



# Application of an Improved Chloroform-Free Lipid Extraction Method to Staghorn Coral (*Acropora cervicornis*) Lipidomics Assessments

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Received: 28 August 2020 / Accepted: 6 December 2020

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## Abstract

Lipids are excellent biomarkers for assessing coral stress, although staghorn coral data (*Acropora cervicornis*) is lacking. Lipid extraction is the most critical step in lipidomic assessments, usually performed using carcinogenic solvents. Efficient alternative using less toxic methods, such as the BUME method using butanol and methanol as extraction solvents, have not been applied to coral lipidomics evaluations. Thus, we aimed to develop a lipidomic approach to identify important coral health biomarkers by comparing different solvent mixtures in staghorn corals. Total lipid extraction was equivalent for both tested methods, but due to its efficiency in extracting polar lipids, the BUME method was chosen. It was then applied to different coral masses (0.33–1.00 g), resulting in non-significant differences concerning number of lipid classes and compounds. Therefore, this method can be successfully applied to coral assessments in a climate change context, with the added benefit of low sample masses, lessening coral sampling impacts.

**Keywords** Lipidomics · BUME method · Staghorn coral · Bioindicator · Puerto rico

Global climate change has become one of the main threats to coral reef ecosystems worldwide (Hoegh-Guldberg et al. 2017; Birkeland 2018), although negative impacts on coral growth and survival have been increasingly well-documented, studies identifying resilience pathways are still poorly understood (Towle et al. 2015). Caribbean reefs are particularly impacted in the Anthropocene, with an

estimated two-thirds of coral species threatened by human activities, and an additional one-third threatened by coastal development (Burke and Maidens 2004). The coral species *Acropora cervicornis* and *A. palmata* constitute critical reef-builders in the Caribbean (M. Dorinda Gilmore and Blaine R. Hall 1976; Gladfelter et al. 1978), providing extensive three-dimensional habitat for fish and other marine life. Unfortunately, Acroporid corals are among the most environmentally sensitive species, and also the most severely affected by global climate change, being currently listed as threatened under the U.S. Endangered Species Act. Indeed, populations of the *A. cervicornis* coral, commonly known as staghorn coral, have decreased significantly in the last three decades in almost all areas of the western Atlantic and Caribbean (Precht et al. 2002), and constitute the main target of restoration efforts. A lack of data concerning the effects of climate change on this species, however, is noted (Towle et al. 2015). Knowledge concerning physiological responses to stress and resilience potential for coral species chosen for restoration efforts is paramount to successfully inform conservation decisions (Towle et al. 2015).

In this context, lipid composition is frequently applied as a biochemical marker and indicator of stress-related changes in coral research, due to their broad involvement

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00128-020-03078-3>.

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in biochemical and physiological processes (Conlan et al. 2017). Lipid distributions serve as an indicator of multiple coral health aspects, such as the effects of environmental and anthropogenic stresses and the ability of coral species to offset the response to climate-associated stress (Conlan et al. 2017).

Lipids are also a diverse category of biomolecules with enormous structural diversity, due to their various combinations of polar headgroups, fatty acyl chains, and backbone structures (Bou Khalil et al. 2010). This presents challenges when qualitatively and quantitatively differentiating between unique molecular species among thousands of different lipid isoforms (Bou Khalil et al. 2010). Lipid extraction, in particular, is the most critical step in lipidomic assessments, as inadequate extraction may lead to several issues, such as low recovery and fatty acid oxidation, consequently affecting fatty acid composition results (Rodríguez-Palmero et al. 1998). The most widely applied methods for lipid isolation and purification are the Folch method and the Bligh and Dyer method (Bligh and Dyer 1959), although SPME has also been applied in this regard (Reyes-Garcés and Gionfriddo 2019; Saliu et al. 2020). Both generate reliable and quantitative data, and use a chloroform/methanol/water system to separate the lipids from their impurities (Breil et al. 2017). However, there are certain drawbacks when employing these methods. The first is the use of a known carcinogen, chloroform, which poses ethical questions regarding researcher health, as well as environmental concerns. The second is that the extracted lipids must be retrieved from the bottom fraction after the two-phase separation. The required penetration of the upper phase and the intermediate may lead to possible extract contamination, which in turn compromises the analysis (Löfgren et al. 2016). In this context, the green chemistry approach aims to substitute toxic solvents, such as chlorinated compounds, with greener alternatives, and has been applied to several lipidomic assessments (Anastas and Warner 1998).

