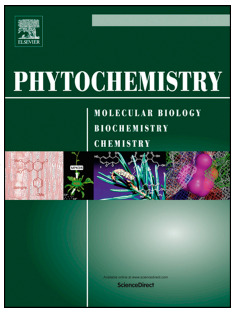

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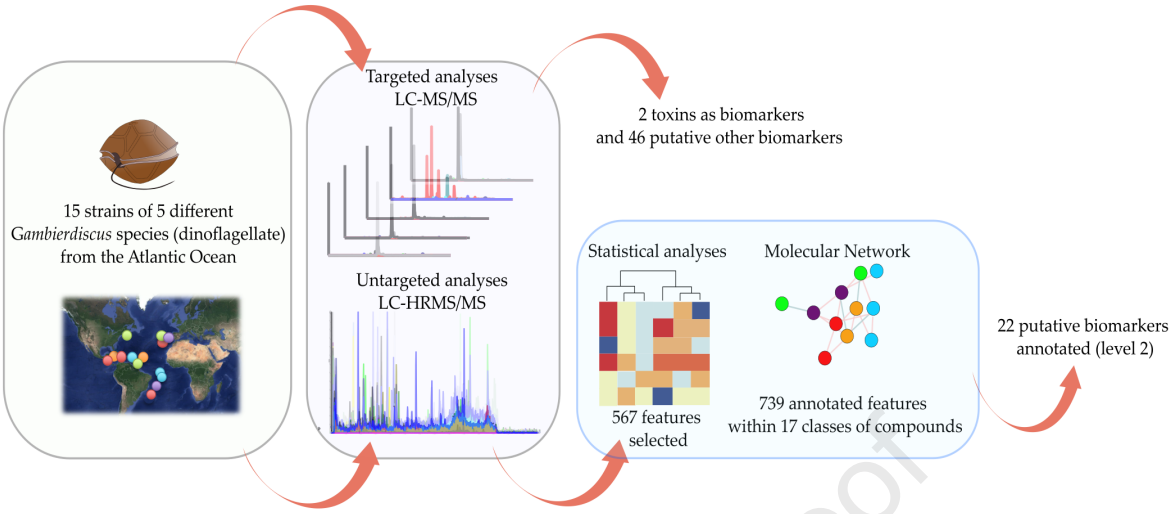

Targeted and non-targeted mass spectrometry to explore the chemical diversity of the genus *Gambierdiscus* in the Atlantic Ocean

Authors

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ABSTRACT

Dinoflagellates of the genus *Gambierdiscus* have been associated with ciguatera, the most common non-bacterial fish-related intoxication in the world. Many studies report the presence of potentially toxic *Gambierdiscus* species along the Atlantic coasts including *G. australes*, *G. silvae* and *G. excentricus*. Estimates of their toxicity, as determined by bio-assays, vary substantially, both between species and strains of the same species. Therefore, there is a need for additional knowledge on the metabolite production of *Gambierdiscus* species and their variation to better understand species differences. Using liquid chromatography coupled to mass spectrometry, toxin and metabolomic profiles of five species of *Gambierdiscus* found in the Atlantic Ocean were reported. In addition, a molecular network was constructed aiming at annotating the metabolomes. Results demonstrated that *G. excentricus* could be discriminated from the other species based solely on the presence of MTX4 and sulfo-gambierones and that the variation in toxin content for a single strain could be up to a factor of two due to different culture conditions between laboratories. While untargeted analyses highlighted a higher variability at the metabolome level, signal correction was applied and supervised multivariate statistics performed on the untargeted data set permitted the selection of 567 features potentially useful as biomarkers for the distinction of *G. excentricus*, *G. caribaeus*, *G. carolinianus*, *G. silvae* and *G. belizeanus*. Further studies will be required to validate the use of these biomarkers in discriminating *Gambierdiscus* species.

The study also provided an overview about 17 compound classes present in *Gambierdiscus*, however, significant improvements in annotation are still required to reach a more comprehensive knowledge of *Gambierdiscus*' metabolome.

Keywords: (5 max)

Toxin profile, *Gambierdiscus* sp., Metabolomics, Molecular networks, chemotaxonomy

1. Introduction

Ciguatera poisoning (CP) is the most commonly reported marine toxin-related foodborne disease with at least between 10,000 to 50,000 cases worldwide annually (Friedman *et al.* 2017). This illness results from the consumption of fish or shellfish that have ingested and accumulated ciguatoxins (CTXs) produced by certain species of dinoflagellates in the genera *Gambierdiscus* and *Fukuyoa*.

Initially considered as a monophyletic group (*i.e.* "*Gambierdiscus toxicus"*), the observation of a strong disparity in terms of morphology and toxicity measured using *in-vivo* or *in-vitro* bioassays (Durand-Clément 1986, Holmes *et al.* 1990) led different research groups to review the classification of *Gambierdiscus* (Chinain *et al.* 1999, Litaker *et al.* 2009, Chinain *et al.* 2020). To date, among the 18 species of *Gambierdiscus* characterized so far by morpho-molecular techniques (Litaker *et al.* 2009, Vandersea *et al.* 2012, Nishimura *et al.* 2016, Kretzschmar *et al.* 2019, Litaker *et al.* 2019, Ott *et al.* 2022), the production of algal ciguatoxins initially identified in *G. toxicus* has been confirmed in *G. polynesiensis* in the Pacific Ocean (Chinain *et al.* 2010, Longo *et al.* 2019, Yon *et al.* 2021a).

Although episodes of ciguatera poisoning are frequent in the Atlantic Ocean, particularly in the Caribbean area, the Canary and Madeira Archipelagos (Boucaud-Maitre *et al.* 2018), the causative ciguatoxins (named C-CTXs for Caribbean ciguatoxins) were, until very recently, only detected in fish (Pottier *et al.* 2002, Ramos-Sosa *et al.* 2022). Searching for toxins potentially involved in ciguatera poisoning has relied on a combination of bioassays and analytical chemistry (Gaiani *et al.* 2020, Gaiani *et al.* 2021, Litaker *et al.* 2017, Reverté *et al.* 2018, Tudó *et al.* 2020a, Tudó *et al.* 2020b, Mudge *et al.* 2023). Importantly, both approaches have demonstrated discrepancies in terms of toxicity or presence of CTX-like and other polyether compounds, between species and also between strains of the same species (see Litaker

et al. (2017); Chinain *et al.* (2020) and references therein). For example, there is a clear contrast between the production of maitotoxin-1 by *G. australes* strains from the Pacific area, but not from the Atlantic Ocean (Chinain *et al.* 1999, Rhodes *et al.* 2014, Pisapia *et al.* 2017b, Estevez *et al.* 2020, Estevez *et al.* 2021). Therefore, the information provided by phylogenetic approaches may not reflect the toxicity and diversity of metabolites produced by certain species/strains of *Gambierdiscus* and it remains important to develop complementary approaches based on chemotaxonomy to complete the understanding of the differences between *Gambierdiscus* species/strains.

Chemical analysis using a targeted approach (*i.e*. monitoring of a reduced number of previously identified compounds or putative closely related analogues) allows the detection and quantification of a set of polyether compounds in *Gambierdiscus* species isolated from the Atlantic Ocean (see Table S1) such as maitotoxins (MTXs) (Pisapia *et al.* 2017b, Estevez *et al.* 2021), gambierones (Rodriguez *et al.* 2015, Murray *et al.* 2019, Yon *et al.* 2021b, Mudge *et al.* 2022) and gambieric acids (Nagai *et al.* 1992a, Nagai *et al.* 1992b). Recently, a compound suggested as an algal C-CTX (named C-CTX5) produced by one strain of *G. silvae* and two strains of *G. caribaeus* has been reported (Mudge *et al.* 2023). This approach is, however, strongly limited by the reduced availability of reference standards (Sibat *et al.* 2018, Estevez *et al.* 2020).

To overcome the lack of reference material and fill in the gaps on the non-toxin metabolites produced by dinoflagellates, the application of more comprehensive untargeted analysis (*i.e*. considering as much as possible the whole set of metabolites) is increasingly used (Gémin *et al.* 2021, Sibat *et al.* 2021). This approach remains challenging due to dinoflagellate rather slow growth, high chemical diversity and largely undescribed metabolomes (probably related to their unusual and complex genomes (Waller and Jackson2009)).

Still, it allowed Malto *et al.* (2022) to report species- and strain-specific features for two *Gambierdiscus* species (*i.e. G. balechii* and *G. carpenteri*), that were tentatively annotated using the Global Natural Product Social Molecular Networking (GNPS) platform (Wang *et al.* 2016).

