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Metabolomic variability of four macroalgal species of the genus Lobophora using diverse approaches

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Abstract

Among comparative metabolomic studies used in marine sciences, only few of them are dedicated to macroalgae despite their ecological importance in marine ecosystems. Therefore, experimental data are needed to assess the scopes and limitations of different metabolomic techniques applied to macroalgal models. Species of the genus Lobophora belong to marine brown algae (Family: Dictyotaceae) and are widely distributed, especially in tropical coral reefs. The species richness of this genus has only been unveiled recently and it includes species of diverse morphologies and habitats, with some species interacting with corals. This study aims to assess the potential of different metabolomic fingerprinting approaches in the discrimination of four well known Lobophora species (L. rosacea, L. sonderii, L. obscura and L. monticola). These species present distinct morphologies and are found in various habitats in the New Caledonian lagoon (South-Western Pacific). We compared and combined different untargeted metabolomic techniques: liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance (\textsuperscript{1}H-NMR) and gas chromatography (GC-MS). Metabolomic separations were observed between each Lobophora species, with significant differences according to the techniques used. LC-MS was the best approach for metatype distinction but a combination of approaches was also useful and allowed identification of chemomarkers for some species. These comparisons provide important data on the use of metabolomic approaches in the Lobophora genus and will pave the way for further studies on the sources of metabolomic variations for this ecologically important macroalgae.

Keywords: Lobophora – Dictyotaceae – macroalgae – metabolomic – fingerprinting – comparative approach – New Caledonia
Specialized metabolites are often considered as low molecular weight molecules, end products of cellular regulatory processes, and final responses of biological systems to genetic and/or environmental changes (Fiehn, 2002). They can be regarded as products of natural selection during evolution. These secondary metabolites play also an important role in shaping algal chemical diversity (Wink, 2003). The set of metabolites present in the organisms can be highly complex and their biosynthesis can also be related to the associated microbiota (Roessner and Bowne, 2009). Traditionally, the chemical composition of an organism is explored through natural product chemistry which includes long and tedious steps of isolation and structure elucidation of metabolites (Robinette et al., 2011). This approach is time consuming and incomplete as it focuses mostly on the major compounds produced. Recent advances in more global approaches called metabolomics allow the analysis of a wider part of the metabolome by the simultaneous detection of hundreds to thousands of the metabolites of a small sample in a short period of time. In environmental sciences, metabolomics has therefore appeared as a quick and useful approach to examine the metabolite diversity of species and study their variations with time, geography, biotic interactions or other environmental factors (Bundy et al., 2009). Compared to the plant kingdom, relatively few environmental metabolomics studies have been reported on marine organisms. Taxonomy-based metabolomics has been applied for marine organisms like sponges (Ivanisevic et al., 2011a; Pérez et al., 2011), zoanthids (Costa-Lotufo et al., 2018; Jaramillo et al., 2018) and microalgae (Mooney et al., 2007). Variability in the metabolomic profiles were explored in time and space for some sponges (Rohde et al., 2012), ascidians (López-Legentil et al., 2006), zoanthids (Cachet et et al., 2015) and corals (Slattery et al., 2001) but also in response to environmental factors like temperature or salinity (Abdo et al., 2007; Bussell et al., 2008).

Among the chemical studies dedicated to macroalgae, only a few used metabolites as a taxonomic tool targeting specific compounds or classes like phenolics (Connan et al., 2004) or diterpenes (Campos De Paula et al., 2007). While these studies traditionally focus on potentially active compounds with pharmaceutical interests, a more global approach using metabolomics can represent a useful tool to explore the metabolome of macroalgae and its fluctuations. For example, metabolomics was applied on the red alga Asparagopsis taxiformis to study the spatio-temporal variation of its metabolome (Greff et al., 2017). Another study on the red alga Portieria hornemannii explored different sources for the variation of non-polar metabolites between cryptic species and life stages (Payo et al., 2011). Metabolomics also appeared as a complementary tool to understand defense or tolerance mechanisms of macroalgae in an ecological context (Rempt et al., 2012; Ritter et al., 2014). Marine brown macroalgae from the genus Lobophora (Family Dictyotaceae) have already been studied chemically. Gerwick & Fenical (1982) first described 1-(2,4,6-trihydroxyphenyl)hexadecan-1-one in L. papenfussii. Three sulfoquinovosyldiacylglycerols (SQDGs) and later lobophorolide were identified from L. variegate (Cantillo-Ciau et al., 2010; Kubanek et al., 2003). Recently, seven nonadecaketides named lobophorols, lobophopyranones and lobophorones (Gutiérrez-Cepeda et al., 2015) were found in the Atlantic L. variegata while the polyunsaturated lobophorenols A, B and C were described in the tropical L. rosacea (Vieira et al., 2016). Abundant in tropical coral reef habitats, some Lobophora species are closely associated with corals and therefore strongly involved in coral-algal interactions (Rasher & Hay, 2010), leading in some cases to negative impacts on corals. The high specific diversity of Lobophora genus has recently been unveiled (Vieira et al., 2014, 2017), with species exhibiting various morphologies and habitats, questioning the link between chemical diversity and
species diversity. Due to this chemical diversity, species of this genus are therefore good candidates to undergo metabolomics-based study to explore the metabolic variability among the different species.