To overcome these drawbacks, researchers have developed alternative lipid extraction methods. For example, Matyash et al. used a methamethyl-tert-butyl ether, methanol and water mixture to extract lipids from animal tissue, obtaining a lipid-enriched upper phase and reporting high lipid recoveries when compared to the Folch method (Matyash et al. 2008). Subsequently, Löfgren et al. developed a new rapid and simple chloroform-free method for total lipid extraction of animal tissues using a butanol/methanol mixture (BUME method), reporting lipid recoveries fully comparable to or even higher than those obtained in the Folch method, with the added advantages of simplicity, throughout and automation, lower solvent consumption, economy due to the use of inexpensive reagents, and health and environment compensations (Löfgren et al. 2016).

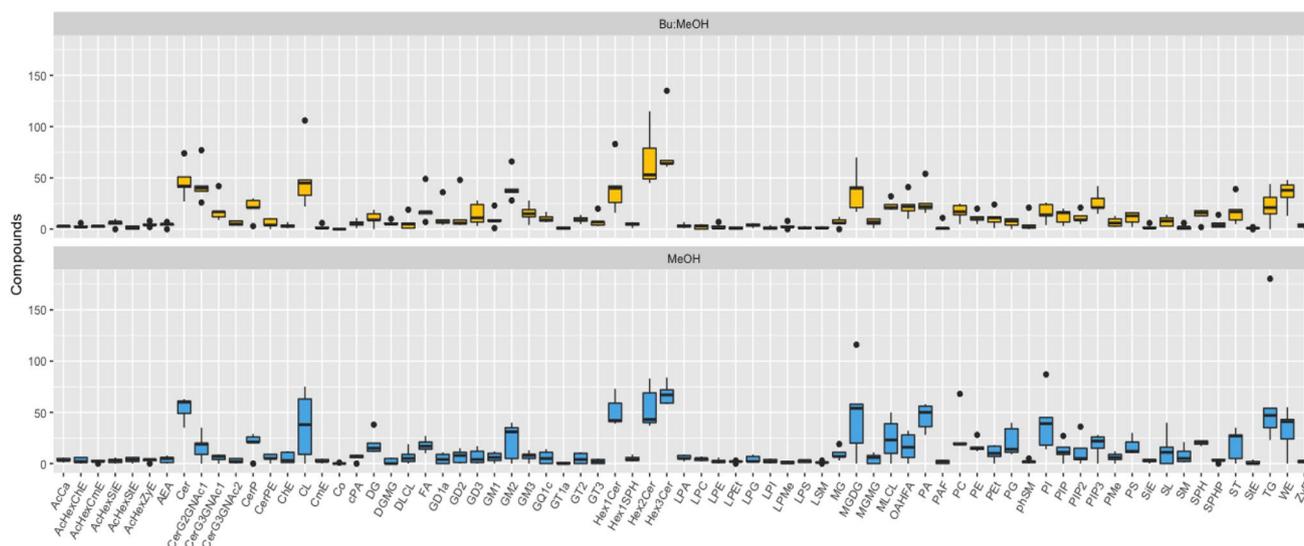
The BUME method, although proven effective, has not yet been widely applied for the analysis of biological systems where lipid assessments are of particular interest, such as corals. In this context, this study aimed to develop and apply a chloroform-free extraction that overcomes the drawbacks of the aforementioned methods followed by a non-targeted screening workflow based on high performance liquid chromatography with high resolution mass spectrometry (LC-HRMS) for lipid determination in staghorn corals (Figure S1).

## Materials and Methods

A total of five coral samples (identified as D35, N2, N46, V50, and V59) were collected from reefs around Culebra Island, Puerto Rico in April 2018 (Fig. 1), under DRNA (Departamento de Recursos Naturales y Ambientales, Puerto Rico) permit number 2018-IC-034 (E) O-VS-PVS15-SJ-00980–20,042,018. Samples were collected from detached coral colonies in two reefs as follows: Los Corchos (N2, V59 coordinates: 18.285733° N, –65.234516° W) and Luis Peña (D35, N46 and V50 coordinates: 18.311256° N, –65.33703° W) after the impact of hurricanes Irma and Maria (details in Rodríguez-Casariago et al. 2020 *submitted*). All fragments correspond to different coral genotypes (genets) as evaluated using microsatellite markers (details in Rodríguez-Casariago et al. 2020, *submitted*). A tissue and skeleton fragment of about 2–4 cm was obtained from each colony using bone cutters. Samples were immediately flash frozen with liquid nitrogen and transported to the laboratory in dry ice for storage at –80°C until analysis.

Samples were pulverized prior to lipid extraction following the *in toto* method which utilizes the coral as a whole, crushing the intact skeleton and tissue while frozen, and the resultant powder was utilized for lipid analysis (Conlan et al. 2017). This method proves to be more efficient for lipid quantification than tissue isolation by air spraying because high amounts of organic material are retained in coral skeletons (Conlan et al. 2017).

A total of 1 g of each pulverized coral sample was weighed and transferred to 15 mL polypropylene centrifuge tubes. The use of methanol (MeOH) as the extracting solvent was compared to the BUME method (Bu:MeOH at a 3:1 ratio). Each sample was soaked in 3 mL of either MeOH or Bu:MeOH (3:1). The samples were left overnight in the chosen solvents, followed by centrifugation at 3500 rpm for 10 min at 24°C in a Sorvall RT6000 refrigerated centrifuge (Dupont, now Thermo Scientific, Waltham, MA, US). The solid residues were then separated from the supernatants and re-suspended for 10 min with 3 mL of the same solvent mixture and re-centrifuged two more times in the same conditions to obtain a total supernatant volume of 9 mL. A



**Fig. 1** Lipid extraction results for the methanol and butanol:methanol methods. *AcCa* Acyl Carnitine, *AcHexChE* AcylGlcCholesterol ester, *AcHexCmE* AcylGlcCampesterol ester, *AcHexSiE* AcylGlcSitos-terol ester, *AcHexStE* AcylGlcStignasterol ester, *AcHexZyE* Acyl-GlcZymosterol ester, *AEA* N-Acylethanolamine, *Cer* Ceramide, *CerG2GNAc1* and *CerG2GNAc2* Neutral glycosphingolipid, *CerP* Ceramides phosphate, *CerPE* Ceramides phosphoethanolamine, *ChE* cholesterol ester, *CL* Cardiolipin, *CmE* Campesterol ester, *Co* Coenzyme, *cPA* Cyclic phosphatidic acid, *DG* Diglyceride, *DGMG* Monogalactosyldiacylglycerol, *DLCL* Dilysocardioli-*pin*, *FA* Fatty acid, *GD1a* GD2, GD3, GM1, GM2, GM3, GQ1c, GT1a, GT2, and *GT3* Ganglioside, *Hex1Cer* Hexosylceramide, *Hex1SPH* Glucosyl-sphingosine, *Hex2Cer* Dihexosylceramide, *Hex3Cer* Trihexosylcer-*amide*, *LPA* Lysophosphatidic acid, *LPC* Lysophosphatidylcholine, *LPE* Lysophosphatidylethanolamine, *LPEt* Lysophosphatidyletha-

*nol*, *LPG* Lysophosphatidylglycerol, *LPI* Lysophosphatidylino-*sitol*, *LPMe* Lysophosphatidylmethanol, *LPS* Lysophosphatidylserine, *LSM* Lysosphingomyelin, *MG* mono-acyl glycerol, *MGDG* Monoga-*lactosyldiacylglycerol*, *MGMG* Monogalactosylmonoacylglycerols, *MLCL* Monolysocardioli-*pin*, *OAHFA* OAcyl-(gamma-hydroxy)FA, *PA* Phosphatidic acid, *PAF* Platelet-activating factor; *PC* Phosphati-*dylcholine*, *PE* Phosphatidylethanolamine, *PEt* Phosphatidylethanol, *PG* Phosphatidylglycerol, *phSM* Sphingomyelin (phytosphingosine); *PI* Phosphatidylino-*sitol*, *PIP* Phosphatidylino-*sitol* phosphate, *PIP2* Phosphatidylino-*sitol* bisphosphate, *PIP3* Phosphatidylino-*sitol* trisphosphate, *PMe* Phosphatidylmethanol, *PS* Phosphatidylserine, *SiE* Sitos-*terol* ester, *SL* Seminolipid, *SM* Sphingomyelin, *SPH* Sphingo-*sine*, *SPHP* Sphingosine phosphate, *ST* Sulfatide, *StE* Stigmasterol ester, *TG* Triglyceride, *WE* Wax ester; *ZyE* Zymosterol ester

1 mL aliquot of each sample was then transferred to an LC vial for analysis.