In an attempt to assess potentially consistent differences in metabolomes of *Gambierdiscus* species, the current study screened 15 strains of five of the seven species of *Gambierdiscus* identified in the Atlantic Ocean so far (Chinain *et al.* 2020). The analytical approach included both targeted analysis and a non-targeted one using a metabolomics workflow followed by feature-based molecular network (FBMN) (Nothias *et al.* 2020) analysis to identify a set of features that could be useful for species discrimination. The laboratory effect, i.e. variation induced by culturing certain identical strains in different laboratories, was evaluated at the toxin- and metabolome levels, using both targeted and non-targeted analyses. This latter approach is critical as it has yet to be established to what extent metabolomes of these species vary according to growth conditions and to ensure the robustness of the selected features on more strains of the different species.

2. Results

In the present study, 15 strains from five species of *Gambierdiscus* originating from the Atlantic Ocean were grown in four different laboratories, along with one strain of *G. australes* originating from the Pacific Ocean that was used as an outgroup (Figure 1). Each laboratory grew between four to six strains, with three of the strains grown in two or three laboratories as biological controls. Chemical profiling was performed by liquid chromatography coupled to tandem low-resolution mass spectrometry (LC-MS/MS) in MRM mode for targeted analysis and toxin quantification and by liquid chromatography coupled to tandem high-resolution mass spectrometry (LC-HRMS/MS) to explore the chemical diversity between the different species in an untargeted approach.

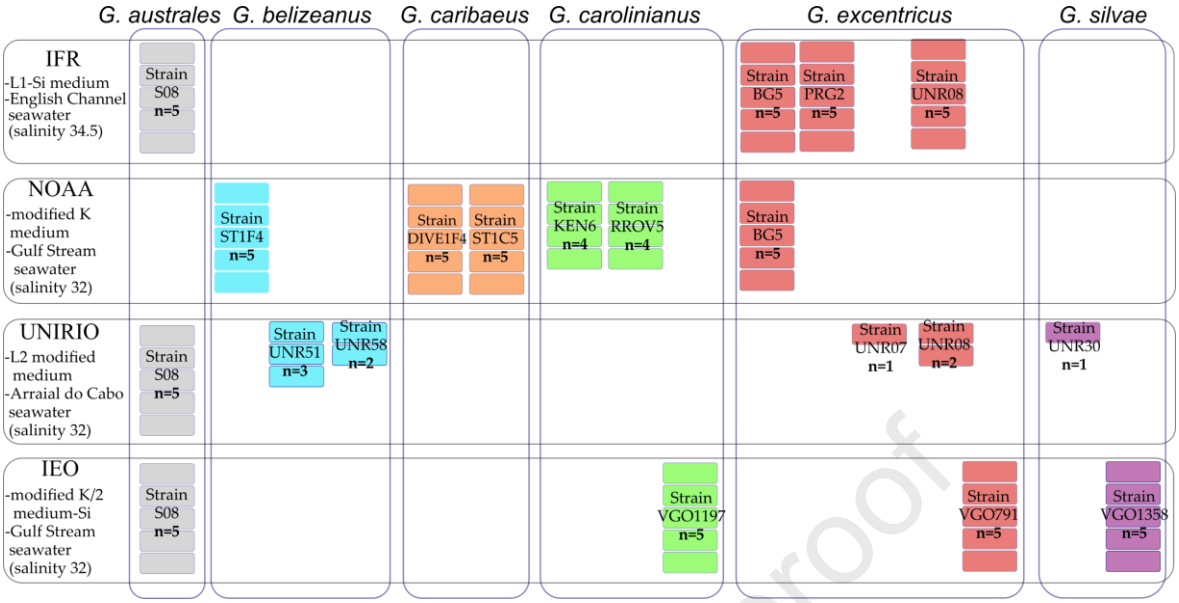

Figure 1. Experimental design for the culture of *Gambierdiscus* strains, including culture media and seawater, strain code and replicates provided by each laboratory: (IFR) METALG Laboratory, Ifremer; (NOAA) Beaufort Laboratory, National Oceanic and Atmospheric Administration; (UNIRIO) Marine Microalgae Laboratory, Federal University of Rio de Janeiro and (IEO) Spanish Institute of Oceanography, Vigo Oceanographic Center. n = number of replicates (also coded by colored-box numbers).

2.1. Targeted analyses

2.1.1. Toxin profiles between strains and laboratories

Toxin profiles were evaluated either by comparison with commercially available standards such as MTX1, gambierone and 44-methylgambierone or with purified solutions of previously identified compounds for MTX4 and sulfo-gambierones (Pisapia *et al.* 2017b, Yon *et al.* 2021b). Using this targeted approach, three different qualitative profiles (presence/absence) were observed (Figure 2 and Figure S1). One toxin profile corresponded to strains of the species *G. silvae*, *G. carolinianus*, *G. caribaeus* and *G. belizeanus* and was characterized by the presence of gambierone and 44-methylgambierone. A different profile was observed for *G. australes* AUS S08, which produced only 44-methylgambierone and MTX1. Finally, the

third toxin profile, shared by all strains of *G. excentricus* consisted of MTX4 and sulfo-gambierones.

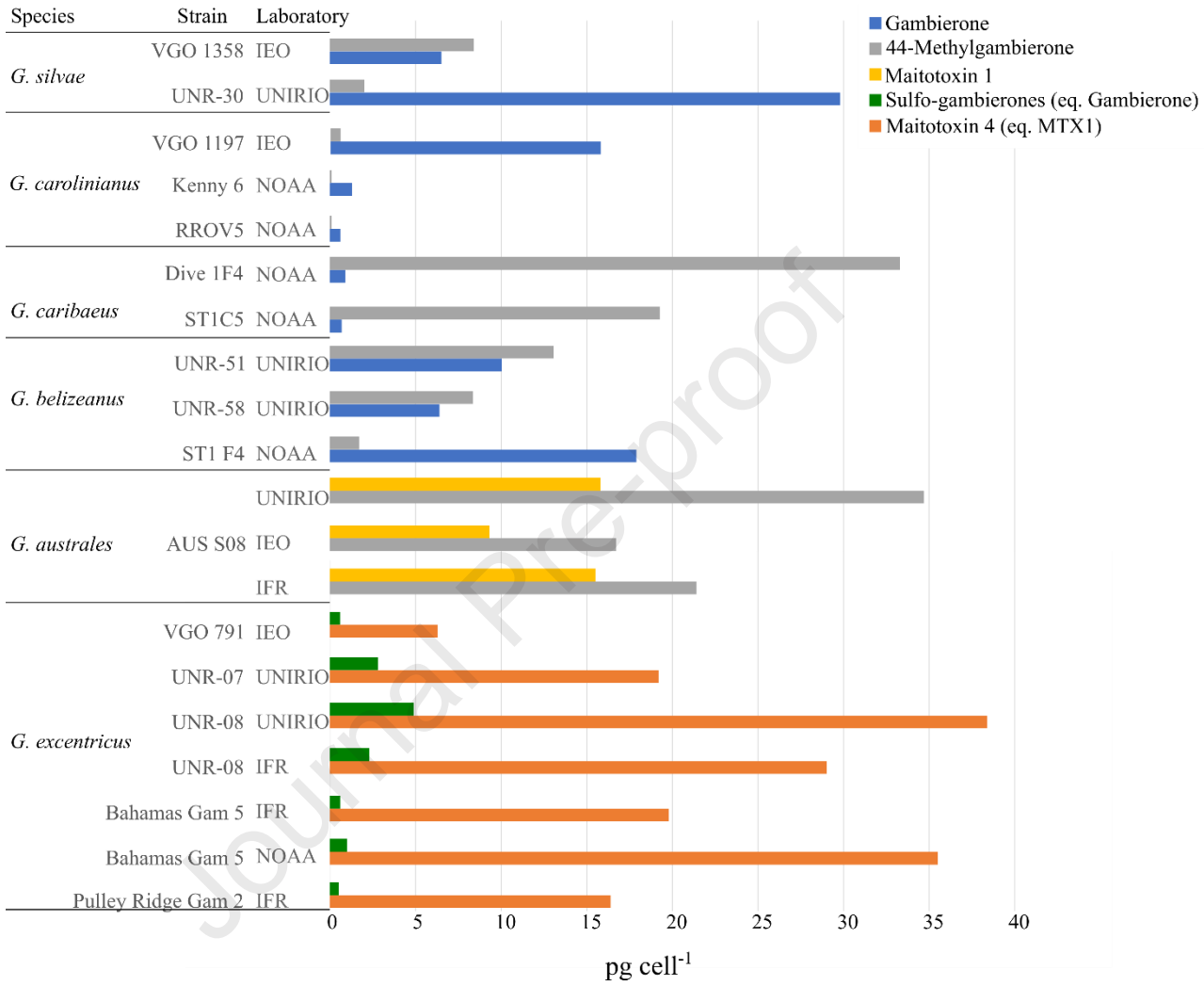

Figure 2. Quantitative toxin profile (in pg toxin equivalent cell^{-1}) obtained by calibration curve of reference standards using LC-MS/MS in negative ESI MRM mode for gambierones (sulfo-gambierones, gambierone, 44-methylgambierone) and maitotoxins (MTX1 and -4) in the different strains (represented by the pools of replicates of strains grown in each laboratory).

