of
556 the isoprostanoids identified and quantified. This study suggests the possible use of
557 isoprostanoid 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 isoprostanoid profiles of macroalgae and heavy metal stress. For instance, the total
560 isoprostanoid 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 isoprostanoids 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_2O_2). Besides nutritional interests,
575 additional studies will be necessary to unravel the biological effects of algal isoprostanoids
576 in humans, since they show very similar structures to the relevant bioactive IsoPs and
577 PGs.
578
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List of abbreviations

AA: arachidonic acid

ALA: linolenic acid

BHT: butylated hydroxytoluene

DHA: docosahexaenoic acid

DW: dry weight

EI: electron ionization

EPA: eicosapentaenoic acid

ESI: electrospray ionization

EY: extraction yield

FSW: filtered sea water

FW: fresh weight

IS: internal standard

IsoFs: isofurans

IsoPs: isoprostanes

LC: liquid chromatography

LOD: limit of detection

LOQ: limit of quantification

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

624 REFERENCES

625

626 Aaronson, S. (1986). A role for algae as human food in antiquity. *Food and Foodways, 1*,
627 311-315.
628 Barbosa, M., Collado-Gonzalez, J., Andrade, P. B., Ferreres, F., Valentao, P., Galano, J.
629 M., Durand, T., & Gil-Izquierdo, A. (2015). Nonenzymatic alpha-linolenic acid
630 derivatives from the sea: aacroalgae as novel sources of phytoprostanes. *Journal of*
631 *agricultural and food chemistry, 63*(28), 6466-6474.
632 Brodie, J., Chan, C. X., De Clerck, O., Cock, J. M., Coelho, S. M., Gachon, C., Grossman,
633 A. R., Mock, T., Raven, J. A., Smith, A. G., Yoon, H. S., & Bhattacharya, D. (2017).
634 The algal revolution. *Trends in plant science, 22*(8), 726-738.
635 Christeller, J. T., & Galis, I. (2014). α-Linolenic acid concentration and not wounding per se
636 is the key regulator of octadecanoid (oxylipin) pathway activity in rice (*Oryza sativa*
637 L.) leaves. *Plant Physiology and Biochemistry, 83*, 117-125.
638 Cock, J. M., & Coelho, S. M. (2011). Algal models in plant biology. *Journal of experimental*
639 *botany, 62*(8), 2425-2430.
640 Collen, J., Pinto, E., Pedersen, M., & Colepicolo, P. (2003). Induction of oxidative stress in
641 the red macroalga *Gracilaria tenuistipitata* by pollutant metals. *Archives of*
642 *environmental contamination and toxicology, 45*(3), 337-342.
643 Craigie, J. S. (2010). Seaweed extract stimuli in plant science and agriculture. *J. Appl.*
644 *Phycol., 23*(3), 371-393.
645 Cuyamendous, C., Leung, K. S., Durand, T., Lee, J. C., Oger, C., & Galano, J. M. (2015).
646 Synthesis and discovery of phytofurans: metabolites of alpha-linolenic acid
647 peroxidation. *Chemical communications, 51*(86), 15696-15699.
648 Dillehay, T. D., Ramirez, C., Pino, M., Collins, M. B., Rossen, J., & Pinot-Navarro, J. D.
649 (2008). Monte Verde: seaweed, food, medicine and the peopling of South America.
650 *Science, 320*, 784-789.
651 Dittami, S., Gravot, A., Renault, D., Goulitquer, S., Eggert, A., Bouchereau, A., Boyen, C.,
652 & Tonon, T. (2011). Integrative analysis of metabolite and transcript abundance
653 during the short-term response to saline and oxidative stress in the brown alga
654 Ectocarpus siliculosus. *Plant Cell Environ., 34*(4), 629-642.
655 Durand, T., Cracowski, J.-L., Guy, A., & Rossi, J.-C. (2001). Syntheses and preliminary
656 pharmacological evaluation of the two epimers of the 5-F2t-isoprostane. *Bioorganic*
657 *& medicinal chemistry letters, 11*(18), 2495-2498.
658 Durand, T., Guy, A., Vidal, J.-P., & Rossi, J.-C. (2002). Total synthesis of 15(R)-and 15(S)
659 -F2t-isoP by a biomimetic process using the cyclization of acyclic dihydroxylated
660 octa-5,7-dienyl Radicals. *J. Org. Chem., 67*(11), 3615-3624.
661 El Fangour, S., Guy, A., Despres, V., Vidal, J.-P., Rossi, J.-C., & Durand, T. (2004). Total
662 syntheses of the eight diastereoisomers of the syn-anti-syn phytoprostanes F1
663 Types I an II *J. Org. Chem., 69*, 2498-2503.
664 El Fangour, S., Guy, A., Vidal, J.-P., Rossi, J.-C., & Durand, T. (2005). A flexible synthesis
665 of the phytoprostanes B1 types I an II. *J. Org. Chem., 70*, 989-997.
666 Galano, J. M., Lee, J. C., Gladine, C., Comte, B., Le Guennec, J. Y., Oger, C., & Durand,
667 T. (2015). Non-enzymatic cyclic oxygenated metabolites of adrenic,
668 docosahexaenoic, eicosapentaenoic and alpha-linolenic acids; bioactivities and
669 potential use as biomarkers. *Biochimica et biophysica acta, 1851*(4), 446-455.
670 Galano, J. M., Lee, Y. Y., Oger, C., Vigor, C., Vercauteren, J., Durand, T., Giera, M., &
671 Lee, J. C. (2017). Isoprostanes, neuroprostanes and phytoprostanes: An overview
672 of 25years of research in chemistry and biology. *Progress in lipid research, 68*, 83-
673 108.