We first decided to assess the potential of different approaches in metabolomic fingerprinting to separate four well-known *Lobophora* species (*L. rosacea*, *L. sonderii*, *L. obscura* and *L. monticola*), with distinct morphology and present in diverse habitats of the New Caledonian lagoon (South-Western Pacific). The systematics of these species being well described, we aimed at providing important insights on the relevance of these approaches to first discriminate species. The results of these preliminary data will then pave the way for deeper metabolomic studies on the presence of cryptic species, the influence of environmental parameters or biotic factors like the reproductive cycles. In terms of reproduction, little is known about *Lobophora* in New Caledonia and this genus is supposed to be reproductive all year round (Vieira, pers. com.). Our knowledge on the main specialized metabolites found in *L. rosacea* was a prerequisite to guide our study as they are presumed to have a taxonomic relevance. We used untargeted metabolomic approaches using three different techniques: UHPLC-MS-QToF, $^1$H-NMR and GC-MS followed by unsupervised and supervised analyses to highlight chemical differences among species.

## 2. Results

### 2.1. $^1$H-NMR

The matrix obtained after data analyses was composed of 7,998 buckets. A value of 33.5% of variance was explained by the two first components of the PCA (Fig. 1a) and mainly due to *L. rosacea* characterized by a different metabolomic fingerprint than the other three species (PPLS-DA, CER = 0.328, $p = 0.001$, post hoc $p < 0.05$, Fig. 1b, Table S1). In the central cluster of the PCA that grouped the three other species, only *L. monticola* and *L. obscura* present significant different metabolomic fingerprints ($p = 0.018$), whereas *L. sonderii* is not chemically different from the two others (Table S1, $p > 0.05$).

The overlay of $^1$H NMR spectra (Fig. 2), indicated that the major signals are shared by all *Lobophora* species: intense signals at $\delta_H$ 1-2 ppm due to the methylenes of long chain fatty acids, and signals at $\delta_H$ 2.8 and 5.3 ppm attributed to carbon-carbon unsaturations. More variable regions containing characteristic signals of the polyunsaturated lobophorenols A, B and C were observed between $\delta_H$ 3.2-4.5 (chlorinated and hydroxylated methines), 4.8-5.2 and 5.0-5.8 ppm (terminal olefinic protons, Fig. 2 and S1). Due to the high number of generated bins, Kruskal-Wallis loading plot (Fig. S2) was used to identify chemical markers, which separate metabolic diversity of *Lobophora* species (with $p < 0.05$, Table S2). The regions corresponding to the signals of lobophorenols are the main markers of differences between species and are mostly present in *L. rosacea*.

### 2.2. UHPLC-QToF

After LC-MS data analyses and filtering, 600 metabolic features were finally considered. The variance on the two first components of the PCA was explained by 38.7% (Fig.1c), a value slightly higher than for NMR analysis. The LC-MS approach permitted a better separation of each species’ metabolome than NMR (CER =
0.115, p = 0.001, p < 0.05 for each tested pair, Fig. 1d, Table S1). The difference between chemical groups was mainly quantitative as shown in the Venn diagram (Fig. S4).

Among chemomarkers, two compounds, lobophorenol B ($m/z$ 334.272 [M + NH$_4$]+, C$_{21}$H$_{32}$O$_2$) and lobophorenol C ($m/z$ 336.287 [M + NH$_4$]+, C$_{21}$H$_{34}$O$_2$), previously isolated in $L$. rosacea (Vieira et al., 2016) were mainly detected in this species (Fig. 3, Table S3). They were also detected in $L$. monticola, but with high variability and not in $L$. sonderii and $L$. obscura. The other chemomarkers of each species were tentatively annotated based on the construction of a molecular network but no other match was found in the current database, with the majority of them appearing as minor intensity ions.

To combine data obtained by LC-MS with those by NMR, a multiple factor analysis (MFA) was performed (Fig. 4). The four $Lobophora$ species were well separated despite a lower variance of 21.7% on the two first dimensions.