Quantitatively, all strains of *G. excentricus* clearly produced more MTX4 than sulfo-gambierones. For the four other species considered in this study, the ratio gambierone/44-methylgambierone showed intra-specific differences. The overall toxin content of gambierones and maitotoxins ranged from 0.10 to 38 pg cell^{-1} (Figure 2 and Table S2), with the lowest concentration measured in *G. carolinianus* RROV5 and the highest in *G. excentricus* UNR08.

When comparing toxin concentrations in the three strains cultivated in different laboratories *(i.e. G. australes, G. excentricus* Bahamas Gam 5 and UNR08), up to a 2-fold difference in toxin content was observed, reflecting the effect of different culture conditions on cellular toxin content.

2.1.2. Variation of chemical profile across species *via* targeted analysis

In addition to the toxins identified, a total of 298 features (*i.e.* a chromatographic peak corresponding to one transition – see Table S3; Table S4; Figure S1 and Figure S2 – at one retention time) were obtained by manual integration. The associated data matrix (*i.e.* area for each feature for each strain) was first analysed with principal component analysis (PCA) to compare the overall chemical profiles. The first two components of the associated PCA (Figure 3) explained 23.1% (PC1) and 14.0% (PC2) of the total variance. The PCA score plot shows that *G. excentricus* (red) and *G. australes* (grey) were clearly discriminated from the other species on PC1 and PC2, respectively. On the other hand, the four-remaining species (*G. belizeanus* – blue, *G. caribaeus* – orange, *G. carolinianus* – green and *G. silvae* – purple) formed a scattered cluster.

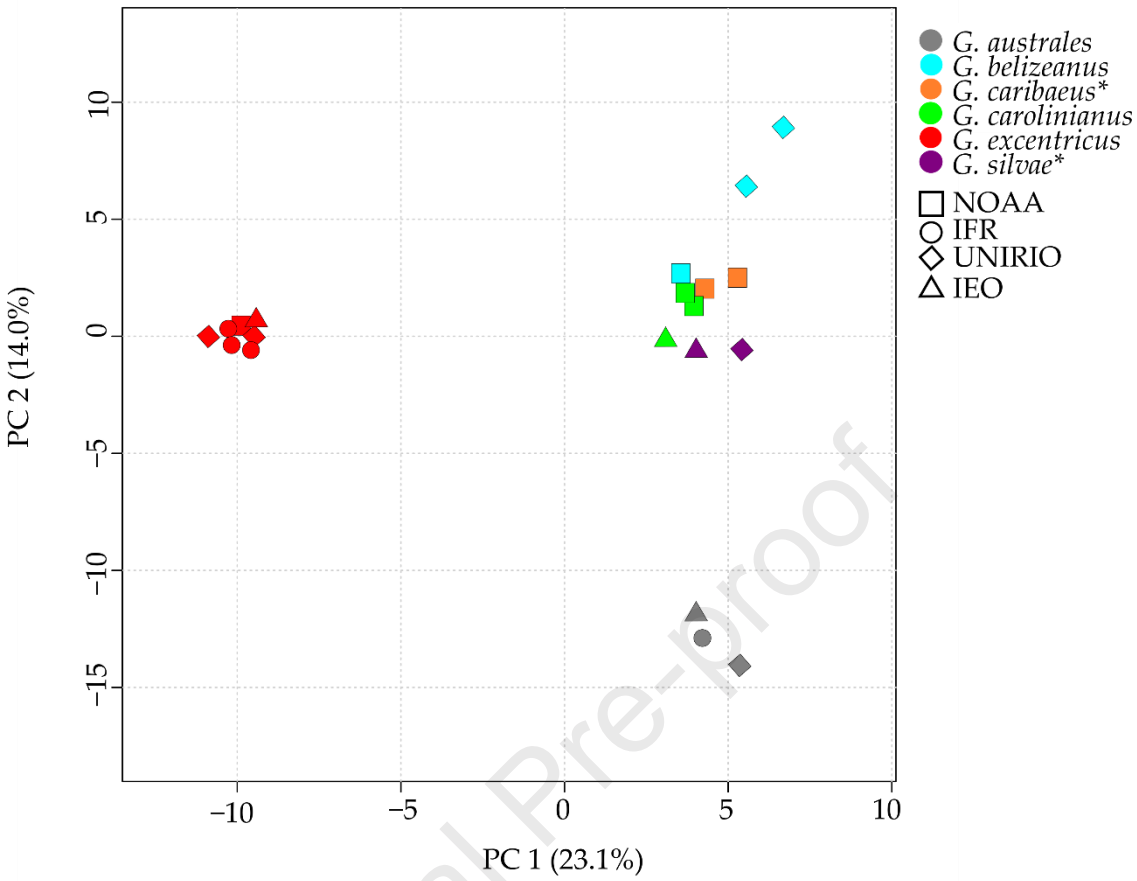

Figure 3. Principal component analysis score plot based on the 298 features integrated from the targeted analysis data acquired on the pool per strain per laboratory. The color coding represents the species while the symbol shapes represent the laboratory. (NOAA) Beaufort Laboratory, National Oceanic and Atmospheric Administration; (IFR) METALG Laboratory, Ifremer; (UNIRIO) Marine Microalgae Laboratory, Federal University of Rio de Janeiro and (IEO) Spanish Institute of Oceanography, Vigo Oceanographic Center.
* When only two strains were available (for *G. silvae* and *G. caribaeus*) a third sample has been virtually constituted using the average intensity of the strains available to build the model and then removed from data visualization.

Once the interlaboratory control (*G. australes*) was removed to only compare the strains isolated from the Atlantic Ocean, the supervised 3-component Partial Least-Squares Discriminant Analysis (PLS-DA) score plot (R^2: 0.97; Q^2: 0.83; *p*-value 0.02 with 100 permutations) showed that the separation between the *G. belizeanus*, *G. caribaeus*, *G. carolinianus* and *G. silvae* species was successfully achieved on component 1 explaining 23.5% of the total variability (Figure S3).

The 46 most discriminant features (*i.e.* features that are important for species discrimination), with a variable importance in projection (VIP) score > 1.5 (*i.e.* a score > 1

corresponds to a significant variable in the model) (Wold *et al.* 2001) are presented in Figure 4.