Galloway, A. W. E., Britton-Simmons, K. H., Duggins, D. O., Gabrielson, P. W., & Brett, M. T. (2012). Fatty acid signatures differentiate marine macrophytes at ordinal and family ranks. *Journal of phycology, 48*(4), 956-965.

Gantar, M., & Svircev, Z. (2008). Microalgae and cyanobacteria: food for thought. *Journal of Phycology, 44*(2), 260-268.

Guy, A., Flanagan, S., Durand, T., Oger, C., & Galano, J. M. (2015). Facile synthesis of cyclopentenone B1- and L1-type phytoprostanes. *Frontiers in chemistry, 3*, 41.

Guy, A., Oger, C., Hepekauzen, J., Signorini, C., Durand, T., De Felice, C., Fürstner, A., & Galano, J. M. (2014). Oxygenated metabolites of n-3 polyunsaturated fatty acid as potential oxidative stress biomarkers: total synthesis of 8-F3t-IsoP, 10-F4t-NeuroP, and [D4]-10-F4t-NeuroP. *Chem. Eur. J, 20*, 6374-6380.

Holdt, S., & Kraan, S. (2011). Bioactive compounds in seaweed: functional food applications and legislation. *Journal of applied phycology, 23*(3), 543-597.

Hurd, C., Harrison, P., Bischof, K., & Lobban, C. (2014). *Seaweed ecology and physiology*: Cambridge University Press.

Jahn, U., Galano, J. M., & Durand, T. (2008). Beyond prostaglandins-chemistry and biology of cyclic oxygenated metabolites formed by free-radical pathways from polyunsaturated fatty acids. *Angewandte Chemie, 47*(32), 5894-5955.

Jimenez-Escrig, A., Gomez-Ordonez, E., & P, R. (2012). Brown and red seaweeds as potential sources of antioxidant nutraceuticals. *J. Appl. Phycol, 24*, 1123-1132.

Kumari, P., Kumar, M., Reddy, C. R. K., & Jha, B. (2013). 3 - Algal lipids, fatty acids and sterols. In H. Dominguez (Ed.), *Functional ingredients from algae for foods and nutraceuticals*, (pp. 87-134): Woodhead Publishing.

Kumari, P., Kumar, M., Reddy, C. R. K., & Jha, B. (2013). *Algal lipids, fatty acids and sterols*.

Küpper, F. C., Gaquerel, E., Boneberg, E.-M., Morath, S., Salaün, J.-P., & Potin, P. (2006). Early events in ther perception of lipopolysaccharides in the brown alga *Laminaria digitata* include an oxidative burst and activation of fatty acid oxidation cascades. *Journal of experimental botany, 57*(9), 1991-1999.

Leung, K. S., Chen, X., Zhong, W., Yu, A. C. H., & Lee, C.-Y. J. (2014). Microbubble-mediated sonoporation amplified lipid peroxidation of Jurkat cells. *Chemistry and Physics of Lipids, 180*, 53-60.