2.3. GC-MS

The richness in non-polar specialized metabolites in $Lobophora$ led us to analyze the CH$_2$Cl$_2$ fractions by GC-MS while attempts to use NMR for those fractions were unfruitful due to intense lipidic peaks. The explained variance on axis 1-2 of the PCA was 35.7%, a value similar to the values obtained with the two other techniques (Fig.1e). All algal metabotypes were differentiated with this technique (CER = 0.304, p = 0.001, Fig.1f) except for $L$. monticola vs $L$. rosacea (p = 0.431, Table S1). Among the chemomarkers contributing to the discrimination of the species metabotypes, we identified a small carboxylic acid: 2-pentenoic acid (M5), an amide: maleimide, 2-methyl-3-vinyl (M18), and two esters: methyl stearate (M38) and hexanoic acid, 2-ethyl-, hexadecyl ester (M48) (Table 1). M5 and M48 are specific to $L$. obscura while this species contained lower amount of M38 compared to the three other species. $Lobophora$ sonderii and $L$. rosacea exhibit higher levels of M18 (Fig. S5).

Moreover, some compounds known as plastic pollutants were found in all species and contribute to differences in their chemical profiles (Phenol, 2,4-di-tert-butyl; tributyl acetyl citrate; o-xylene; naphthalene, 2,6-dimethyl-; p-cresol, 2,6-di-tert-butyl-; N-methyl-N-benzyltetradecanamine).

3. Discussion

Even though LC-MS is a method largely employed in metabolomics studies due to its high sensitivity, it is not suitable for all metabolites and more appropriate for polar, weakly polar and neutral compounds (Wang et al., 2015). Moreover, it relies on ionization process, limiting the study of poorly-ionizable compounds. GC-MS is then more suitable when non-polar metabolites are found as the main major specialized metabolites. On the contrary and even if much less sensitive, NMR is more universal and does not rely on ionization processes nor the separation of analytes by HPLC, and solubilization of the metabolites is the only limitation. NMR spectra are able to provide a better snapshot of what are the major metabolites and their relative concentrations in the studied specimens. This information is highly relevant for major metabolites that may correspond to specialized
metabolites providing useful information concerning species discrimination. NMR would therefore be less affected by environmental changes often linked to minor metabolites (Ivanišević et al., 2011b).

The four species of Lobophora are well described morphologically and their phylogeny resolved in 2014 using mitochondrial gene Cox3 (Vieira et al., 2014). Lobophora monticola, L. obscura, L. rosacea and L. sonderii are distantly-related species. They are distributed evenly across the Lobophora evolutionary tree, with L. rosacea the most basal species of the four (Fig. S6)(Vieira et al., 2014, 2017). In the current study, the algal species have been separated based on their metabolomic fingerprints, and depending on the used technique. The LC-MS approach was the most effective technique for the separation of the four species of this genus (PPLS-DA, CER = 0.115), but NMR and GC-MS (CER$_{NMR}$ = 0.328, CER$_{GC/MS}$ = 0.304) also provided interesting complementary results. While less sensitive than LC-MS or GC-MS, NMR provides the highest reproducibility among metabolomic measuring platforms (Farag et al., 2012a).

In our study, NMR analyses performed on the same methanolic fractions as LC-MS allowed a clear distinction of L. rosacea from the other three species, in agreement with phylogenetic data where this species appears as the most basal. Like LC-MS, NMR highlighted lobophorenols as discriminating metabolites in L. rosacea, with most of the signals responsible for the chemical divergence corresponding to characteristic signals of these molecules. Lobophorenol A was not detected by LC-MS while it was observed as marker in NMR because of some characteristic signals at δH 3.70 (H-4) and 6.02 ppm (H-2; see Fig. S1). The absence of detection of lobophorenol A by LC-MS might stem from a high reactivity of the chlorinated derivative during ionization. These lobophorenols have been shown to present allelopathic effects against the coral Acropora muricata (Vieira et al., 2016). Molecular networking based on MS$_2$ spectra analyses did not allow identification of additional chemomarkers. Annotation of compounds from marine organisms, especially macroalgae, is still challenging with the lack of specific databases. Altogether, LC-MS and NMR provide complementary approaches to analyze the metabolome and their combination is highly relevant to discriminate Lobophora species, as supported by the MFA.

NMR analyses also showed the rich composition in lipidic derivatives of Lobophora species, which may be problematic because intense long-fatty-chain signals may mask other signals. However, this issue could be resolved using GC-MS, allowing the study of the non-polar part of the metabolome. Compared to the poor specialized LC-MS database, the available GC-MS databases enabled the annotation of some chemomarkers. However, the discrimination between species metabotypes was partly explained by compounds presumably identified as pollutants from plastic origin and present in the coastal sites, which were likely differently accumulated in the algae or at their surface as seen in Sargassum spp. and Fucus vesiculosus (Chan et al., 2004; Gutow et al., 2016). This result potentially highlights more different adhesion capacities of microplastics between species rather than metabolic differences. Other markers evidenced by the NMR method include: 2-pentenoic, which is a small unsaturated fatty acid previously found in plants (Wu and Chen, 1992). Pentenoic acid was found up-regulated under salinity stress in the halophyte Aeluropus lagopoides (Paidi et al., 2017). Maleimide, 2-methyl-3-vinyl was also detected in the algal samples. This metabolite may be a transformation product of chlorophylls and bacteriochlorophylls (Naeher et al., 2013). We hypothesize that these compounds originate in part from micro-organisms associated to Lobophora species. Indeed, epiphytes often colonized the
algal surface (Egan et al., 2013). Then, by extracting the algal metabolome, we may also extract compounds from bacteria or epiphytes and metabotypes observed in our study may arise, at least in part, from the algal-associated organisms. Even if we carefully removed epiphytes from their surface, microorganisms are still present and may contribute to the global metabolome of the specimens. For example, methyl stearate has been found in bacteria and plants but also microalgae, ascidians and macroalgae (De Rosa et al., 2001; Sharmin et al., 2016; Takeara et al., 2008; Terekhova et al., 2010) and is assumed to have antibacterial and cytotoxic activities (Elshafie et al., 2017; Takeara et al., 2008). The influence of species-specific microbial communities, which can produce minor compounds, may also explained the better result obtain by LC-MS in the discrimination of *Lobophora* species.