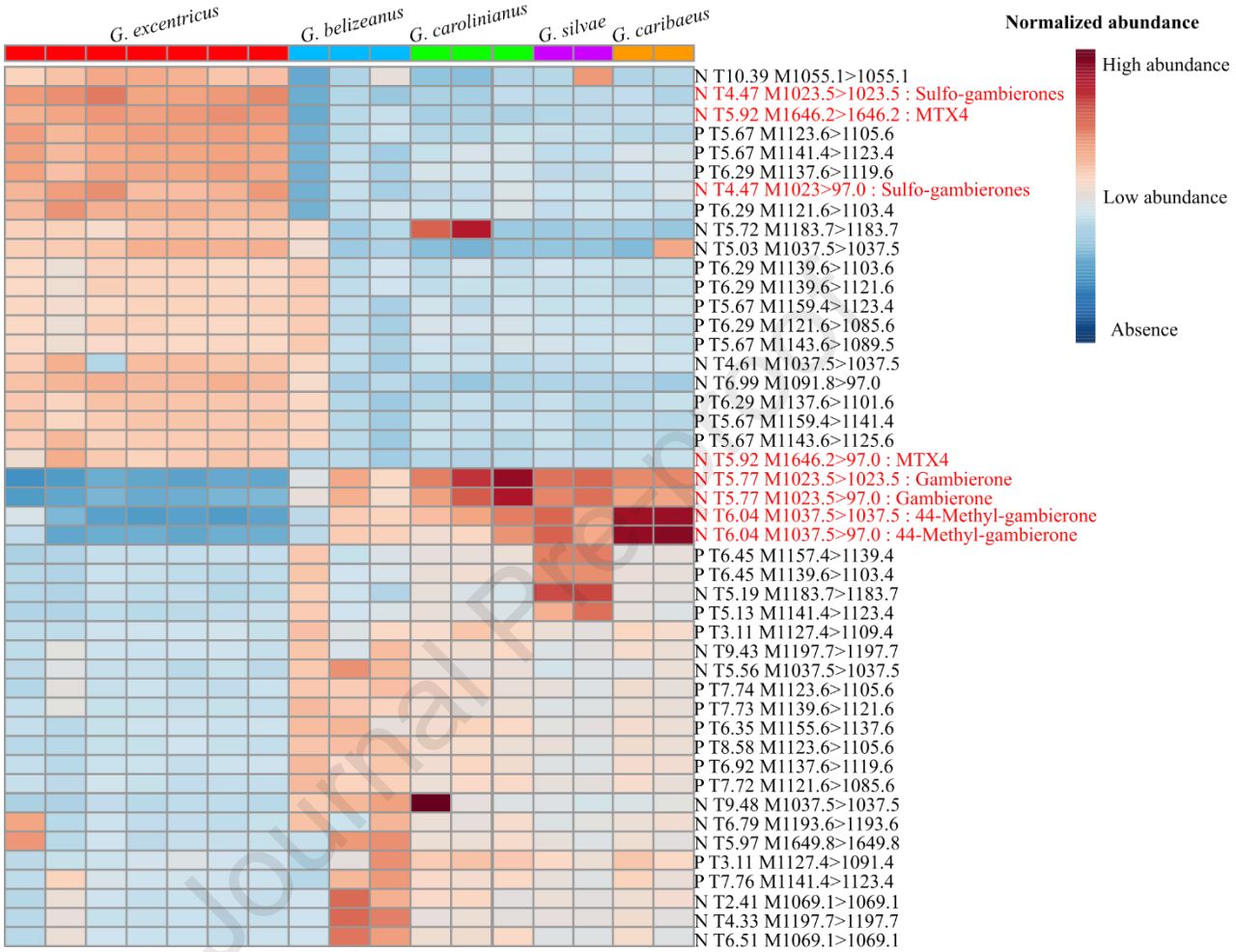

Figure 4. Heat map of normalized abundance of the 46 most important features in species separation (VIP score > 1.5) selected by the PLS-DA model from the 298 features obtained by LC-MS/MS. N: negative ionization mode; P: positive ionization mode; T: retention time in minute; M: transition with precursor ion > product ion. In red the identified compounds and in black the features that could not be identified.

Interestingly, on the heatmap (figure 4), 21 features were either only present or more abundant in all *G. excentricus* strains compared to all the other strains and among them, two features corresponded to sulfo-gambierones and two others to MTX4. The remaining 25 features were more abundant in all other species compared to *G. excentricus* although there was

no clear species-specific pattern between the four other *Gambierdiscus* species. Still, four features corresponded to gambierone and 44-methylgambierone (by comparing to standards).

The attempt to confirm the polyether-like identity of the 38 features (in black on figure 4) by LC-HRMS was unsuccessful as the transition giving signal by LC-MS/MS corresponded to either an isotope of the theoretical mass, a different exact mass (mass error > 10 ppm) or a compound which does not exhibit a typical polyether pattern on the mass spectrum (*i.e*. adducts and water losses, data not shown). Thus, so far, these features could not be associated with any known polyether produced by *Gambierdiscus* species (Pisapia *et al.* 2017b, Yon *et al.* 2021b).

2.2. Untargeted analysis

2.2.1. Exploring chemical divergence between *Gambierdiscus* species.

To more deeply explore the intra- and inter-species differences within *Gambierdiscus* metabolomic profiles, extracts were further profiled by LC-HRMS and subjected to a metabolomic workflow (Want *et al.* 2010). The final data matrices after data filtering (removing blank and inconsistent signals) and merging of positive and negative ionization modes (using a multi-block approach) contained the integration of 11,424 features across the 82 samples and were first analysed by an unsupervised ComDim approach. The first two components of the ComDim score plot (Figure 5A) accounted for 16.6% and 10.2% of the total variance, however, unexpectedly, the main clustering seemed to be related to the laboratory origin of the samples. This laboratory effect is clearly visible when looking at the outgroup (*G. australes* samples in grey). However, exploring components 1 to 5 (Figure S4) allowed to observe a structured representation of the samples according first to the laboratory and then to the *Gambierdiscus* species.

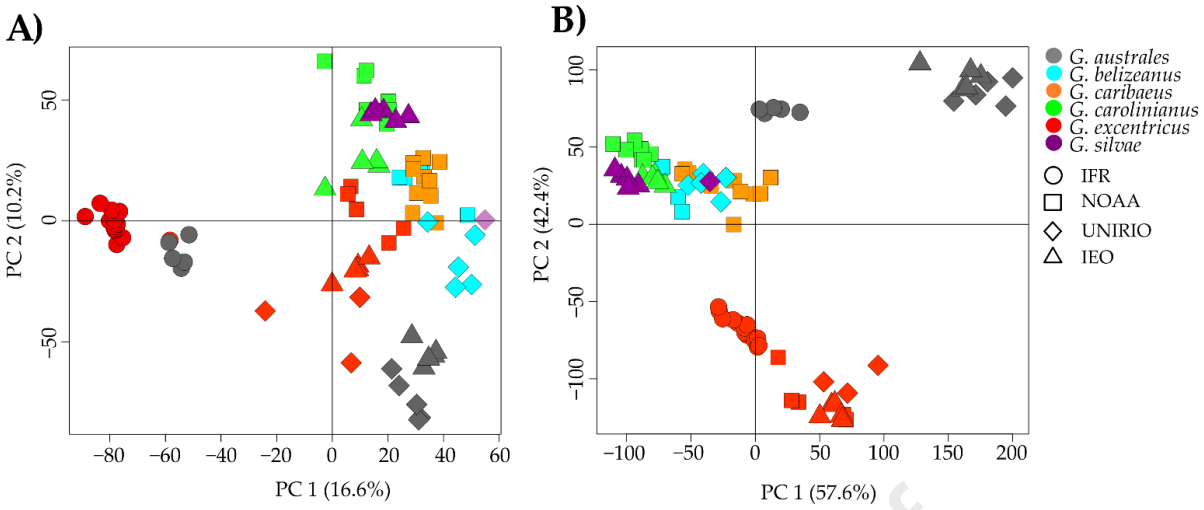

Figure 5. (A) ComDim score plot and (B) OSC-ComDim score plot of features obtained by untargeted analysis using full scan mode in both polarities (log-transformed, pareto-scaled data and multiblock correction) illustrating the strong laboratory effect in (A) and its correction after OSC (B). The color coding represents the species while the symbol shapes represent the laboratory. (IFR) METALG Laboratory, Ifremer; (NOAA) Beaufort Laboratory, National Oceanic and Atmospheric Administration; (UNIRIO) Marine Microalgae Laboratory, Federal University of Rio de Janeiro and (IEO) Spanish Institute of Oceanography, Vigo Oceanographic Center.

As interlaboratory variations impair the exploration of the data by ComDim, it was decided to tentatively remove such an effect from the data to confirm the species effect on the clustering. Several strategies were attempted and are detailed in Supplementary paragraph 1. Orthogonal Signal Correction (OSC) was finally performed to remove the interlaboratory effect from the original data matrix, providing two new data matrices: one related to the interlaboratory effect and one orthogonal to this effect (*i.e.* formerly the residue) where such effect is reduced. The latter residual matrix was finally explored to highlight species differences.

While some interlaboratory effect was still noted, the resulting OSC-ComDim (ComDim after OSC correction) score plot (Figure 5B) shows a significantly improved discrimination according to the *Gambierdiscus* species, with three well-defined clusters on the first two PCs: one for *G. australes* (grey), one for *G. excentricus* (red) and one for the other species (*G. belizeanus*, *G. caribaeus*, *G. carolinianus* and *G. silvae*). As the OSC correction revealed the species-dependent structure of the data, supervised statistical analysis was performed to highlight the contributing features.

2.2.2. Supervised data analysis highlights signals related to Atlantic species discrimination

Using the initial merged data matrix, a multi-block partial least squares discriminant analysis (MB-PLS-DA) model (Figure S5) was constructed based on 9 components (R^2X cumulated 0.55, R^2Y cumulated 0.99, Q^2Y 0.95) and validated (1000 permutations, *p*-value 0.001). From this model, where *G. excentricus* was well separated on component 1 while the other species were separated on component 2, a list of 567 features with a VIP score > 1.5 (Supplementary file 2) was selected, mostly from the positive ionization mode.

2.2.3. Molecular networking for compound annotation

Among the 567 VIPs selected, only one could be annotated by standard injection, *i.e.* 44-methylgambierone (VIP score 1.5) eluting at 7.7 min with the two *m/z* 1037.4765 ($[M-H]^-$, -1.9 ppm) and 1039.4945 ($[M+H]^+$, +1.3 ppm). In an attempt to identify the remaining features, the FBMN approach was performed (Aron *et al.* 2020).