Loeffler, C., Berger, S., Guy, A., Durand, T., Bringmann, G., Dreyer, M., von Rad, U., Durner, J., & Mueller, M. J. (2005). B1-phytoprostanes trigger plant defense and detoxification responses. *Plant Physiol, 137*(1), 328-340.

Medina, S., Miguel-Elizaga, I. D., Oger, C., Galano, J. M., Durand, T., Martinez-Villanueva, M., Castillo, M. L., Villegas-Martinez, I., Ferreres, F., Martinez-Hernandez, P., & Gil-Izquierdo, A. (2015). Dihomo-isoprostanes-nonenzymatic metabolites of AdA-are higher in epileptic patients compared to healthy individuals by a new ultrahigh pressure liquid chromatography-triple quadrupole-tandem mass spectrometry method. *Free radical biology & medicine, 79*, 154-163.

Milne, G. L., Gao, B., Terry, E. S., Zackert, W. E., & Sanchez, S. C. (2013). Measurement of F2- isoprostanes and isofurans using gas chromatography-mass spectrometry. *Free Radical Biology and Medicine, 59*(0), 36-44.

Minghetti, L., Salvi, R., Lavinia Salvatori, M., Ajmone-Cat, M. A., De Nuccio, C., Visentin, S., Bultel-Ponce, V., Oger, C., Guy, A., Galano, J. M., Greco, A., Bernardo, A., & Durand, T. (2014). Nonenzymatic oxygenated metabolites of alpha-linolenic acid B1- and L1-phytoprostanes protect immature neurons from oxidant injury and promote differentiation of oligodendrocyte progenitors through PPAR-gamma activation. *Free radical biology & medicine, 73*, 41-50.

Mueller, S., Hilbert, B., Dueckershoff, K., Roitsch, T., Krischke, M., Mueller, M. J., & Berger, S. (2008). General detoxification and stress responses are mediated by

oxidized lipids through TGA transcription factors in Arabidopsis. *Plant Cell, 20*(3), 768-785.

Newton, L. (1951). *Seaweed Utilization*. London: Sampson Low.

Noschka, E., Moore, J. N., Peroni, J. F., Lewis, S. J., Morrow, J. D., & Robertson, T. P. (2009). Thromboxane and isoprostanes as inflammatory and vasoactive mediators in black walnut heartwood extract induced equine laminitis. *Veterinary Immunology and Immunopathology, 129*(3,Äì4), 200-210.

Oger, C., Brinkmann, Y., Bouazzaoui, S., Durand, T., & Galano, J. M. (2008). Stereocontrolled access to isoprostanes via a bicyclo[3.3.0]octene framework. *Organic letters, 10*(21), 5087-5090.

Oger, C., Bultel-Poncé, V., Guy, A., Balas, L., Rossi, J.-C., Durand, T., & Galano, J. M. (2010). The handy use of Brown's catalyst for a skipped diyne deuteration: application to the synthesis of a d4-labeled-F4t-neuroprostane. *Chem. Eur. J, 16*, 13976-13980.

Pereira, H., Barreira, L., Figueiredo, F., Custodio, L., Vizetto-Duarte, C., Polo, C., Resek, E., Engelen, A., & Varela, J. (2012). Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. *Marine Drugs, 10*(9), 1920-1935.

Pinto, E., Sigaud-Kutner, T. C. S., Leitao, M. A. S., Okamoto, O. K., Morse, D., & Colepicolo, P. (2003). Heavy metal-induced oxidative stress in algae. *Journal of phycology, 39*(6), 10.

Plaza, M., Cifuentes, A., & Ibanez, E. (2008). In the search of new functional food ingredients from algae. *Trends in food science and technology, 19*(1), 31-39.

Ritter, A., Dittami, S. M., Goulitquer, S., Correa, J. A., Boyen, C., Potin, P., & Tonon, T. (2014). Transcriptomic and metabolomic analysis of copper stress acclimation in *Ectocarpus siliculosus* highlights signaling and tolerance mechanisms in brown algae. *BMC plant biology, 14*, 116.

Ritter, A., Goulitquer, S., Salaun, J., Tonon, T., Correa, J., & Potin, P. (2008). Copper stress induces biosynthesis of octadecanoi and eicosanoid oxygenated derivatives in the brown algal kelp *Laminiaria digitata*. *New Phytol., 180*(4), 809-821.

Roncarati, F., Sáez, C. A., Greco, M., Gledhill, M., Bitonti, M. B., & Brown, M. T. (2015). Response differences between *Ectocarpus siliculosus* populations to copper stress involve cellular exclusion and induction of the phytochelatin biosynthetic pathway. *Aquatic toxicology, 159*, 8.