Even if less important than interspecific differences, intraspecific variability can be explained by their development stage, life history traits and evolution and may also result from their environment. *Lobophora* life cycle is not documented in New Caledonia lagoon and reproductive state has been seen all year round (Vieira, personal observation). Gametophytes and sporophytes are not easily dissociable but may potentially present different chemical profiles as seen in other macroalgae, like the red algae *Portieria hornemannii* in the Philippines (Payo et al., 2011). The close association of *L. rosacea* and *L. monticola* with corals could lead to chemical adaptation or specification in the algal chemistry, notably against coral associated microbiome. On the other hand, growing in algal beds, *L. sonderii* is more exposed to herbivores and its chemistry probably evolved differently, notably to repulse predators. *Lobophora obscura* is also exposed to other organisms but its encrusting form with thick and coarse thalli, may deter predators. Less effort in metabolites production may be balanced by its protective morphology. Because very few chemomarkers were identified, these hypotheses should be further investigated and tested. Moreover, regarding the good discrimination observed with LC-MS in this work, this method may be useful to separate cryptic species of *Lobophora*, as successfully applied for *Portieria dioli* (Payo et al., 2011).

The choice of the technique to accurately distinguish species is pivotal and only few studies comparing these techniques have been published to date, most of them being applied to plants. For example, untargeted LC-MS was the most effective to discriminate several green tea (Kellogg et al., 2017). Other authors used multiple approaches to study the metabolomic fingerprint in zoanthids (Costa-Lotufo et al., 2018) or in the plant kingdom (Agnolet et al., 2010; Farag et al., 2012b). Multiple metabolomics approaches are rare on macroalgae. Notably, LC-MS and HR-MAS NMR were used to evaluate the relevance for taxonomical purpose in five species of the genus *Cystoseira* (Jégou et al., 2010). Due to the high diversity in metabolites, with diverse physico-chemical properties and different concentration ranges, the global analysis in metabolites is challenging. Using multiple metabolomics approaches allow a broader analysis. It is a good tool to appreciate the chemical diversity among species and can bring complementary information to the phylogenetic data, the unavoidable base for classification. These approaches enabled a better exploration of the chemical speciation or evolution among genus or even at a broader scale, as realized by Belghit et al.(2017) on 21 species belonging to red, green and brown algae. With the increase of shared metabolomics platforms, metabolomic fingerprinting might be applied to other macroalgae and marine organisms, and when coupled with genomics or transcriptomics, it will greatly improve our understanding of adaptive mechanisms involved in multi-stressors environments. This coupling has been recently applied to macroalgae, like in the model *Ectocarpus siliculosus* where transcriptomic and genomic data available allowed to better understand the metabolic changes during saline and oxidative stress (Dittami et
al., 2011) or under different CO₂ and O₂ concentrations (Gravot et al., 2010). While Lobophora genus is not a typical model organism, the decreased cost and increased sequencing capabilities of Next Generation Sequencing make it possible to examine species beyond traditional models (Konotchick et al., 2013; Unamba et al., 2015). In particular, it is an example of common brown alga widely distributed in tropical waters and producing major non-polar metabolites and therefore can represent a model for other metabolomic studies applied to brown algae. A coupling between metabolomics and meta-genomics could also help to understand the diversity of associated bacteria and better assess their contribution to the algal metabolome. Because associated microorganisms are commonly species-specific, this could partly explain the better results obtained with LC-MS. While a multiple metabolomic approach is promising for several applications in macroalgae, data interpretation remain the biggest challenge to date and more metabolomics studies on macroalgae are needed.

4. Conclusion

Metabolic fingerprinting with LC-MS was the most appropriate technique in the discrimination of different Lobophora species, but the coupling with NMR is also useful as the main metabolites can be observed with these methods and identified as chemomarkers. Indeed, lobophorenols, previously identified specialized metabolites in L. rosacea, were detected as chemomarkers with both LC-MS and NMR while they were not detected by GC-MS, which appeared a less useful technique for analyzing the Lobophora genus. This study demonstrates that an untargeted metabolomic approach via LC-MS/NMR will be helpful for further ecological studies in Lobophora. Notably, this technique is appropriate to explore the sources of metabolomic variations in this genus at the temporal and spatial scales, influenced by environmental factors, and also in response to different biotic interactions.

5. Experimental

5.1. Sampling

Lobophora rosacea, L. sonderii and L. obscura were collected by SCUBA during summer 2016 at Ricaudy (22°18.956’S; 166°27.405’E, Nouméa, New Caledonia). Lobophora monticola was collected during summer 2016 at Sainte-Marie (22°18.269’S; 166°28.791’E, Nouméa, New Caledonia). Species identifications were performed by combining morphological and genetic analyses following Vieira et al. (2014). Vouchers for each species are kept at IRD herbarium (IRD10213, IRD10195, IRD10187, IRD10199). Lobophora rosacea has a thin fan-shaped thallus, growing fixed by the basal part within coral branches like Acropora spp. Lobophora monticola is also found associated to branching corals, and thalli grows partially or completely in contact with them. Lobophora sonderii forms dense erected blades, mixed with other brown seaweeds in Sargassum beds. Conversely, L. obscura has encrusting and thick leather-like thalli, strongly attached to dead corals or coral rubbles (Fig. 5). Specimens (six for L. rosacea, L. monticola and L. obscura and five for L. sonderii) were placed in separate ziplock plastic bags, immediately placed into ice and stored at -20 °C until sample grinding.

5.2. Metabolite extractions
Algae were freeze-dried and manually ground with liquid nitrogen in a mortar. Samples were then stored in silicagel until chemical extractions. For each replicate, a mass of 250 mg was extracted 3 times with 5 mL of MeOH/CH₂Cl₂(1:1) during 5 min in an ultrasonic bath. Supernatants were pooled and filtered from samples. The extracts were concentrated to dryness in the presence of C18 silica powder (100 mg, Polygoprep 60-50, Macherey-Nagel®) using a rotary evaporator, and the solid was then fractioned by Solid Phase Extraction (SPE, Strata C18-E 500 mg/6 mL, Phenomenex®) by the successive elution of H₂O (6 mL), MeOH (6 mL) and CH₂Cl₂ (6 mL) after cartridge cleaning (6 mL MeOH/CH₂Cl₂) and conditioning (6 mL H₂O). The MeOH fractions were then filtered on syringe filters (PTFE, 0.20 μm, Phenomenex®), dried in a speedvac and further used for UHPLC-QToF and NMR analyses. The CH₂Cl₂ fractions were only analyzed by GC/MS.

5.3. Metabolomic analyses

5.3.1. NMR

Dry samples were dissolved in 0.5 mL CDCl₃. ¹H-NMR spectra were recorded on a cryoprobe-equipped 600 MHz Agilent spectrometer. The following parameters were used for data acquisition: 16 ppm spectral width, 1 s relaxation delay with water pre-saturation (PS), number of scans 32, acquisition time 1.7 s, 16 K complex data points, 90° pulse angle.

5.3.2 UHPLC-QToF

Metabolomic fingerprints were recorded on an UHPLC (Dionex Ultimate 3000, Thermo Scientific®) coupled to an accurate mass spectrometer equipped with an ESI source (QqToF Impact II, Bruker Daltonics®). Metabolite separation were performed on a C18 UHPLC column (Acclaim™ RSLC 120 C18 150 x 2.1 mm, 2.2 μm, Thermo Scientific®) at 40 °C. The mobile phase consisted in a mix of H₂O + 0.1 % formic acid + 10 mM ammonium formate (solvent A) and acetonitrile/H₂O (95:5) + 0.1 % formic acid + 10 mM ammonium formate (solvent B). Injection volume was set to 3 μL and elution flow to 0.4 mL min⁻¹. The elution gradient was programmed as follows: 40 % B during 2 min, increased up to 100 % B from 2 to 8 min, followed by an isocratic step of 100% B during 4 min. The initial conditions were gradually recovered from 12 to 14 min, and hold 3 min for column equilibration for a total runtime of 17 min. MS parameters were set as follows: nebulizer gas N₂ at 40 psig, gas temperature 300 °C, drying gas N₂ flow 4 L min⁻¹, capillary voltage 3500 V. Mass spectra were acquired in positive ionization mode from 50 to 1,200 amu at 2 Hz. Auto-MS² spectra were acquired according to the same conditions then previously. A quality control sample (QC) was prepared with 25 μL of each sample. It was used to check MS shift over time and to normalize data according to injection order. The run started with three blank injections, followed by 10 injections of the QC for mass spectrometer stabilization. Samples were then randomly injected, inserting one QC every five samples. A final blank was injected to check any memory effect of the compounds on the column.

5.3.3 GC-MS

CH₂Cl₂ fractions were analyzed on a gas chromatograph (7890B GC System - 7693 autosampler, Agilent Technologies®) coupled to a mass selective detector (5977A MSD, Agilent Technologies®). Separation of metabolites was performed on a HP-5MS 5% Phenyl-Methyl Silox column (30 m x 0.25 mm, 0.25 μm, Agilent Technologies®) with helium as mobile phase. The run started at 40 °C for 5 min and increased by 10 °C min⁻¹ up
to 300°C for a total runtime of 31 min. A constant flow rate was set to 1 mL min⁻¹. A volume of 1 μL of each sample was injected in splitless mode at 250°C. A solution with a mix of C8-C20 and C21-C40 alkanes (Fluka Analytical) was also injected for the determination of compound retention index.

### 5.3.4 Data treatment

¹H-NMR spectra were automatically Fourier-transformed and processed on MesReNova 11. Spectra baselines were automatically corrected followed by the Whittaker smoother correction. An equal width bucketing of 0.001 ppm was applied between 0-8 ppm to finally obtain the data matrix. Data were auto-scaled and log-transformed before statistical analyses.

LC–MS raw data files were first calibrated before converting them to netCDF files (centroid mode) using Bruker Compass DataAnalysis 4.3. NetCDF files were processed using the package XCMS for R software (version 3.3.2, XCMS version 1.50.1). Optimized parameters for XCMS were used as follows: peak picking (method= "centwave", peakwidth= c(2,20), ppm= 15, mzdif= 0.05, prefilter= c(0,0)), retention time correction (method = "obiwarp"), matching peaks across samples (bw= 30, mzwid= 0.015, minfrac= 0.3) and filling in missing peaks. The matrix was then cleaned according to blanks and pooled samples to remove analytical variability. Molecular network based on MS² spectra were constructed with GNPS (Wang et al., 2016) and managed under Cytoscape 3.5.0 (Shannon et al., 2003).

Agilent data files acquired from GC-MS analysis were exported into CDF files using MSD Chemstation (F.01.001903, Agilent Technologies®). CDF files were then processed using the package eRah (version 1.0.5, Domingo-Almenara et al., 2016) under R performing preprocessing, peak deconvolution (min.peak.width = 2.5, min.peak.height=2500, noise.threshold=500, avoid.processing.mz=c(73,149,207)), peak alignment (min.spectra.cor=0.90, max.time.dist=60, mz.range=40:500) and missing compound recovery (with presence required in 3 samples at least). Compound annotation was performed manually by comparing mass spectra with NIST 2011 database completed with the calculation of Kováts' index (Van Den Dool and Kratz, 1963). The matrix obtained was finally filtered according to the blank. Data from LC-MS and GC-MS were normalized by log-transformation before statistical analyses.

### 5.4. Statistical analyses

Principal component analysis (PCA) was used to visualize the metabolome variation according to species (ade4 package for R). Powered Partial Least-Squares-Discriminant Analysis (PPLS-DA) identified the maximum covariance between our data set and their class membership and permutational tests based on cross model validation (MVA.test and pairwise.MVA.test) were used to test differences between groups (RVAideMemoire package). Discriminating compounds were then identified according to the PPLS-DA loading plots (correlation circles; RVAideMemoire package). Multiple Factor Analysis (MFA, variables scaled to unit variance) was used to combine data obtained from LC-MS and NMR (FactoMineR and factoextrapackages for R). Kruskal-Wallis tests were performed in MetaboAnalyst 3.0 and R (PMCMR package). Post-hoc Conover’s test was done on R software (PMCMR package). Venn diagram was constructed with Venny 2.1 (Oliveros 2007-2015).
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**Figure and Table captions**

**Fig. 1.** Discriminant power of the three chemical approaches via unsupervised (Principal Component Analysis, PCA, a, c, e) and supervised discriminant (Powered Partial Least-Squares-Discriminant Analysis, PPLS-DA, b, d, f) analyses of *Lobophora* species metabolome analyzed by (a, b) NMR for MeOH fractions and (c, d) LC-MS for MeOH fractions and (e, f) GC-MS for CH2Cl2 fractions (LO: *L. obscura* in red, LR: *L. rosacea* in orange,

**Fig. 2.** Overlay of $^1$H-NMR (600 MHz) spectra of the four *Lobophora* species (one representative sample per species was chosen, the full overlay spectra is available in Fig. S3. *L. monticola* in red, *L. obscura* in green, *L. rosacea* in blue and *L. sonderii* in purple). Regions of discriminating signals are highlighted by black rectangles.

**Fig. 3.** Box plots of lobophorenols B and C (and chemical structure of lobophorenols A-C) among the four *Lobophora* species and blank (log-transformed data, y-axis), detected by LC-MS, expressed as mean normalized intensities ± SD (n = 3 for blank, n = 6 for LM, LO, LR and n = 5 for LS)(LM: *L. monticola* in green, LO: *L. obscura* in red, LR: *L. rosacea* in orange and LS: *L. sonderii* in blue, x-axis). The statistical analyses were performed using Kruskal-Wallis (KW) followed by post-hoc Conover’s test. Letters indicate significant differences between groups based on post-hoc pairwise comparisons (p < 0.05).

**Fig. 4.** Multiple Factor Analysis (MFA) obtained with LC-MS and NMR data from the MeOH fractions of *Lobophora* species (LM: *L. monticola* in green, LO: *L. obscura* in red, LR: *L. rosacea* in orange, and LS: *L. sonderii* in blue). Confidence level used to construct the ellipses = 0.95, variables scaled to unit variance.

**Fig. 5.** Pictures of *Lobophora* species: (a) *L. rosacea*, (b) *L. sonderii*, (c) *L. obscura* and (d) *L. monticola*. Arrows indicate algal thalli (algae were collected at Ricaudy for (a), (b), (c) and Sainte-Marie for (d); images by G. Boussarie).

**Table 1.** Chemomarkers detected by GC-MS in the CH$_2$Cl$_2$ fraction of *Lobophora* species, annotated with NIST 2011 database (COMP. = compound, RI = Van Den Dool and Kratz Retention Index, EXP. = experimental, LIT. = literature).
(CER = 0.328, p = 0.001) (CER = 0.115, p = 0.001) (CER = 0.304, p = 0.001)
lobophorenol B

lobophorenol C

Normalized intensity

blank LM LO LR LS

(KW = 16.44, p = 0.0024)

(KW = 18.34, p = 0.0011)
Table 1. Chemomarkers detected by GC-MS in the CH$_2$Cl$_2$ fraction of *Lobophora* species, annotated with NIST 2011 database (COMP. = compound, RI = Van Den Dool and Kratz Retention Index, EXP. = experimental, LIT. = literature).

<table>
<thead>
<tr>
<th>COMP.</th>
<th>MOLECULAR NAME</th>
<th>CHEMICAL FAMILY</th>
<th>CAS NUMBER</th>
<th>RAW FORMULA</th>
<th>% MATCH</th>
<th>LIT. RI</th>
<th>EXP. RI</th>
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<tbody>
<tr>
<td>M5</td>
<td>2-pentenoic acid carboxylic acid</td>
<td>626-98-2</td>
<td>C$_5$H$_8$O$_2$</td>
<td>94</td>
<td>873</td>
<td>921</td>
<td></td>
</tr>
<tr>
<td>M18</td>
<td>maleimide, 2-methyl-3-vinyl amide</td>
<td>21494-57-5</td>
<td>C$_7$H$_7$NO$_2$</td>
<td>91</td>
<td>1261</td>
<td>1262</td>
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<tr>
<td>M38</td>
<td>methyl stearate ester</td>
<td>112-61-8</td>
<td>C$<em>{19}$H$</em>{38}$O$_2$</td>
<td>98</td>
<td>2130</td>
<td>2129</td>
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<tr>
<td>M48</td>
<td>hexanoic acid, 2-ethyl-, hexadecyl ester</td>
<td>59130-69-7</td>
<td>C$<em>{24}$H$</em>{48}$O$_2$</td>
<td>64</td>
<td>-</td>
<td>2468</td>
<td></td>
</tr>
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</table>
SUPPLEMENTARY INFORMATION

Metabolomic variability of four macroalgal species of the genus *Lobophora* using diverse approaches

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*corresponding authors

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Olivier P. THOMAS: Tel 0035391493563, olivier.thomas@nuigalway.ie
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Fig. S1. Chemical structure of lobophorenols A-C.

<table>
<thead>
<tr>
<th>Lobophorenonl</th>
<th>X</th>
<th>Δ1,2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cl</td>
<td>yes</td>
</tr>
<tr>
<td>B</td>
<td>OH</td>
<td>yes</td>
</tr>
<tr>
<td>C</td>
<td>OH</td>
<td>no</td>
</tr>
</tbody>
</table>

Fig. S2. Kruskal-Wallis test loading plots with bins varying among species in red (p < 0.05). Data were obtained with NMR.