In a molecular network (MN), chemical features (ions with a mass, retention time and MS/MS spectrum) are referred to as nodes (when connected to other features) or singletons (when unconnected to other features). The connections between chemical features (or nodes) are called edges and correspond to the user-defined minimum i) number of common fragment ions and ii) cosine value (*i.e* similarity) between the MS/MS spectra of the features. The FBMN obtained in this study was composed of 6,615 nodes, 16,942 edges and 4,224 singletons and provided an overview of metabolites or metabolite families and their relative abundance (*i.e.* chemodiversity) within the different *Gambierdiscus* species. In addition, the use of MolNetEnhancer (Ernst *et al.* 2019) permitted annotation of 739 features across 40 clusters (Figure 6) at an annotation level 3 according to the classification of annotation levels proposed by Sumner *et al.* (2007), thus providing structural family information. The comparison with

databases (cosine score > 0.9 and exact mass error < 10 ppm) also permitted to identify 18 compounds at an annotation level 2 (Supplementary part 2). Out of the 567 features selected by the MB-PLS-DA, 175 features could be sufficiently fragmented to be integrated into the MN but none of them could be annotated by comparison with GNPS databases when considering a relative retention time consistent to the polarity of the putative identity and an accurate mass error lower than 10 ppm. Altogether, 10 features with a VIP score < 1.5 were present in 4 annotated clusters (*i.e.* classes of compounds) corresponding to aliphatic, betaine lipid, phenylpropanoic acid and glycerolipid.

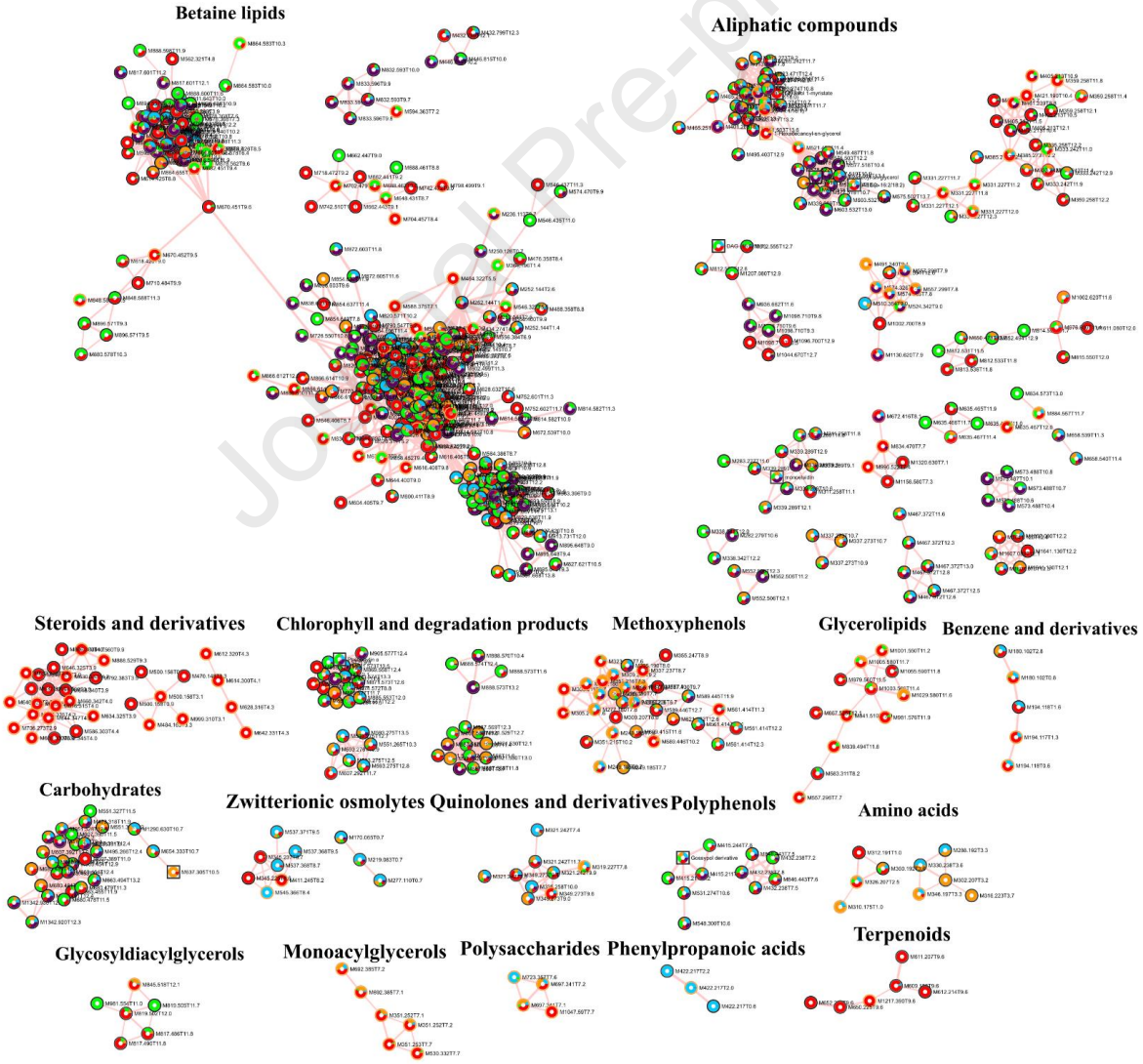

Figure 6. Selection of the 40 annotated clusters (739 features) belonging to 17 chemical classes annotated using the molecular network built with GNPS from iterative auto-MS/MS data acquired on the *Gambierdiscus* extracts. 68 clusters of more than 3 nodes, 212 clusters of 2 nodes and 4224 singletons were excluded from the MN. Each node corresponds to a precursor ion with a color-coded diagram representing its relative abundance within the different species (blue for *G. belizeanus*, orange for *G. caribaeus*, green for *G. carolinianus*, red for *G. excentricus* and purple for *G. silvae*). The border color of each node corresponds to the VIP score of the feature (<1: black, between 1 and 1.5: orange and > 1.5: red). The shape of each node corresponds to the annotation level (circle: annotation level 2, square: annotation level 3). The annotated molecular network is provided in supplementary File 3.

On the heatmap of the normalized abundance of 567 features within the samples (Figure 7), five distinct groups of features were observed. The first group corresponding to the first 70 features (from top to bottom on figure 7) was characterized by a normalized abundance more important in all *G. carolinianus* and *G. caribaeus* strains while the second (features 71 to 220) consisted of features more present in only *G. caribaeus* strains. The same trend was observed for features 221 to 300 that were more abundant in all *G. silvae* strains and features 301 to 494 that were more abundant in *G. belizeanus* strains. Finally, the features 495 to 567 were more present in *G. excentricus* strains but some of them were also present in *G. belizeanus*, *G. caribaeus* or *G. silvae*.