Roy, J., Fauconnier, J., Oger, C., Farah, C., Angebault-Prouteau, C., Thireau, J., Bideaux, P., Scheuermann, V., Bultel-Poncé, V., Demion, M., Galano, J. M., Durand, T., Lee, J. C.-Y., & Le Guennec, J.-Y. (2017). Non-enzymatic oxidized metabolite of DHA, 4(RS)-4-F4t-neuroprostane protects the heart against reperfusion injury. *Free Radical Biology and Medicine, 102*, 229-239.

Saez, C. A., Roncarati, F., Moenne, A., Moody, A. J., & Brown, M. T. (2015). Copper-induced intra-specific oxidative damage and antioxidant responses in strains of the brown alga *Ectocarpus siliculosus* with different pollution histories. *Aquatic toxicology, 159*, 81-89.

Thoma, I., Loeffler, C., Sinha, A. K., Gupta, M., Krischke, M., Steffan, B., Roitsch, T., & Mueller, M. J. (2003). Cyclopentenone isoprostanes induced by reactive oxygen species trigger defense gene activation and phytoalexin accumulation in plants. *Plant J, 34*(3), 363-375.

Tseng, C. K. (1981). Commercial cultivation. In C. S. Lobban & M. J. Wynne (Eds.), *The Biology of Seaweeds*, (pp. 680-725). Oxford: Blackwell Science Publications.

Turner, N. J. (2003). The ethnobotany of edible seaweed (*Porphyra abbottae* and related species; Rhodophyta: Bangiales) and its use by first nations on the pacific coast of Canada. *Canadian Journal of Botany, 81*(4), 283-293.

778 Wielgosz-Collin, G., Kendel, M., & Couzinet-Mossion, A. (2016). Chapter 7 - Lipids, Fatty
779 Acids, Glycolipids, and Phospholipids. In *Seaweed in health and disease*
780 *prevention*, (pp. 185-221). San Diego: Academic Press.
781 Yonny, M. E., Rodriguez Torresi, A., Cuyamendous, C., Reversat, G., Oger, C., Galano, J.
782 M., Durand, T., Vigor, C., & Nazareno, M. A. (2016). Thermal stress in melon
783 plants: phytoprostanes and phytofurans as oxidative stress biomarkers and the
784 effect of antioxidant supplementation. *Journal of agricultural and food chemistry,*
785 *64*(44), 8296-8304.
786 Yruela, I. (2005). Copper in plants. *Brazilian Journal of Plant Physiology, 17*(1), 11.
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Figure captions:

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

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

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

Table captions:

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

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

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 ± 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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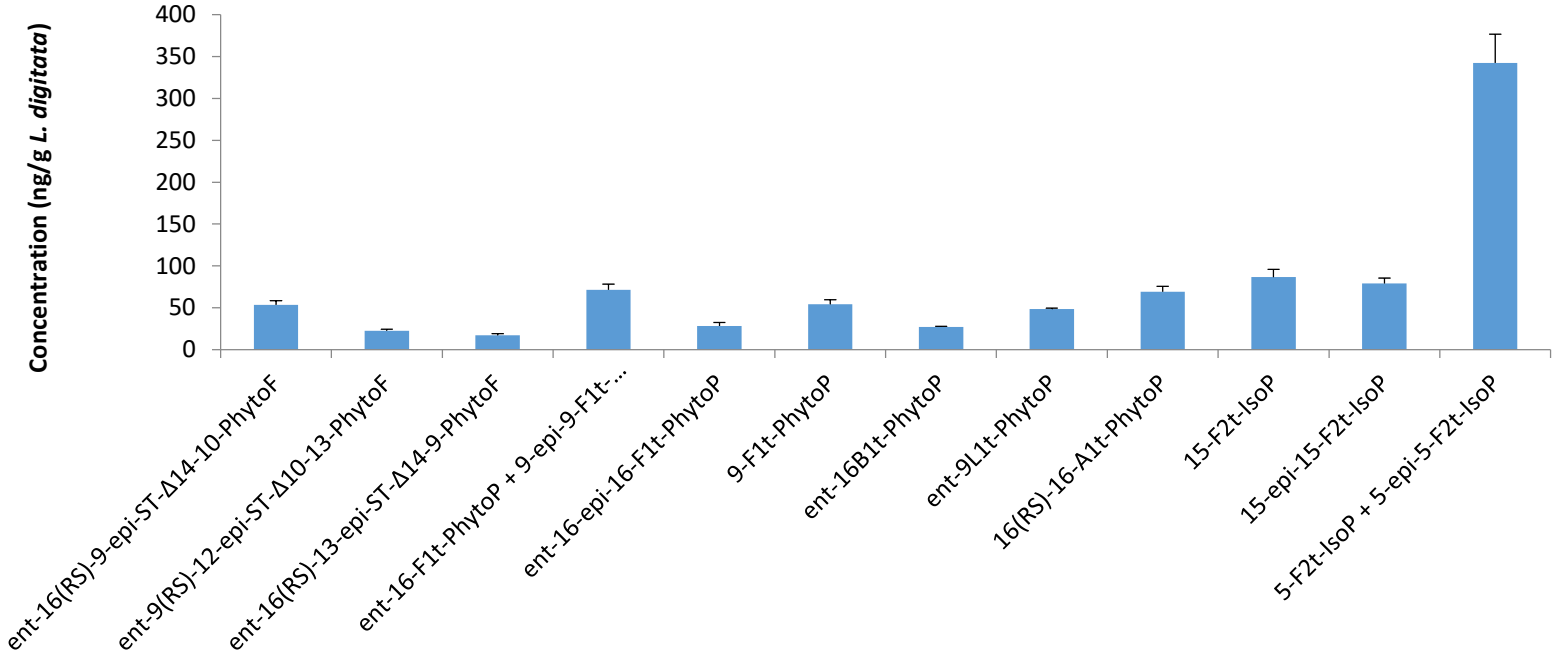
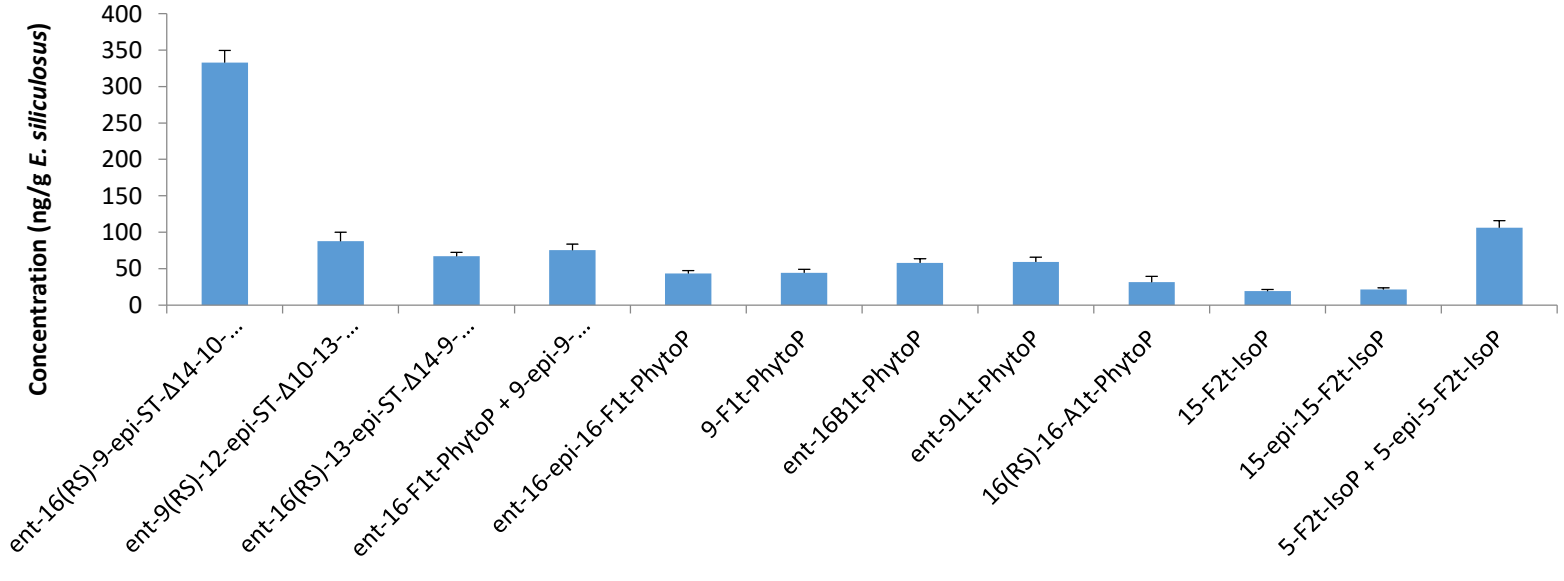
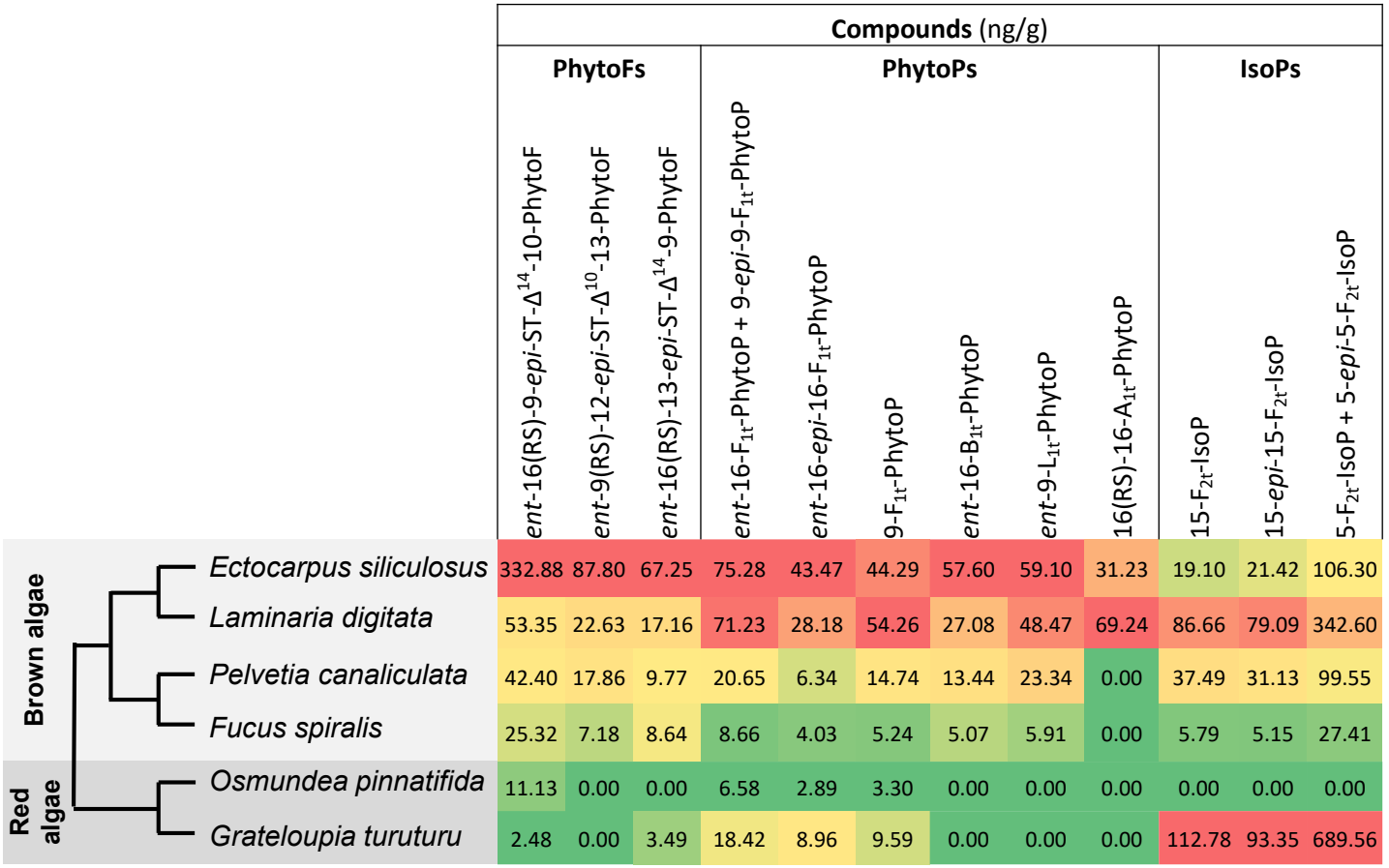
L. digitata