Fig. S3. Overlay of 1H-NMR (600 MHz) spectra of the four Lobophora species (L. monticola in red, L. obscura in green, L. rosacea in blue and L. sonderii in purple). Regions of discriminating signals are highlighted by black rectangles.
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<table>
<thead>
<tr>
<th></th>
<th>NMR</th>
<th></th>
<th></th>
<th>LC-MS</th>
<th></th>
<th></th>
<th>GC-MS</th>
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<tr>
<td></td>
<td>LM</td>
<td>LO</td>
<td>LR</td>
<td>LM</td>
<td>LO</td>
<td>LR</td>
<td>LM</td>
<td>LO</td>
<td>LR</td>
</tr>
<tr>
<td>LO</td>
<td>0.018</td>
<td>-</td>
<td>-</td>
<td>LO</td>
<td>0.006</td>
<td>-</td>
<td>-</td>
<td>LO</td>
<td>0.006</td>
</tr>
<tr>
<td>LR</td>
<td>0.022</td>
<td>0.006</td>
<td>-</td>
<td>LR</td>
<td>0.044</td>
<td>0.006</td>
<td>-</td>
<td>LR</td>
<td>0.431</td>
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<tr>
<td>LS</td>
<td>0.276</td>
<td>0.276</td>
<td>0.018</td>
<td>LS</td>
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<td>0.018</td>
<td>0.006</td>
<td>LS</td>
<td>0.012</td>
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</table>

Table S2. Selection of the most significant regions in the spectra varying among Lobophora species (from Kruskal-Wallis test, with p-value < 0.05). Characteristic signals (ppm) of lobophorenols A, B and C are also assigned to the corresponding chemical shift range.

<table>
<thead>
<tr>
<th>Max ppm area</th>
<th>Range ppm area</th>
<th>Characteristic signals (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.678</td>
<td>0.662 - 0.68</td>
<td>-</td>
</tr>
<tr>
<td>0.892</td>
<td>0.823 - 0.997</td>
<td>Lobophorenol C: 0.97</td>
</tr>
<tr>
<td>1.214</td>
<td>1.237 - 1.253</td>
<td>-</td>
</tr>
<tr>
<td>1.446</td>
<td>1.407 - 1.501</td>
<td>Lobophorenol C: 1.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lobophorenol A, B &amp; C: 1.46</td>
</tr>
<tr>
<td>2.05</td>
<td>2.035 - 2.077</td>
<td>Lobophorenol B: 2.06, A &amp; C: 2.07</td>
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<tr>
<td>2.3</td>
<td>2.08 - 2.386</td>
<td>Lobophorenol A: 2.10/2.25, B: 2.14, C: 2.24</td>
</tr>
<tr>
<td>2.412</td>
<td>2.318 - 2.433</td>
<td>Lobophorenol B &amp; C: 2.36</td>
</tr>
<tr>
<td>2.816</td>
<td>2.747 - 2.836</td>
<td>Lobophorenol A, B &amp; C: 2.82</td>
</tr>
<tr>
<td>2.956</td>
<td>2.85 - 3.08</td>
<td>Lobophorenol A: 2.86/2.87</td>
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<td>3.374</td>
<td>3.240 - 3.442</td>
<td>Lobophorenol C: 3.32</td>
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<td>3.512</td>
<td>3.365 - 3.595</td>
<td>Lobophorenol B: 3.48, C: 3.46</td>
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<td>3.714</td>
<td>3.536 - 3.721</td>
<td>Lobophorenol A: 3.70</td>
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<td>4.004</td>
<td>3.854 - 4.302</td>
<td>Lobophorenol B: 3.94</td>
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<td>4.37</td>
<td>4.103 - 4.398</td>
<td>Lobophorenol A: 4.38</td>
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<td>5.014</td>
<td>4.927 - 5.102</td>
<td>Lobophorenol A, B &amp; C: 4.94 and 5.00</td>
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<tr>
<td>5.291</td>
<td>5.244 - 5.397</td>
<td>Lobophorenol A: 5.34, A, B &amp; C: 5.37 and 5.38</td>
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<td>5.488</td>
<td>5.398 - 5.667</td>
<td>Lobophorenol A: 5.48/5.49, B &amp; C: 5.45 and Lobophorenol B: 5.51, C: 5.52</td>
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<tr>
<td>5.599</td>
<td>5.399 - 5.74</td>
<td>-</td>
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<td>5.708</td>
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<td>Lobophorenol B &amp; C: 5.82</td>
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<td>5.837 - 6.013</td>
<td>Lobophorenol B: 5.92</td>
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<td>6.365 - 6.534</td>
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<td>6.961</td>
<td>6.682 - 7.12</td>
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<tr>
<td>7.715</td>
<td>7.684 - 7.779</td>
<td>-</td>
</tr>
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Table S3. Lobophora ions responsible for the difference according to species after LC-MS analysis. The mSigma (mS) value is a measure for the goodness of fit between experimental mass and isotopic pattern with theoretical ones: lower is the mS, better is the annotation.

<table>
<thead>
<tr>
<th>m/z</th>
<th>rt</th>
<th>Ion assignment</th>
<th>Ion formula</th>
<th>error (ppm)</th>
<th>mS</th>
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<td>334.2741</td>
<td>606</td>
<td>[M + NH₄⁺]</td>
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<td>[M + NH₄⁺]</td>
<td>C₂₁H₂₆NO₂</td>
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<td>21.5</td>
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