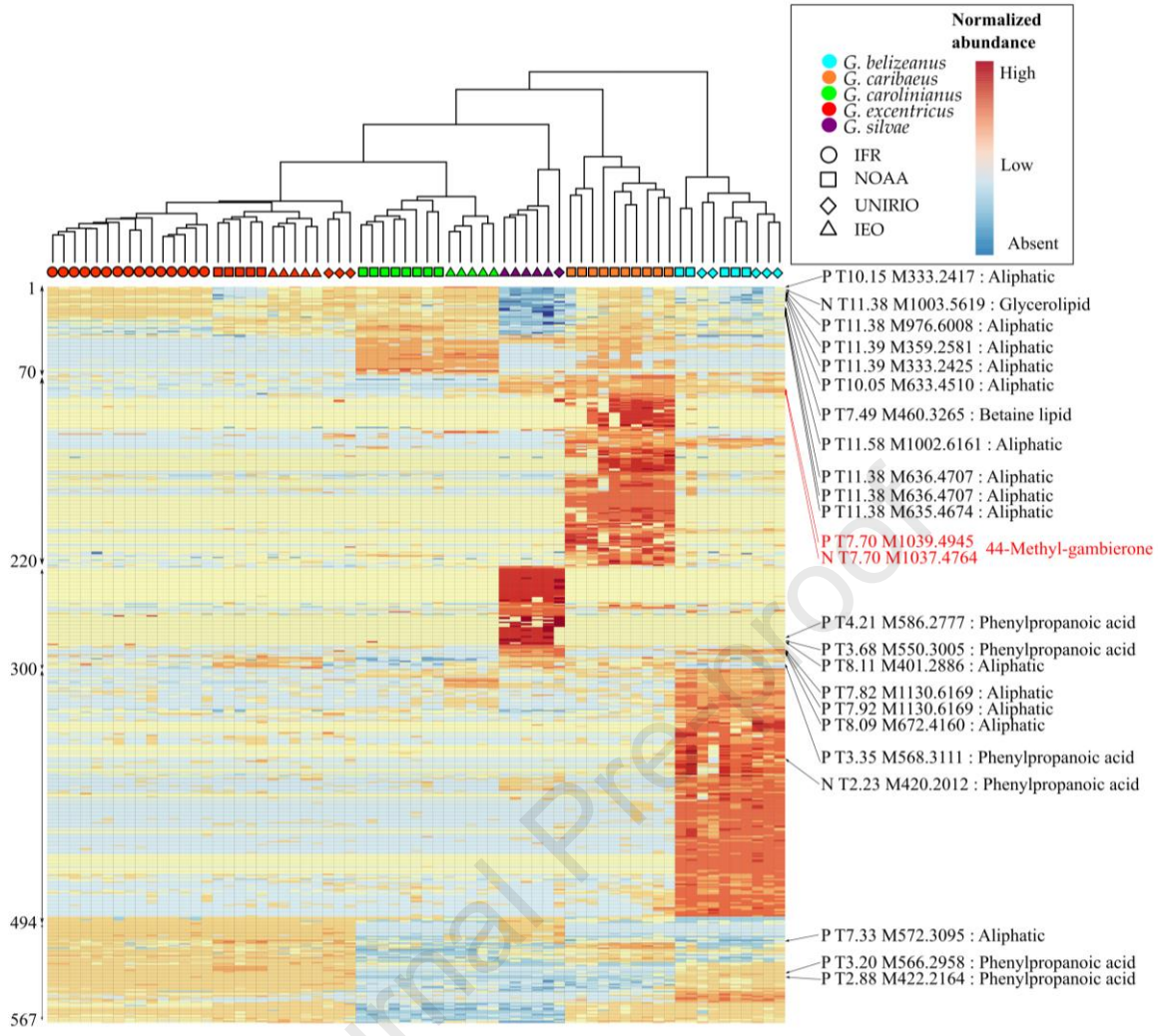

Figure 7. Heatmap based on normalized abundance of the 567 more important features (VIP score upper than 1.5) for species differentiation selected by MB-PLS-DA and hierarchical classification of the different samples based on the 567 selected features (the list of features is available in supplementary File 2). The color coding represents the species while the symbol shapes represent the laboratory. N: negative ionization mode; P: positive ionization mode; T: retention time in minute; M: measured *m/z*. Features identified (level 1 annotation) are color coded in red and features with annotation level 3 are color coded in black.

3. Discussion

In this study, targeted and non-targeted high-resolution mass spectrometry were used to evaluate and increase the knowledge about the chemodiversity (*i.e*. polyether toxins and metabolomic profiles annotated using molecular networking) of five *Gambierdiscus* species originating from the Atlantic Ocean. Studying the intra and inter-specific variations of these

profiles demonstrated that the effect of different cultivation conditions depended on the analytes considered, and also enabled the selection of a series of candidate biomarkers putatively useful for species discrimination.

3.1. Exploration of *Gambierdiscus* chemodiversity through targeted and untargeted mass spectrometry

The current knowledge of metabolites produced by *Gambierdiscus* is quite limited (Table S1) and mostly encompasses toxins (Chinain *et al.* 2010, Pisapia *et al.* 2017b, Murray *et al.* 2018, Boente-Juncal *et al.* 2019, Estevez *et al.* 2020, Murray *et al.* 2020, Murray *et al.* 2021, Yon *et al.* 2021a), and to a lesser extent pigments (Durand and Berkaloff, 1985, Zapata *et al.* 2012). In addition, Malto *et al.* (2022) also provided insights into *Gambierdiscus* metabolome with the annotation of 303 features (i.e. 33 at level 2 based on GNPS library hits and 270 at level 3) corresponding to lipids, sterols, carbohydrates, peptides, terpenes and polyhydroxylated compounds.

In this study, the consideration of most recently reported toxin analogues associated with the growing but still limited access to reference standards allowed to provide the most up-to-date quantitative toxin profile of maitotoxins (MTX1 and MTX4) and gambierones (gambierone, 44-methylgambierone, sulfo-gambierones) for 15 strains corresponding to five of the seven species of *Gambierdiscus* found in the Atlantic Ocean so far (Chinain *et al.* 2020). The toxin profile of the *G. australes* strain used as an outgroup was also provided even though this strain was isolated from the Pacific Ocean (Nishimura *et al.* 2013). A similar toxin profile was observed for all strains of *G. silvae*, *G. belizeanus*, *G. carolinianus* and *G. caribaeus* although the strains were isolated at different periods and locations across the Atlantic Ocean and then cultivated in laboratory conditions which shows that toxin production capacity

(presence or absence) is not significantly influenced by the strain's environment. These strains contained various amounts of gambierone and 44-methylgambierone (Figure 2 and Table S2) while no other polyether toxins reported in *Gambierdiscus* were detected. Similarly, all strains of *G. excentricus* regardless of isolation area, produced MTX4 and sulfo-gambierones while no gambierone or 44-methylgambierone were detected. Such differences between one species of *Gambierdiscus* and the other species found in a same area was also reported in the Pacific where only *G. polynesiensis* was demonstrated as a ciguatoxin producer among the seven species found in French Polynesia (Stuart *et al.* 2022).

Surprisingly, while recent studies reported the presence of putative toxins (*i.e.* partial structural identification and lack of confirmation in the literature) such as 29-methylgambierone in *G. silvae* (Mudge *et al.* 2022), MTX5 in *G. australes* (Estevez *et al.* 2021) and C-CTX5 in *G. silvae* and *G. caribaeus* (Mudge *et al.* 2023), none of them could be detected in this study. In the present work, the capacity of a strain to produce toxins was not strongly affected by its culture conditions, so it might suggest that the lack of detection of these toxins was linked to a combination of low toxin content in the cell studied and low sensitivity of the analytical method, however, as there is a lack of quantitative data on these compounds in the literature, it was not possible to know whether it would have been detectable.

As the targeted approach was limited by knowledge on polyehter toxins previously reported, a more comprehensive analysis of the metabolites produced by the different strains was undertaken in this work through untargeted analysis followed by molecular networking as data exploration. Hence, the FBMN obtained in this study permitted to annotate 739 features within 17 different classes of compounds with an annotation level 3 (Figure 6). In addition to the chemical classes already reported by Malto *et al.* (2022), phenolic compounds (methoxy phenols, polyphenols), glycerols (glycerolipids, acylglycerols), zwitterionic osmolytes, quinolones and steroids were annotated in the course of this study using MolNetEnhancer (Ernst

et al. 2019) illustrating the capacity of *Gambierdiscus* for producing a large variety of compounds as previously reported for other micro-algal organisms (Koester *et al.* 2022). As expected, constitutive and primary metabolites such as lipids, carbohydrates, pigments and amino acids, that could be annotated, were detected in all strains. However, features in clusters corresponding to steroids and terpenoids were almost exclusively detected in *G. excentricus* while features annotated as phenylpropanoic acids were only detected in *G. belizeanus* suggesting a specific production by these strains that could be interesting to study for specie-specific biomarker selection.

3.2. Laboratory effects depending on the analytical approach

An important culture effort was carried out in this study. To reduce the time consuming and challenging acclimation process of *Gambierdiscus* strains, only three parameters were fixed for the culture in all laboratories: irradiance, light: dark cycle and growth phase at sample collection. The remaining culturing conditions (seawater, media, type of flask, source of light, etc.) were defined by each laboratory to maintain optimal growth of *Gambierdiscus* strains. Thus, the observation of a laboratory effect was therefore expected and was investigated and tentatively corrected using the *G. australes* outgroup and the two *G. excentricus* strains that were grown, on purpose, in more than one laboratory.

Variation of toxin content (evaluated for MTX1, MTX4 and sulfo-gambierones) did not exceed a factor of 2.1 between replicates of the same strains cultivated in different laboratories (Figure 2). As for the variation within one strain kept in a given laboratory, the amount of 44-methylgambierone produced by the five biological replicates of *G. australes* was in the range of 10-18% variation (*i.e*. data obtained from three laboratories, based on the integration of the feature NT7.70M1037.4764 from untargeted analysis).

Similarly, the laboratory effect was low for the targeted analysis demonstrating that the toxin profile or the 298 features resulting from the integration of the 56 MRM transitions were less subjected to laboratory variation.

While the literature contains many toxicity comparisons (using either bioassays or chemical profiles) between *Gambierdiscus* species or strains of the same species (Litaker *et al.* 2017, Estevez *et al.* 2020, Rossignoli *et al.* 2020, Tudó *et al.* 2020b), studies focusing only on the laboratory effect remain scarce. Generally, other variations could have impaired interpretation of the laboratory effect through use of different strain, temporality, measurement methods or age of the strains. Only one study, performed with an N2a cell-based assay on *G. pacificus,* concluded that differences in culture conditions did not affect the toxicity (11% of variation) (Pisapia *et al.* 2017a), a result consistent with the findings obtained by targeted analyses in this study.