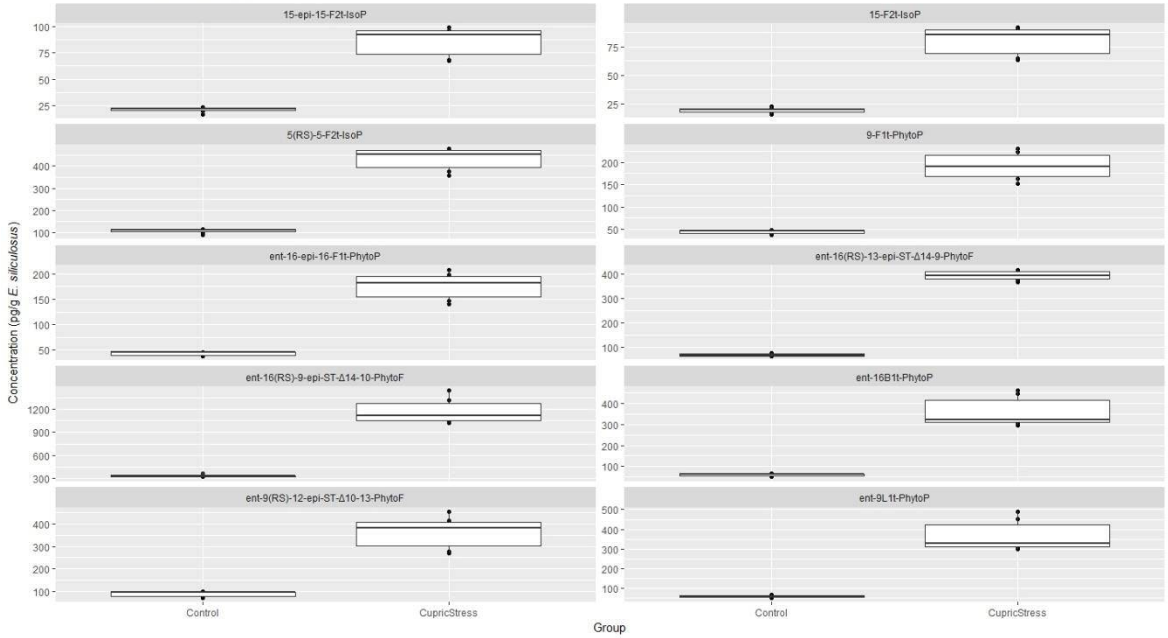


E. siliculosus

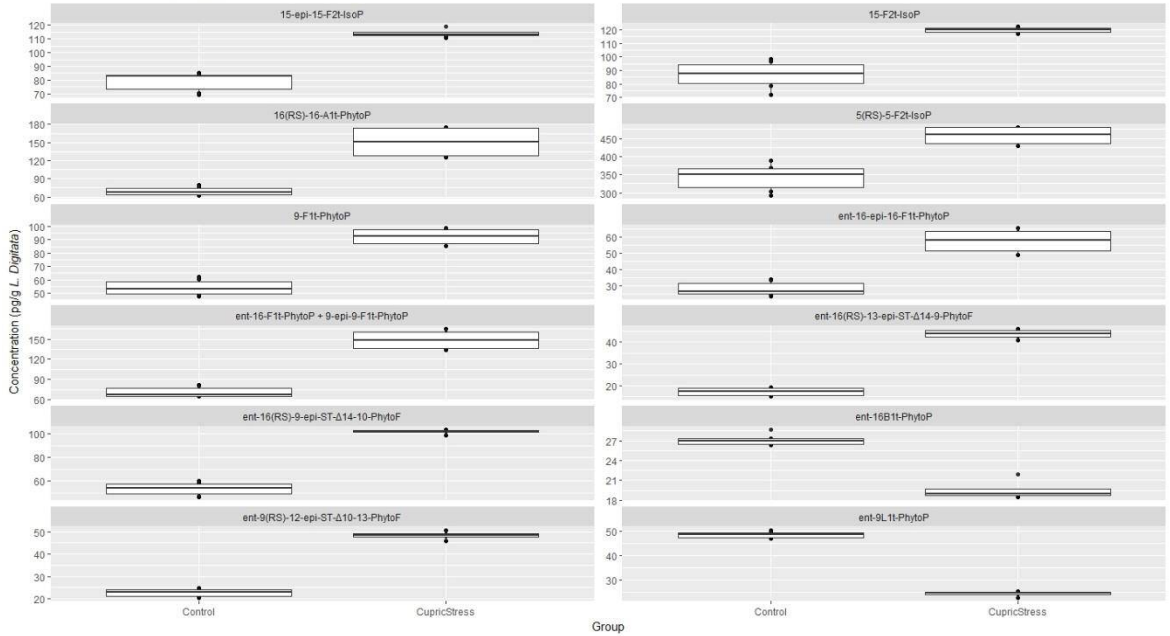


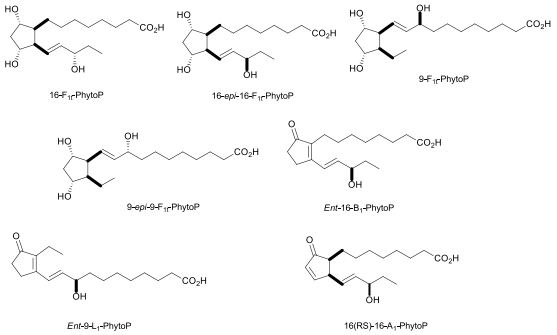
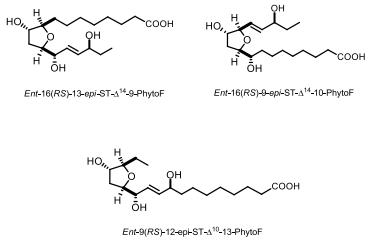
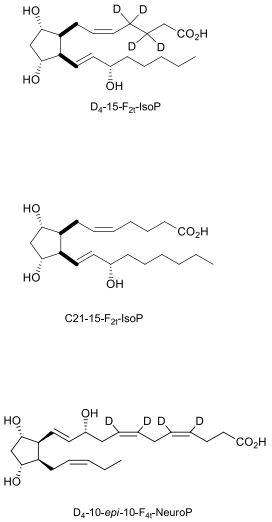
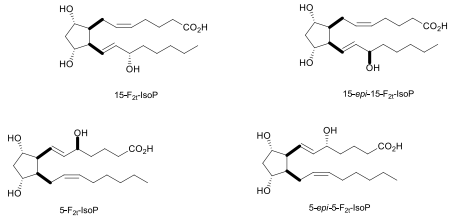

Compounds (ng/g)
PhytoFs
PhytoPs
IsoPs
ent-16(RS)-9-epi-ST-Δ14-10-PhytoF
ent-9(RS)-12-epi-ST-Δ10-13-PhytoF
ent-16(RS)-13-epi-ST-Δ14-9-PhytoF
ent-16-F1t-PhytoP + 9-epi-9-F1t-PhytoP
ent-16-epi-16-F1t-PhytoP
9-F1t-PhytoP
ent-16-B1t-PhytoP
ent-9-L1t-PhytoP
16(RS)-16-A1t-PhytoP
15-F2t-IsoP
15-epi-15-F2t-IsoP
5-F2t-IsoP + 5-epi-5-F2t-IsoP
Brown algae
Red algae
Ectocarpus siliculosus 332.88 87.80 67.25 75.28 43.47 44.29 57.60 59.10 31.23 19.10 21.42 106.30
Laminaria digitata 53.35 22.63 17.16 71.23 28.18 54.26 27.08 48.47 69.24 86.66 79.09 342.60
Pelvetia canaliculata 42.40 17.86 9.77 20.65 6.34 14.74 13.44 23.34 0.00 37.49 31.13 99.55
Fucus spiralis 25.32 7.18 8.64 8.66 4.03 5.24 5.07 5.91 0.00 5.79 5.15 27.41
Osmundea pinnatifida 11.13 0.00 0.00 6.58 2.89 3.30 0.00 0.00 0.00 0.00 0.00 0.00
Grateloupia turuturu 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)
α-linolenic Acid (ALA)			
Arachidonic Acid (AA)			

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

Compounds	Group	*L. digitata* ng/g	±	SD	p-value	*E. siliculosus* ng/g	±	SD	p-value	*F. spiralis* ng/g	±	SD	p-value	*P. canaliculata* ng/g	±	SD	p-value	*O. pinnatifida* ng/g	±	SD	p-value	*G. turuturu* ng/g	±	SD	p-value
ent-16(RS)-9-*epi*-ST-Δ^{14}-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^{10}-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^{14}-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_{1t}-PhytoP + 9-*epi*-9-F_{1t}-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_{1t}-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_{1t}-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_{1t}-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_{1t}-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_{1t}-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_{2t}-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_{2t}-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_{2t}-IsoP + 5-*epi*-5-F_{2t}-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	