All chemicals were at least HPLC grade. Water, MeOH, butanol and ammonium formate were purchased from Fisher Scientific. Native fatty acid (FA) standards were bought from Fisher Scientific (Acros Organic and Alfa Aesar) and Cayman Chemical (Ann Arbor, MI, US). Control samples containing native FA standards were used in the analyses and are listed in Table 1. A mixture containing all reference substances were combined to form a 1 mg L<sup>-1</sup> solution in MeOH.

The samples were analyzed using a Thermo Q-Exactive (UPLC-HRMS) Orbitrap equipped with a heated electro-spray ionization (HESI) source in both negative and positive modes. MS conditions were as follow: capillary temperature (T) of 350°C, auxiliary (aux) gas heater T of 450°C, spray voltage of 5 kV, sheath gas flow rate of 40 arbitrary units (au), aux gas flow of 10 au and S-lens RF level of 80). The coral samples (n = 5) were initially run in full scan MS (resolution of 140,000 and scan range of 100 to 800 m/z) for positive and negative mode. To test the reproducibility and

**Table 1** List of native standards used as control in the LC-HRMS analysis

| Native FA stand-ards     | Molecular for-<br>mula                         | Monoisotopic<br>Mass (g mol <sup>-1</sup> ) | Monitored<br>ion [M+H] <sup>+</sup> |
|--------------------------|--|---|-------------------------------------|
| α-Linolenic acid         | C <sub>18</sub> H <sub>30</sub> O <sub>2</sub> | 278.2246                                    | 277.2173                            |
| Palmitoleic acid         | C <sub>16</sub> H <sub>30</sub> O <sub>2</sub> | 254.2246                                    | 253.2173                            |
| Palmitic acid            | C <sub>16</sub> H <sub>32</sub> O <sub>2</sub> | 256.2402                                    | 255.2329                            |
| Stearic acid             | C <sub>18</sub> H <sub>36</sub> O <sub>2</sub> | 284.2715                                    | 283.2642                            |
| Myristic acid            | C <sub>14</sub> H <sub>28</sub> O <sub>2</sub> | 228.2089                                    | 227.2016                            |
| Eicosenoic acid          | C <sub>20</sub> H <sub>38</sub> O <sub>2</sub> | 310.2872                                    | 309.2799                            |
| Docosahexaenoic<br>acid  | C <sub>22</sub> H <sub>32</sub> O <sub>2</sub> | 328.2402                                    | 327.2329                            |
| Linoleic acid            | C <sub>18</sub> H <sub>32</sub> O <sub>2</sub> | 280.2402                                    | 279.2329                            |
| Eicosapentaenoic<br>acid | C <sub>20</sub> H <sub>30</sub> O <sub>2</sub> | 302.2245                                    | 301.2173                            |
| Arachidonic acid         | C <sub>20</sub> H <sub>32</sub> O <sub>2</sub> | 304.2402                                    | 303.2329                            |
| Oleic acid               | C <sub>18</sub> H <sub>34</sub> O <sub>2</sub> | 282.2559                                    | 281.2486                            |

increase accuracy in the identification of lipids, experiments using lower coral sample amount of N46, were analyzed in Full Scan (70,000 resolution) with MS2 data dependent (17,500 resolution and  $m/z$ : 200–1000) for positive and negative mode. Chromatographic separations were performed on a Hypersil Gold aQ column (50 × 2.1 mm, 1.9 μm, Thermo Scientific, West Palm Beach, FL, US) using a binary gradient program with MeOH and ammonium formate as mobile phases. The linear gradient started at 80% MeOH and increased to 95% MeOH within 2 min. In the following 10 min, MeOH percentage was increased to 99% and was kept at this level for 5 min. In the remaining 3 min MeOH was decreased to 80% to equilibrate for the next run (total of 20 min). A total of 10 μL of the sample was injected at a flow rate of 500 μL/min. Mass tolerance was always set to below 5 ppm. Blank samples consisting of each tested solvent and control samples were injected every 10 samples, in the beginning and in the end of each run to verify instrument performance. Lipid identification and relative quantitation was performed using the Lipid Search Software™ (Thermo Scientific, version 4.2.21). This database includes approximately 1.5 million lipids and predicted fragmentation ions. The processed data resulted in a list of identified lipids generated from the software. Manual post-processing was conducted by filtering in excel and eliminating all data with signal to noise ratio < 3 and a delta ppm of ± 10.

Based on previous literature assessments performed in coral, eight lipid classes were identified as the major lipids

in coral tissues and selected as the focus of this research: cholesterol esters (ChE), fatty acids (FA), monoacylglycerols (MG), monogalactosyldiacylglycerols (MGDG), phosphatidylcholines (PC), phosphatidylethanolamines (PE), triglycerides (TG), and wax esters (WE) (Harland et al. 1993; Imbs et al. 2010; Lin et al. 2013; Rosset et al. 2019; Stien et al. 2020). The Lipid Search parameters were adjusted to include only these classes in the MS2 negative and positive modes. All data were blank subtracted. Thus, compounds identified in higher amounts in blanks compared to the samples were eliminated, e.g. peak areas of identified compounds within each class must be at least 10 times higher in the samples in comparison to the blanks. Native FA standards used as control samples were identified in the negative mode by the Lipid Search software, except for eicosenoic acid ( $m/z = 311.2378$ ) and docosahexaenoic acid ( $m/z = 329.2480$ ), which were detected in the positive mode. Table 2 exhibits the adduct ions considered and software search options applied for lipid identification.

After selection of appropriate extraction solvent, additional experiments were conducted to assess whether the BUME method would be equally effective with varying sample masses under 1 g, in order to lessen coral sampling impacts. Therefore, the same procedure used for 1 g of sample was followed for sample N46 using varied sample masses and solvent volumes (Table 3). The solvent-to-mass ratio was maintained consistent for all samples.

**Table 2** Software search parameters applied for lipid identification in staghorn coral samples

| Adduct ions                            |  |
|--|--|
| Negative polarity                      | -H<br>+ HCOO<br>+ CH <sub>3</sub> COO              |
| Positive polarity                      | + HCOO<br>+ NH <sub>4</sub><br>+ Na<br>+ Li<br>+ K |
| Search options                         |  |
| Experiment type                        | LC-MS  |
| Parent tolerance                       | 0.1 Da   |
| Neutral loss (NL)/ precursor tolerance | 0.5 Da   |
| Precursor tolerance                    | 5.0 ppm  |
| Product tolerance                      | 0.5 Da   |
| Minimum merge range                    | 2.0  |
| Minimum peak width                     | 0.0  |
| Relative intensity threshold           | Parent – 0.01<br>Product ion – 1.0%                |
| M-score threshold                      | 5.0  |

**Table 3** Mass to volume sample preparation of coral samples under 1 g

| Sample number | Sample mass (g) | Solvent (Bu:MeOH mixture, mL) |
|---------------|-----------------|-------------------------------|
| 1             | 0.33            | 1.0                           |
| 2             | 0.4             | 1.2                           |
| 3             | 0.5             | 1.5                           |
| 4             | 0.66            | 2.0                           |
| 5             | 0.83            | 2.5                           |

Statistical analyses were conducted in the R v.3.5.0. software (R Core Team 2020) considering an alpha of 0.05. A Shapiro–Wilk normality test was conducted to verify the distribution of the variables assessed in this study. Since data displayed non-normal distribution, non-parametric tests were conducted. A Mann–Whitney–Wilcoxon test was performed to determine whether the methods were significantly different. In order to determine if the different masses used in this study influence in the lipid extraction, a Kruskal–Wallis test was conducted.

## Results and Discussion

A total of 73 lipid classes were extracted in both MeOH and BUME methods, as displayed in Fig. 1, with a means of 1003 compounds extracted in the methanol only method, and 995 in the BUME method. When considering the eight major lipid classes, a mean of 229 compounds was extracted from the five samples in the methanol only method, and 154 in the BUME method. No significant differences were observed when comparing the methods for all lipid classes (Mann–Whitney test:  $W = 62,180$ ,  $p = 0.12$ ) and for the eight major lipid classes (Mann–Whitney test:  $W = 925$ ,  $p = 0.23$ ), indicating that both are able to efficiently extract lipids from coral samples. However, considering that butanol is capable of more efficiently extracting polar lipids (Baker et al. 2001; Scherer et al. 2009), the BUME method was chosen for further analyses.

Concerning only the eight lipid classes extracted by the BUME method, MGDG were the most predominant, at 24.6%, followed by WE (22.5%), TG (14.45%), FA (13.67%), PC (10.94%), PE (7.29%), MG (4.3) and, lastly, ChE (2.2%). A previous study on *Acropora* sp. corals from coastal waters off Vietnam using the traditional Bligh and Dyer method with chloroform indicates a different lipid profile, comprising 40.2% WE, 27.5% TG, 17.9% polar lipids, 4.1% MGDG and 1.6% free FA (Imbs 2013).

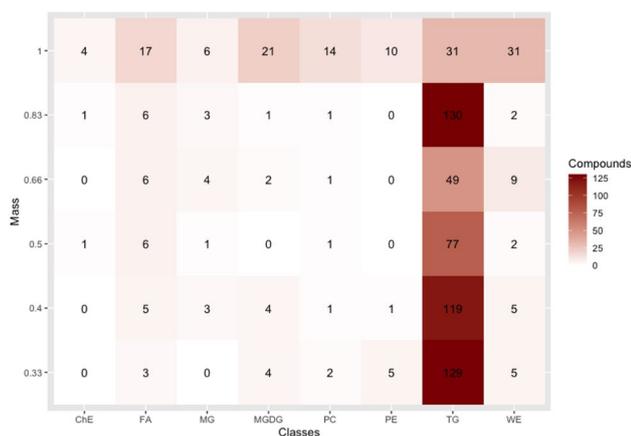
The presence of MGDG galactolipids is noted herein due to the obligatory symbiotic relationship between single-celled photosynthetic algae (Symbiodinaceae family) and

staghorn coral (Rosset et al. 2019), as this class is one of the main component of photosynthetic cell thylakoid membranes (Harwood and Guschina 2009). Increased MGDG have been reported in response to increased water temperatures, in an attempt to prevent membrane leakage events (Gombos et al. 1994; Rosset et al. 2019). WE, on the other hand, are one of the main classes of reserve lipids in all coral species, and are directly affected by diseases and increased energy consumption under adverse habitat conditions, such as bleaching due to high water temperatures (Yamashiro et al. 2001, 2005; Fleury et al. 2004; Grottoli et al. 2004).

TG are storage lipids used for respiration and are an integral part of the cell membrane constitution (Yamashiro et al. 2001), and, as observed herein, are usually present in high amounts in corals. The FA group, essential in many physiological processes, such as energy use and storage, cell membrane structure and gene regulation (Gurr et al. 2002) has been reported as the major lipid storage class in other coral assessments (Treignier et al. 2008; Tolosa et al. 2011), in contrast to the present study, where it was the fourth most predominant class. Phosphatidylcholines (PC) and Phosphatidylethanolamines (PE), both polar structural lipids, are responsible for lipid membrane fluidity (Pekiner 2002). Higher PC and PE levels in deeper environments have been reported compared to shallow water corals, postulated as due to higher pressure and lower temperatures at greater depths (Lin et al. 2013), and decreased levels have been noted in bleached corals, due to cell damage (Grottoli et al. 2004). MG, non-polar storage lipids have been reported as decreasing after bleaching events carried out in vitro (Solomon et al. 2020), although some species (e.g., *Porites compressa*) do not seem to exhibit high amounts of this lipid class (Rodrigues et al. 2008). ChE, also structural lipids, tend to increase in corals exhibiting increased zooplankton feeding, as zooplankton are a dietary cholesterol source (Grottoli et al. 2004).

Non-targeted lipidomics can be applied to expand lipid knowledge. The strength of this technique lies in the development of a simple and fast extraction method, coupled with the minimization of background contaminants (Tumanov and Kamphorst 2017). The described extraction method uses a Bu:MeOH mixture, which yields quite satisfactory results, and the HESI-HPLC-HRMS technique has the capability of detecting compounds without derivatization with a high degree of sensitivity and selectivity based on the unique mass/charge ( $m/z$ ) of each compound, considered adequate for lipidomic assessments (Pham et al. 2019).

Previous studies have indicated that increased lipid concentrations of WE and TG is a good survival predictor for *Acropora intermedia* corals undergoing bleaching events (Anthony et al. 2007), as lipids stored in coral tissue represent significant energy reserves that can be used in times of stress (Grottoli et al. 2004; Teece et al. 2011). In the present



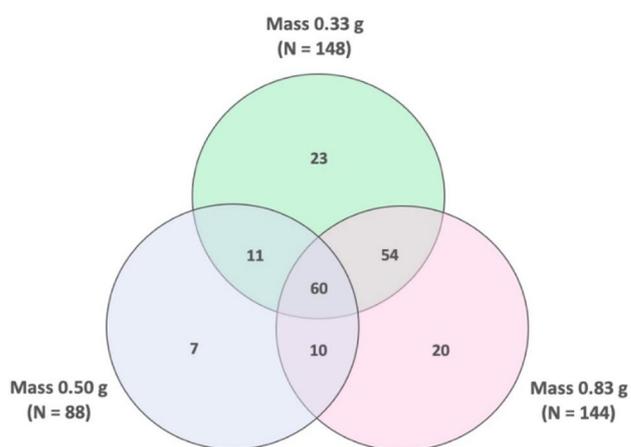
**Fig. 2** Heat map displaying the comparison of total lipids found per class in coral samples using varying masses

study, the identity of the eight most abundant lipid classes was consistent throughout the sample batch for both the MeOH and the MeOH:Bu extraction methods.

Focusing only on the selected eight lipid classes (ChE, FA, MG, MGDG, PC, PE, TG, and WE), varying masses (from 0.33 to 1.00 g) of sample N46 were analyzed in Full Scan MS (positive and negative) and MS2 (positive and negative), following same procedures described previously for the BUME extraction method. The total number of identified compounds varied by mass and lipid class (Fig. 2). The lipid class composition results per sample were subtracted from background compounds found in the blank samples. The most predominant class for all varying masses was TG. Even though extraction for the 1 g aliquot seems to result in a generally higher number of lipid classes, no significant differences were detected between the six different masses used in this study (Kruskal–Wallis test:  $X^2 = 9.89$ ,  $df = 5$ ,  $p = 0.08$ ), indicating that lower amount of coral samples can be used for lipidomic analysis without compromising the analytical quality of the results and leading to less environmental impacts due to coral sampling.

To evaluate the number of overlapping compounds detected in the samples with varying masses, a Venn Diagram (Fig. 3) was constructed comparing the results for the 0.33, 0.50 and 0.83 g coral masses. This information is important to understand the advantages and limitations of using reduced quantity of samples.

A total of 60 compounds (FA = 3, PC = 1, and TG = 56) were found in common for the different masses. More PE, MGDG and WE were found unique to 0.33 g (MGDG = 4, WE = 3 and PE = 5). At the same time, lower amounts of FA, TG and MG were found when using less than 0.83 g of coral tissue. Even though using less sample volume may benefit or limit certain classes, we demonstrate herein that the total amount of lipid classes, as well as the number of overlapping



**Fig. 3** Venn diagram displaying the number of overlapping compounds among different mass samples

compounds is similar between the samples extracted using varying masses of corals. Thus, it is expected that changes in sample mass within the parameters determined in this experiment will not affect the potential identification or quantification of lipid classes and that MS2 information will offer the required accurate lipid identification for future bio-monitoring assessments. In summary, both tested methods efficiently extracted a variety of lipid classes in corals. The selected and employed BUME method was proved adequate for lipidomic assessments in staghorn coral (even using lower coral masses) and can be used to evaluate physiological stress in corals by assessing variations in WE, TG, MG and FA lipid distributions. Therefore, this method can be successfully applied to coral assessments in a climate change context, addressing coral health impacts due to anthropogenic sources.

**Acknowledgements** This material is based upon work supported by the National Science Foundation under Grant No. HRD-1547798. This NSF Grant was awarded to Florida International University as part of the Centers for Research Excellence in Science and Technology (CREST) Program. The authors would like to acknowledge the support of Thermo Scientific Corporation for providing analytical columns and the demo version of the Lipid Search software and from NSF grant IOS1810981 supporting coral sample collection. This is contribution number 996 from the Southeast Environmental Research Center in the Institute of Environment at Florida International University.

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