Concerning untargeted analysis, the ComDim score plot (Figure 5A) demonstrated that differences between laboratories largely outweighed the inter-species effect, as could be expected at the metabolome levels when dealing with different culture conditions, because the metabolome represents the ultimate phenotypic response of an organism to a modification of its environment or functioning. This is why this study included the *G. australes* strain as a control group, to allow for the search for suitable signal correction to reduce such effect, and subsequently to focus on the inter-specific differences. Several strategies for correcting this effect were evaluated, and the OSC approach (Fig. 5B) was the most effective without substantially reducing the number of features. Future inter-laboratory comparison studies should take into consideration the need of standardising culture conditions (e.g. seawater, medium, type of flask, source of light) to avoid the use of a signal correction step that proved only partially helpful in reducing the laboratory effect.

3.3. Exploration of intra- and inter-species chemotaxonomic variability for species classification

Comparison of toxin profiles confirmed the singularity of *G. excentricus* toxin production but did not permit to differentiate the other four species. In addition, the possibility of using low-resolution mass spectrometry in MRM mode to search for other putatively known polyethers or isobaric compounds was explored. This semi-targeted approach revealed that the majority (*i.e.* 58%) of features were common to several species, however, a selection of 7 to 38 features were present in only one species and could therefore represent putative biomarker candidates. The selection of the 46 most discriminant features using PLS-DA model demonstrated that 21 features could be useful to discriminate *G. excentricus* from the other species (including features corresponding to MTX4 and sulfo-gambierones), the 25 other features were not specific to a single specie making them less interesting as biomarker candidates.

Previously suggested as a potential biomarker of the presence of *Gambierdiscus* (Murray *et al.* 2020), 44-methylgambierone has also been detected in other genera (Tibiriçá *et al.* 2020, Murray *et al.* 2021). Since 44-methylgambierone has not been found in *G excentricus* so far, its robustness as a biomarker of this genus is questionable.

Based on these results, the monitoring of sulfo-gambierones in addition to MTX4 should be performed to facilitate the report of *G. excentricus*, as the response factor of sulfo-gambierones was significantly higher than the one of MTX4 (Table S9). Both compounds are, to date, interesting biomarkers of the *G. excentricus* species.

The use of untargeted analysis and supervised statistical analysis was then evaluated for the search of putative biomarkers able to distinguish between the five Atlantic species. Hence, relying on MB-PLS-DA that successfully separated the samples according to the species, it was possible to select 567 features (VIPs) that were related to species discrimination. Some VIPs

were clearly more abundant in certain species compared to the others (Figure 7). Hence a set of 150 features appeared worthy of searching for the presence of *G. caribaeus* (including features corresponding to 44-methylgambierone, this result being consistent with targeted analysis) while a set of 80 features were more abundant in *G. silvae* and a selection of 194 features seemed more abundant in *G. belizeanus*. Finally, 73 features (from 494 to 567) may help in the detection of *G. excentricus*, however, no features were demonstrated as much higher in abundance in that latter species.

3.4. Study limitations

The vast majority of features selected in this study remain unknown due to the poor knowledge of metabolites (except toxins) produced by *Gambierdiscus* as well as micro-algae in general (Zendong *et al.* 2016, Sibat *et al.* 2021). Among the 567 VIP-selected features from untargeted analysis, only 44-methylgambierone (corresponding to two features) was formally identified by comparison to the commercially available standards. For the other VIPs, the FBMN with GNPS database annotations (completed using MolNetEnhancer) provided chemical class annotation for 48 clusters. Unfortunately, only 10 of the 565 VIPs could be annotated in that way due to the low annotation coverage. While the use of MNs for structural annotation has been growing steadily, and the GNPS database is continually accumulating reference mass spectra (more than 700,000 MS/MS spectra in 2024 https://external.gnps2.org/gnpslibrary), less than 10% of the features could be annotated at a level 3 in this study and less than 0.2% at a level 2, reflecting the considerable effort required to improve annotation capabilities for complex marine organisms. Promising approaches were recently reported to increase the annotation rate of metabolites from poorly described organisms. For example, by using a targeted approach (isolation and structural characterization) on a selection of metabolites to build an additional in-house database, Carriot *et al.* (2021) were able to annotate up to 212 metabolites and by combining actual databases with predictive

databases generated by *in-silico* approaches. Koester *et al.* (2022) increased from 4.7% of the MN annotated using GNPS database (typical for marine organisms) to over 75% of the annotated network via chemical classes propagation using Sirius (Dührkop *et al.* 2019).

In addition, since the metabolome is influenced by many biotic and abiotic parameters, more studies using larger number of strains, species and replicates are mandatory to confirm the hypotheses on features that are important to discriminate species and to build robust discrimination models.

4. Conclusions

This study reports the toxin and metabolomic profiles of 15 strains from 5 species of *Gambierdiscus* found in the Atlantic Ocean and cultivated in four different laboratories. There was no significant difference in targeted analysis across laboratories, however, the metabolome of *Gambierdiscus* was highly influenced by culture conditions. Such a laboratory effect within the metabolomic data could be reduced with a proper signal correction (i.e. OSC). The level of annotation in this study was limited to known toxins and several ubiquitous chemical classes (*e.g*. lipids) while the dense MN demonstrated the breadth of the effort required to achieve a comprehensive knowledge of the metabolome of *Gambierdiscus.*

Nevertheless, *G. excentricus* appeared to be sensitively discriminable using its singular toxin profile (MTX4 and sulfo-gambierones). For the other species, untargeted analysis with supervised multivariate statistics permitted the selection of 567 features representing promising candidates to help in the differentiation of *G. caribaeus*, *G. silvae* and *G. belizeanus*.

Further studies are needed to identify the candidate biomarkers and to test their robustness, including how they are affected by culturing conditions and their specificity (by screening other strains and species and natural samples).

5. Materials and Methods

5.1. Culture of *Gambierdiscus* strains

In the present study, 15 strains of *Gambierdiscus* representing five species were assayed. Cultivation of these strains was conducted in four separate laboratories. Each laboratory grew its available strains (culture condition, species and number of replicates are synthesized in figure 1). In addition, the *G. australes* strain AUS S080911_1 isolated from the Pacific Ocean and provided by M. Adachi was grown in three of the four laboratories (Nishimura *et al.* 2013). This latter strain served as a normalizing outgroup to evaluate interlaboratory variations.

While irradiance (70-100 µmol photons m^{-2} s^{-1}) and light/dark cycle (12h:12h) could be standardized between the different laboratories, making the other cultivation conditions consistent would have resulted in long acclimation times given the slow growth rates of *Gambierdiscus* species or even risks of strain loss. For this reason, the other culturing conditions utilized by each laboratory to maintain optimal growth of *Gambierdiscus* strains were not varied. All details, such as the origin of the strains, culture conditions used in the different laboratories, and harvesting methods employed by each laboratory are provided in Table S5.

5.2. Cell pellet extraction

Freeze dried cell pellets (i.e. a total of 82) obtained from the replicate cultures for each isolate grown under various conditions in the different laboratories were extracted twice in methanol 90%. The extraction volume was standardized, and based on a ratio of 2 mL of solvent per million cells. The extraction cycle was as follows: vortex 30 s, ultrasonic bath (25 kHz on ice, 15 min), vortex 30 s and centrifugation (4,000 g, 2 min). The extracts resulting from the two extraction cycles were pooled into an amber glass vial and stored at -80 °C (final cell concentration 250,000 cells mL^{-1}). The number of cells, the dry weight of each extract and the final mass concentration are provided in Table S6.

To reduce as much as possible manipulation artifacts, all samples were processed randomly over two days using the same batch of solvents and plastic containers. In addition, experimental blank extracts were produced using this extraction procedure on 3 empty 50 mL plastic conical tubes (Falcon®, FisherBrand®, Nunc®).

5.3. Targeted LC-MS/MS analyses

To reduce both analysis time and data treatment, pooled sub-samples were created by taking and pooling a third of the total volume of extracts generated from each replicate to create 20 average samples representative of each strain grown by the four laboratories. The 20 samples and 4 blanks were filtered through 0.2 µm filters (Nanosep, modified nylon) prior to analysis by LC-MS/MS. The blank extractions were included to allow the identification of any extraneous signals derived from containers.

5.3.1. Instrumental conditions

Targeted analyses were performed using an ultra-fast liquid chromatography system (UFLC, Nexera, Shimadzu, Japan) coupled to a hybrid triple quadrupole-linear ion-trap mass spectrometer (4000 QTRAP, Sciex, CA, USA) operating in MRM mode of acquisition. The chromatographic column was a Kinetex C18 (50 x 2.1 mm, 2.6 µm, 100 Å, Phenomenex, CA, USA) with a flow rate of 0.4 mL min^{-1} Two methods were used in this study:

- the negative ionization (ESI^-) method as described in Yon *et al.* (2021b) with MRM transitions corresponding to maitotoxins, gambierones, gambieric acids and gambieroxide (Table S3).

- the positive ionization (ESI^+) method was modified from Estevez *et al.* (2019) by adding the transitions corresponding to C-CTX1 to -4 and I-CTX3 to -6 based on molecular formula reported in the literature (Table S4).

5.3.2. Data processing

The two MRM methods used in this study were able to detect 23 toxins based on 56 MRM transitions (2-4 transitions per toxin). This allowed us to: i) describe qualitative and quantitative toxin profiles for the 6 species and ii) assess the usefulness of the different signals/transitions (irrespective of the retention time) in species discrimination to extend the possibilities of the MRM mode and to potentially detect new analogous or isobaric compounds to the known toxins reported in *Gambierdiscus* species. Chromatographic peaks responding to at least one transition and having an intensity higher than 1,000 in ESI^+ and 10,000 in ESI^- have been manually integrated regardless of the retention time. A chromatographic peak observed on a defined transition at a defined retention time is considered as a feature.

Limits of detection (LOD) and quantification (LOQ) for gambierone and 44-methylgambierone were determined graphically (Vial and Jardy, 1999) using signal-to-noise ratio calculated by Analyst software (S/N > 3 for the LOD and S/N > 10 for LOQ) (Table S7).

Known toxins were either directly quantified by external 6-point calibration curves for available standards (MTX1 from Wako (FUJIFILM Wako, Japan): 200-5000 ng/mL; gambierone and 44-methylgambierone from Cifga (Cifga laboratory, Spain): 50-1250 ng/mL) or estimated in MTX1 equivalent for MTX4 and in gambierone equivalent for sulfo-gambierones, assuming that the structural analogy resulted in similar response factors (Table S2). The instrument control, data processing and analysis were conducted using Analyst software 1.7.2 (Sciex, CA, USA).

5.3.3. Statistical analyses

Statistical analyses were performed using Metaboanalyst 5.0 (Pang *et al.* 2021), on Log_{10}-transformed and Pareto-scaled (mean-centered and divided by the square root of the standard deviation of each variable) data. Data matrices obtained from ESI^- and ESI^+ acquisitions were

merged and strains of the same species were grouped. A principal component analysis (PCA) was first used to explore the dataset, followed by a partial least square discriminant analysis (PLS-DA), after removing the interlaboratory control sample (*G. australes*) and checking for significance (permutation testing). Finally, the features showing the highest variable importance in projection (VIP) score (*i.e.* >1.5) were selected.

5.4. Untargeted LC-HRMS(/MS) analyses

For full scan acquisition, aliquots of extract obtained from all the replicates were filtered through 0.2 µm filters (Nanosep, modified nylon) prior to analysis (82 samples and four blanks) and as recommended for good practice in metabolomic analysis, a quality control (QC) sample was prepared by pooling 10 µL of each extract in an additional vial.

For Auto-MS/MS acquisition, the same 20 pooled samples as used in the targeted analysis (See 2.3) were analysed to reduce the time-consuming data acquisition process (*i.e*. 20 samples and four blanks).

5.4.1. Instrumental conditions

Untargeted analyses were performed using an ultra-high-performance liquid chromatography (UHPLC 1290 Infinity II, Agilent Technologies, CA, USA) coupled to a 6550 Ion Funnel Q-TOF (Agilent Technologies, CA, USA) operating in positive and negative ionization and in full scan and Auto-MS/MS modes. The stationary phase was a Kinetex C18 column (100 x 2.1 mm 1.7 µm, Phenomenex, CA, USA) equipped with a suited guard column. The methods used were described in Gémin *et al.* (2021). Briefly, mobile phases consisted of water (A) and acetonitrile/water (95:5, *v*:*v*) (B), both containing 2 mM ammonium formate and 50 mM formic acid. The flow rate was 0.4 mL min^{-1} and the injection volume was 5 µL. The following elution gradient was used: 5% B (0–1 min), 5–100% B (1–11 min), 100% B (11–13 min), 5% B (13–18 min). Mass spectra were recorded in both positive and negative full-scan

modes from *m/z* 100 to 1700 at a mass resolving power of 25 000 full-width at half-maximum (fwhm, *m/z* = 922.0099) and an acquisition rate of 2 spectra s^{-1}. Auto-MS/MS was performed iteratively: each sample was injected 5 times in ESI^+ mode and 4 times in ESI^- mode. At every iteration, the previously fragmented ions (*m/z* at t_R) were manually excluded from the following analyses (Koelmel *et al.* 2017).

The analyses complied with good practices of metabolomics (Broadhurst *et al.* 2018) with the use of QC injected 10 times at the beginning of the injection sequence and after every 5 samples to assess signal drift during analysis. Sample injections were performed randomly. Experimental and instrumental blanks were injected at the beginning of the sequence and used for data curation.

5.4.2. Data treatment

The raw MS and MS/MS data files were converted into .mzXML format using MS-Convert 3.0 (Chambers *et al.* 2012). Data processing was carried out using the open source software MZmine 2.53 (Pluskal *et al.* 2010) and consisted of: mass detection; peak detection; chromatogram deconvolution; removing of isotopes; peak alignment; gap filling and removing of duplicates. Details of parameters used for each step are provided in Table S8.

Integrated signals (*i.e.* features) were then manually filtered based on three criteria: i) intensity greater than ten times the intensity found in blanks in at least three replicates (or in all replicates if n < 5) per group, ii) presence in at least 2/3 of replicates within each group, iii) minimum intensity of 50,000 counts.

Full-Scan and Auto-MS/MS spectra are available here https://doi.org/10.12770/844d1dae-7544-4f91-90c6-d82ca01657cb. And .mgf files obtained after data curation are available here ftp://massive.ucsd.edu/v07/MSV000094172/.

5.4.3. Statistics

For metabolomics, the multivariate statistical analyses were performed using R 4.1.1 (CRAN https://cran.r-project.org/). The positive and negative matrices were log_{10}-transformed and pareto-scaled (van den Berg *et al.* 2006) and analysed simultaneously after high-level data fusion (Boccard and Rudaz, 2014) by normalizing the total inertia of each block to 1 (Qannari *et al.* 2000), providing a multiblock (MB) data matrix of 11,424 features detected in the 82 samples. The removal of the laboratory effect was performed using orthogonal signal correction (OSC) with the package "mt" (https://cran.r-project.org/package=mt) and the following parameters: Sjoblom method (Sjöblom *et al.* 1998, Svensson *et al.* 2002), no centering (completed during data normalization), 2 OSC components, 3 PLS components, a tolerance of 10^{-3} and 20 iterations. Further analyses by ComDim (equivalent to PCA after multiblock correction (Qannari *et al.* 2000, Rosa *et al.* 2017)) and partial least square discriminant analysis (here MB-PLS-DA) were performed using the package "ropls" (Thévenot *et al.* 2015) (multiblock correction based of normalization of total block variance was performed manually in R prior to multivariate analysis). Clustering and heatmaps, performed on the features selected by MB-PLS-DA, were obtained using the "stats" package (https://cran.r-project.org/package=STAT) to visualize sample clusters and features simultaneously, aiding the identification of variables potentially characteristic of each sample cluster.

5.4.4. Molecular networking

A MN was constructed from the converted Auto-MS/MS (see section 5.4.1 and 5.4.2) data using the GNPS platform (Aron *et al.* 2020) with the following parameters for both polarities: MS^1 tolerance 0.01 Da, MS^2 tolerance 0.01 Da, minimum cosine score 0.75, minimum of six common fragment ions, minimum cluster size of one without MS-cluster and a TopK set at 1000. Both MNs were merged using the "merge polarity workflow" of GNPS based on a mass error tolerance of 20 ppm and a retention time tolerance of 10 s. Data from GNPS were imported

into Cytoscape (version 3.9.1) for network visualization and layout. A color code was mapped to each node according to the average peak area of the feature within the different species (blue for *G. belizeanus*, orange for *G. caribaeus*, green for *G. carolinianus*, red for *G. excentricus* and purple for *G. silvae*). Border paint of node was coded according to the VIP score of features selected by the MB-PLS-DA (black for VIP score < 1, orange between 1 and 1.5 and red > 1.5) and the shape node was set to circle for features with annotation level 3 and 4 (according to Sumner et al., (Sumner *et al.* 2007)) and to square for features with annotation level 2. The width of edges was defined according to the cosine score (thin for a score of 0.75 and wide for a score of 1) and the color of edges was coded on the origin of the data (ESI^+ in red and ESI^- in blue).

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Highlights

- MTX4 and sulfo-gambierones are useful biomarkers for *Gambierdiscus excentricus*.
- Toxin content vary up to 2.1-fold for a strain grown in different laboratories.
- 46 and 567 species-specific features selected by LC-MS/MS and LC-HRMS/MS
- 17 classes of *Gambierdiscus* metabolites annotated using molecular network

Toxin and metabolomic profiles of 17 *Gambierdiscus* strains were obtained by low and high-resolution mass spectrometry. Mutivariate statitics and molecular network were performed to select and annotate putative species-specific biomarkers.

Declaration of interests

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

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: