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State of art and best practices for fatty acid analysis in aquatic sciences

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1
2 33 **Abstract**
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4 34 Determining the lipid content and fatty acid composition of aquatic organisms has been of major
5
6 35 interest in trophic ecology, aquaculture and nutrition for over half a century. Although protocols for
7
8 36 lipid analysis are well-described, their application to aquatic sciences often requires modifications to
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11 37 adapt to field conditions and to sample type. Here, we present the current state of knowledge of
12
13 38 methods dedicated to both marine and freshwater lipid analyses, from sampling to data treatment. We
14
15 39 review: (i) sample preservation, storage and transport protocols and their effects on lipids, (ii) lipid
16
17 40 extraction, separation of polar and neutral lipids, derivatisation and detection methods, and (iii)
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19 41 available tools for statistical analysis of fatty acid data. We provide recommendations for best-
20
21 42 practices in field situations and advocate for protocol standardisation and inter-laboratory calibration.
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25 43 **Keywords:** Marine and freshwater lipids, fatty acids, methods, recommended practices,
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27 44 biomarkers, aquatic nutrition
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46 **Abbreviations:**

4	4-HHE	4-Hydroxy-2-hexenal	HPLC-	High performance liquid
5	4-HNE	4-Hydroxy-2-nonal	ELSD	chromatography-
6	ALC	Fatty alcohols		Electrospray light scattering
7	AV	Acid value		detector
8	BCl ₃	Boron trichloride	HPTLC	High performance thin layer
9	BF ₃	Boron trifluoride		chromatography
10	BHT	Butylated hydroxytoluene	LC	Liquid chromatography
11	BSTFA	Bistrimethylsilyl	LC-MS/MS	Liquid chromatography-mass
12		trifluoroacetamide		spectrometry/mass
13				spectrometry
14	CH ₂ Cl ₂	Dichloromethane		
15	CHCl ₃	Chloroform	LI	Lipolysis index
16		(Trichloromethane)	MAG	Monoacylglycerols
17	CI	Chemical ionisation	MeOH	Methanol
18	CITES	Convention on International	MDA	Malondialdehyde
19		Trade in Endangered Species	MUFA	Monounsaturated fatty acid
20			NCI	Negative chemical ionisation
21	COP	Cholesterol oxidation	O-TMSi	O-Trimethylsilyl
22		products	pAV	Para-Anisidine value
23	DAG	Diacylglycerol	PEG	Polyethyleneglycol
24	DHA	Docosahexaenoic acid	PFE	Pressurised fluid extraction
25		(22:6n-3)	PL	Phospholipids
26	DMA	Dimethyl acetal	PUFA	Polyunsaturated fatty acid
27	DMDS	Dimethyl disulphide	PTV	Programmed temperature
28	DMOX	4,4-Dimethyloxazolazine		vaporising injection
29	FA	Fatty acid	PV	Peroxide value
30	FAME	Fatty acid methyl ester	SCOT	Support coated open tubular
31	FATM	Fatty acid trophic markers	SE	Steryl ester
32	FFA	Free fatty acids	SFA	Saturated fatty acid
33	FID	Flame ionisation detector	SIM	Selected ion monitoring
34	FTIR	Fourier transform infra-red	SPE	Solid phase extraction
35	GC-FID	Gas chromatography-flame	TAG	Triacylglycerol
36		ionisation detection	TBARS	Thiobarbituric acid reactive
37				substances
38	GC-MS	Gas chromatography-mass	TIC	Total ion chromatogram
39		spectrometry	TLC	Thin layer chromatography
40	GF/C GF/F	Glass microfiber filters	WCOT	Wall coated open tubular
41	H ₂ SO ₄	Sulfuric acid	WE	Wax ester
42	HCl	Hydrochloric acid		
43	HI	Hydrolysis index		
44	HPLC	High performance liquid		
45		chromatography		

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49 **Introduction**

50 Lipids and their fatty acids (FA) are ubiquitous components of all living organisms. They are essential
51 compounds for the formation of cell and tissue membranes, act as major sources of metabolic energy,
52 provide thermal insulation and serve as chemical messengers. Fatty acids generally consist of a linear
53 hydrocarbon chain with a carboxyl group (-COOH) at its end. They are characterised by the length
54 of their carbon chain, generally from 14 to 24 (up to 36) atoms, as well as by the position and the
55 number of double bonds (usually from 0 to 6) that determine their degree of unsaturation. Fatty acids
56 are constituents of lipids such as wax and sterol esters (WE, SE), triacylglycerols (TAG),
57 diacylglycerols (DAG), monoacylglycerols (MAG), glycolipids and phospholipids (PL), which show
58 different levels of regulation from ingestion to incorporation. One or several FA are linked either with
59 an ester (or ether / vinyl-ether) bond to an alcohol, mainly glycerol, or with an amide linkage to a
60 long-chain nitrogen base, mainly sphingosine. All of these molecules are categorised as either neutral
61 lipids or polar lipids, with reference to chromatographic methods of lipid class separation but also to
62 their metabolic roles (Christie, 1984). Among neutral lipids, FA-containing compounds include TAG
63 and WE, which are the main storage lipids, whereas DAG, free FA (FFA), and MAG are generally
64 minor constituents. Sterols (both in their free and esterified form) and fatty alcohols (ALC) are also
65 found in neutral lipids. Free FA are rarely present in living organisms, as they may be toxic in high
66 concentrations (Jüttner, 2001). Polar lipids include PL, glycolipids, and sphingolipids. Polar lipids
67 are characterised by a hydrophilic polar head group which, in contrast to their hydrophobic aliphatic
68 chains, allows for the formation of lipid bilayers found in cell membranes. Polar lipid properties are
69 determined by the nature of the polar head groups, the nature of the linkage between the polar head
70 group and aliphatic chain, the length of the carbon chain, as well as the position and the number of
71 double bonds within the FA. These different properties influence the functioning of membrane
72 receptors, the fluidity of membranes, and the stability of the bilayer structure (Hazel and Williams,
73 1990).

1
2 74 Fatty acids have been analysed in aquatic sciences for over 50 years (Ackman *et al.*, 1968) with
3
4 75 the first evidence that FA composition of consumers resembles that of its food (Lee *et al.*, 1971).
5
6 76 Over the next 30 years, the concept of FA trophic markers (FATM) gained in popularity in aquatic
7
8 77 trophic ecology (Dalsgaard *et al.*, 2003). According to this concept, FA composition of some primary
9
10 78 producers is characterised by specific compounds that can be transferred to upper trophic levels (e.g.
11
12 79 Kelly and Scheibling, 2012). The FA composition of a consumer is therefore expected to reflect the
13
14 80 FA composition of its food sources ('you are what you eat' principle). FA have accordingly been
15
16 81 used to study energy flow in food webs in freshwater, estuarine, coastal and deep-sea, environments
17
18 82 in all latitudes, and to examine functional responses to stressors (Kelly and Scheibling, 2012). More
19
20 83 recently, the FATM concept has culminated in quantitative FA-based diet modelling (Iverson *et al.*,
21
22 84 2004; Galloway *et al.*, 2014; Bromaghin *et al.*, 2017), and recent methodological developments allow
23
24 85 for accurate estimation of prey contribution to consumer diet (Litmanen *et al.*, 2020). However, diet
25
26 86 modeling is based on the fact that aquatic consumers generally lack the ability to synthesise long-
27
28 87 chain polyunsaturated FA (LC-PUFA, \geq C20), and have limited capacity for bioconversion of short-
29
30 88 chain PUFA into long-chain PUFA (Castell *et al.*, 1972; Langdon and Waldo, 1981; Sargent *et al.*,
31
32 89 1999; Taipale *et al.*, 2011). This view has recently been challenged (Kabeya *et al.*, 2018).
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39 90 Diet is not the only determinant of the FA composition of aquatic consumers. FA composition
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41 91 varies with intrinsic factors such as phylogeny and developmental/reproductive stages (e.g. Galloway
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43 92 and Winder, 2015), and extrinsic factors such as temperature, salinity and hydrostatic pressure (e.g.
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45 93 Wodtke, 1981; Cossins and Macdonald, 1989; Hazel and Williams, 1990), thus providing information
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47 94 about physiological condition and habitat type of aquatic organisms (e.g. Meyer *et al.*, 2019).
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50 95 The analysis of the FA composition of aquatic organisms is based on procedures originally
51
52 96 developed for applications in food science and medicine. Briefly, once a sample is collected, it is
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54 97 immediately stored in the dark at the lowest possible temperature for the shortest period of time with
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56 98 added antioxidants, and under a nitrogen atmosphere or vacuum. This is to avoid alteration caused by
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58 99 enzymes, heat, oxygen and light. Lipids are then extracted, and lipid classes may be separated using
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1
2 100 chromatography. Next, derivatisation of FA is performed (methylation in most cases) and FA
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4 101 composition is determined by gas chromatography. Finally, data are treated to obtain the quantitative
5
6 102 and qualitative FA profiles of the sample (Figure 1).
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8
9 103 There are likely to be as many variations of procedures as there are laboratories (Parrish, 1999).
10
11 104 Yet, not all may be applicable to aquatic samples. Procedures involved in sample collection, handling
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13 105 and storage, as well as extraction and transesterification steps, can cause biochemical modifications
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15 106 of FA and differential loss, leading to false or misleading interpretations. Identifying the most
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17 107 appropriate protocols and the best-practices in FA analysis of aquatic organisms may prove to be a
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19 108 difficult task for novices, as available literature is widespread and highly diverse, and many studies
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21 109 do not fully describe their methodology (e.g., “modified Folch extraction”).
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25 110 Our objective is to provide an up-to-date review of the procedures used for a large number and
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27 111 variety of aquatic samples and laboratories around the world. This review emerged from technical
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29 112 workshops of the conference “*Lipids in the oceans*” held in Brest (France) in November 2018. We
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31 113 answer practical questions that are not necessarily formulated in papers and textbooks, but essential
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33 114 to obtain a reliable FA profile. For example, which organ/tissue of particular specimens should be
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35 115 analysed to be representative in the context of trophic ecology? How are samples properly collected,
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37 116 transported or shipped? What are the most appropriate extraction/transesterification methods or
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39 117 solvent systems? How can one ensure reliable FA identification and quantification? How are FA data
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41 118 analysed? This paper aims to guide both novice and advanced scientists in identifying the most
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43 119 appropriate protocols and best-practices for FA analysis of aquatic samples. We hope that this
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45 120 comparative synthesis will stimulate further international collaborations and facilitate standardisation
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47 121 of lipid analyses.
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1 Sample handling and storage before lipid extraction

The choice of biological material, its collection and handling techniques, as well as storage methods, are among the most important steps in lipid analysis (Fig. 1). Lipid degradation can occur by hydrolysis (release of FFA from acyl lipids through the enzymatic activity of lipases) or oxidation of carbon-carbon double bonds. In view of the labile nature of lipids, these processes often occur very rapidly, leading to analytical errors and increased sample variability (Christie, 1984). Handling and storage conditions and methods can help minimise sample degradation and analytical bias. Ideally, lipids should be extracted immediately after collection. The second-best option is to flash-freeze samples in liquid nitrogen immediately after collection. As sampling of aquatic organisms is often performed in the field and in remote areas, optimal conditions can rarely be achieved, but samples need to be stored adequately. A broad set of procedures exists, including: (i) sample maintenance on ice, (ii) frozen sample storage from $-18\text{ }^{\circ}\text{C}$ (standard freezer), to $-80\text{ }^{\circ}\text{C}$ (laboratory freezers) and/or to $-196\text{ }^{\circ}\text{C}$ (liquid nitrogen), (iii) freeze-drying of samples, or (iv) sample storage in organic solvents for varying storage periods, from a few hours to years prior to analysis. Adequate storage conditions will prevent (or at least limit) the degradation of lipids through hydrolysis and oxidation processes induced by oxygen, temperature, light and/or enzymatic processes (Rudy *et al.*, 2016). The rate of oxidation and hydrolysis processes (hereafter referred to as “lipid degradation”) is likely to vary greatly across species and tissue types. These processes can be revealed by an increase in FFA and a decrease in glycerolipids and polyunsaturated FA (PUFA) in samples (Christie, 1984, see **section 4**). Here, we provide an overview of different methods used in the literature and discuss their strengths and limitations.

1.1 Sample type/ sampling strategy

Aquatic samples are highly diverse, ranging from bacteria to whales, and sampling procedures need to be adapted to the nature of the studied organisms/functional groups. Samples of seston and

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2 146 phytoplankton are generally collected through water filtration on pre-combusted glass microfiber
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4 147 filters (GF/C or GF/F), while zooplankton can be sorted manually. Such small organisms or functional
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6 148 groups are often directly extracted as whole organisms and stored in solvent. For other organisms
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9 149 such as larger invertebrates, fish, and aquatic mammals, tissue dissection or biopsy is usually
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11 150 required. In the case of multi-purpose samples, it is possible to grind them under liquid nitrogen and
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13 151 subsample the powder (Soudant *et al.*, 1996). In the case of a quantitative study where the mass of
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16 152 lipid or FA is reported per gram of sample, the sample will have to be weighed before or after storage.
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18 153 Sample mass can be measured from fresh or dry tissue. In the latter case, the dry mass may be
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20 154 measured on a dedicated subsample. All material should be lipid-cleaned prior any sampling to avoid
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23 155 cross contamination (see section 3.1). Because it is not possible to describe all sampling strategies
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25 156 here, we highly recommend that any study involving lipid and FA analysis must start with a literature
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27 157 review of available information on targeted organisms or on species that are phylogenetically
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30 158 comparable.

33 159 1.2 Sample handling

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37 160 Maintaining samples on ice is often the first and only practical option available to limit lipid
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40 161 degradation between collection and further processing. Whenever possible, long-lasting dry ice is
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42 162 preferable to water ice. Ideally, samples should be immediately flash-frozen in liquid N₂ in the field
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44 163 and transported to a freezer using a dry shipper. Crushed ice is useful for transportation between
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47 164 locations such as sample transport from the ocean (e.g., small and large vessels) to land, or transport
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49 165 between laboratories. Crushed ice should preferably be used only for a few hours, as this method only
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51 166 slows, but does not stop, lipid and FA degradation (Wood and Hintz, 1971; Chaijan *et al.*, 2006), and
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53
54 167 samples should therefore be frozen as soon as possible. As an alternative, deep-frozen, long-lasting
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56 168 ice packs are a convenient option for short-term storage in the field (e.g., TechniIce™). These ice
57
58 169 packs maintain temperatures around 0 °C for a few hours in a well-insulated container (L. Couturier
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60 170 pers. obs.). In the particular case of studies on thermal acclimation or adaptation, it may be preferable

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2 171 to maintain samples at environment temperature rather than to cool them before freezing as it may
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4 172 induce a short-term cold-response. Freezing / thawing samples more than once should be avoided.
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8 173 1.3 Storage 9

12 174 1.3.1 Frozen storage (−20 °C to −196 °C) 13 14 15

16 175 Storing samples at cryogenic temperature is likely the most common method of preservation before
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18 176 performing FA analyses. Yet, depending on the temperature, storage duration and type of biological
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20
21 177 material, this may not be sufficient to limit lipid degradation. It can lead to increased FFA and
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23 178 products of oxidation (e.g., Christie, 1984; Gullian-Klanian *et al.*, 2017; Tenyang *et al.*, 2019). Lower
24
25 179 freezing temperatures and short-term storage are generally associated with lower lipid degradation
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27
28 180 (e.g., Baron *et al.*, 2007; Rudy *et al.*, 2016; Gullian-Klanian *et al.*, 2017). The level of lipid
29
30 181 degradation in samples is highly dependent on initial freezing temperature and temperature variation
31
32 182 during storage, as well as storage duration prior to analyses (Han and Liston, 1987; Ramalhosa *et al.*,
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34 183 2012; Romotowska *et al.*, 2017). When long-term storage is required, the safest approach is to freeze
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37 184 samples at −80 °C immediately after collection. Although not ideal, long-term storage at −20 °C can
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39 185 be suitable for some species and sample/tissue types, in particular when samples are kept frozen
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42 186 within the organic extraction solvent. Levels of *n*-3 and *n*-6 PUFA in fish muscle, stored at −30 °C
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44 187 and −80 °C, did not change for up to 12 months in both TAG and phospholipid fractions (Passi *et al.*,
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46 188 2005). Shark muscle samples archived for up to 16 years at −20 °C retained their FA profiles, while
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48 189 lipid class composition was not analysed (Meyer *et al.* 2017). Impacts of frozen storage on FA content
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50
51 190 and composition are species-specific and can depend on the total lipid content of the organism and
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53 191 tissue (Rudy *et al.*, 2016). Species with high total lipid content (>10% wet weight) must be treated
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55 192 with increased care (low temperature, and short storage period) to avoid changes in FA content during
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58 193 freezing (Rudy *et al.*, 2016; Sardenne *et al.*, 2019).
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2 194 Based on the literature cited, samples should be stored for less than 6 months prior to analysis at
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4 195 temperature lower than $-20\text{ }^{\circ}\text{C}$ when no information is available on the collected biological material.
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6 196 Note that lipid analysis is also possible for samples stored using brine immersion freezing technique
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9 197 (e.g., fisheries-issued material) (Bodin *et al.*, 2014). The sensitivity of lipids to storage varies
10
11 198 according to species and tissues. We therefore recommend evaluating the sensitivity of the samples
12
13 199 for which there is no information available before the start of the study. For example, a temporal
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15 200 monitoring of the lipid composition of the biological material of interest at the available storage
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17 201 temperatures will make it possible to define the optimal conservation strategy.
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21 202 1.3.2 Freeze-drying

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25 203 Freeze-drying (or lyophilisation) is a low temperature dehydration process of frozen material that is
26
27 204 used to reduce enzymatic activity and thus lipid degradation in samples. Freeze-drying samples
28
29 205 immediately prior to extraction does not affect lipid class or FA compositions of aquatic animal
30
31 206 tissues (e.g., Dunstan *et al.*, 1993; Murphy *et al.*, 2003; Sardenne *et al.*, 2019). This can be a good
32
33 207 alternative to frozen storage, as freeze-dried tissues can be easier to grind and to transport than frozen
34
35 208 tissues. Rehydration may be required to improve the lipid extraction of TAG (e.g., Dunstan *et al.*,
36
37 209 1993) and to comply with extraction procedures that rely on a particular proportion of water in the
38
39 210 sample. Nonetheless, freeze-dried tissues still need to be carefully stored to prevent lipid degradation.
40
41 211 Storage duration can also have detrimental effects on lipid composition of samples. In fish, crustacean
42
43 212 and molluscs, the total duration between freeze-drying and lipid extraction should not exceed one
44
45 213 month at $-20\text{ }^{\circ}\text{C}$ for quantitative analysis (Sardenne *et al.*, 2019). Similarly, freeze-dried microalgae
46
47 214 samples should not be stored for extended period of time (but see Rudy *et al.* 2015). The PUFA
48
49 215 proportion of filtered *Isochrysis galbana* biomass decreased from 36% to 26% after 3 months storage
50
51 216 at $-76\text{ }^{\circ}\text{C}$ (Babarro *et al.*, 2001). Proportions of FFA in freeze-dried *Phaeodactylum tricornutum*
52
53 217 increased from 4 mg/g to 6 mg/g DW after 35 days at $-20\text{ }^{\circ}\text{C}$ while the degree of oxidation was
54
55 218 unaffected (Ryckebosch *et al.*, 2011). Variability in freeze-dried sample stability/viability for lipid
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2 219 analysis is likely to reflect differences of biological material and length/duration of the freeze-drying
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4 220 process.
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6 221 Overall, the quality of the freeze-dried storage depends on: (i) the storage temperature (lower
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8
9 222 temperatures are the best), (ii) the storage duration (shorter durations are better) and (iii) the lipid
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11 223 content of samples (lean samples are conserved better than lipid-rich samples). Lipid oxidation in
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13 224 food products occurs more rapidly when water activity is high (Labuza *et al.*, 1972). Conversely,
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15
16 225 lowering water activity below 0.2 through freeze-drying process may be a factor promoting lipid
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18 226 degradation through oxidation (Labuza *et al.*, 1972), and specific testing should be considered for
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20 227 aquatic samples. Freeze-drying may offer some benefits when used as a short-term preservation
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23 228 method (e.g., during transport), but it can be expensive (necessary equipment) and time-consuming,
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25 229 while potentially shortening the viability of samples for lipid analyses.
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28 230 1.3.3 Solvent and fixative storage

32 231 1.3.3.1 Organic solvent

35 232 One common storage strategy is to directly immerse small organisms such as phytoplankton collected
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37 233 on filters or small-bodied zooplankton into the lipid extraction solvent (e.g., Marty *et al.*, 1992;
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39
40 234 Soudant *et al.*, 1998; Windisch and Fink, 2018). If samples in the extraction solvent (generally
41
42 235 chloroform CHCl_3 and methanol MeOH) are kept in the dark and at low (preferably freezing)
43
44 236 temperatures, lipid degradation is minimal. To our knowledge, maximum storage duration of
45
46 237 extracted lipids has not been formally investigated. However, samples are likely to remain useable
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48
49 238 for months to years. For microalgae samples, it is advised to deactivate lipases by a rapid addition of
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51 239 boiling water or isopropanol prior to extraction (Berge *et al.*, 1995; Budge and Parrish, 1999;
52
53 240 Ryckebosch *et al.*, 2012).

55
56 241 Synthetic antioxidants (0.01% butylated hydroxytoluene, BHT or 0.1% tertiary
57
58 242 butylhydroquinone, TBHQ) can be added to solvent-stored samples to reduce radical formation and
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60 243 oxidative losses of unsaturated lipids (Christie, 2003; Ryckebosch *et al.*, 2012). However, addition

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2 244 of synthetic antioxidant is unnecessary in samples containing large amount of natural antioxidants
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4 245 such as microalgae (Ryckebosch *et al.*, 2012). Note that BHT elute with short-chain FA and when
5
6 246 present in large quantities, it can overlap with FA of interest.
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10 247 1.3.3.2 *Fixatives*

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12 248 Fixatives such as ethanol or formalin are commonly used for biodiversity, taxonomy, morphological,
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14
15 249 or DNA analyses. The use of fixed samples opens new research opportunities, by making it possible
16
17 250 to analyse historical samples collected for purposes other than lipid analysis. However, fixatives
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19 251 influence the chemical composition of the samples and should therefore not be used as a routine
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21
22 252 procedure for lipid analyses. For example, FA composition of crustacean tissues is altered in
23
24 253 formalin-preserved samples, but not in ethanol preserved samples (Phleger *et al.*, 2001). Direct
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26 254 evaluation of fixative effects on lipids are scarce and preliminary tests on different sample types are
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29 255 highly recommended.
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2 Transport and shipping

The packaging, transport and shipping of samples for lipid analysis is a complex process, driven by safety laws, financial costs, stringent storage conditions, and limited duration of storage. Specific legal regulations, including quarantine, must be followed and they can vary, depending on whether the samples are shipped domestically or internationally, and whether there are stopovers during long distance travels. Shipping recommendations to ensure that samples arrive at their destination in good condition include triple packaging with a rigid outer layer and absorbent material, appropriate cooling, accurate labelling (i.e., name, address, phone number, e-mail address, size and weight of the package), and complete legal documentation (e.g., itemised list of content, permits) (IATA, 2010). This section reviews different options of packaging for shipping and traveling, and gives an overview of the main flying regulations and key legal information.

2.1 Shipping samples

Long-distance shipping from the sampling site to the laboratory is challenging. Multiple issues need consideration, such as the conservation state of the samples prior to transport (cooled, frozen, deep-frozen, freeze-dried), the distance, the duration and the type of transport. As samples are generally prone to degradation, temperatures must be kept low and oxidative conditions reduced as much as possible. Regardless of the method used, we recommend only to ship a part of the original sample and keep a backup sample when possible, and to take great care in the packaging (minimum empty space, adapted container to accommodate samples and cooling methods).

When possible, frozen samples should be shipped using dry shippers, which are aluminium containers flooded with liquid nitrogen. They contain adsorbent material that prevents spills of liquid nitrogen during shipment and have attached temperature loggers. Samples can be stored in these conditions for several weeks. However, dry shippers may not be readily available at sampling locations or in laboratories. Restrictions for the transport of liquid nitrogen also need to be considered

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2 280 (e.g. in airplanes or closed vehicles). With transporter authorisation, frozen samples can also be
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4 281 shipped on dry ice depending on transport duration (about 24 h for 2.5 kg of dry ice). Alternatively,
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6 282 deep frozen (-80 °C) aqueous ice packs or reusable technical ice packs (e.g. TechniIce™, 2019) can
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9 283 be used. A cost-effective option is to freeze-dry samples prior to shipping, as it reduces both the
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11 284 volume to be shipped and the risk of lipid hydrolysis by removing water (see section 1). When dry
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13 285 ice packaging or deep-frozen ice packs are not available at the sampling location, freeze-dried
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16 286 samples should be kept cool and in an oxygen-free atmosphere by flushing containers with nitrogen
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18 287 gas and then sealing prior to packaging and shipping. It is also possible to ship samples with small
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20 288 amounts of solvents with respect to international regulations. Note however that depressurisation in
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23 289 aircrafts promotes solvent leakage (F. Pernet pers. obs). To limit this phenomenon, tubes and caps
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25 290 can be coated with Teflon. Alternatively, preserved and/or extracted samples in solvent may be
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27 291 evaporated and flushed with nitrogen so that dried extracts can be air-transported as frozen biological
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30 292 material. Dried lipid extracts will have to be re-immersed in solvent as soon as possible upon arrival.
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32 293 We however recommend to perform preliminary testing before proceeding with this method.
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34 294 The gold standard for shipping samples are specialised transport companies that maintain samples
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36 295 on dry ice and guarantee express delivery to the final destination. However, these options may not be
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39 296 practical for some laboratories and/or available for all sampling sites and destinations. Care should
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41 297 be taken when selecting the shipping company, as several unfortunate experiences have shown that
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43 298 some may not be reliable in maintaining the cold transportation chain.
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47 299 2.2 Flying with samples

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51 300 Although courier is preferable, one might consider putting samples in checked luggage. One of the
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53 301 safest options to transport samples as checked luggage is to use a dry shipper. However, airlines may
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56 302 have different regulations in transporting liquid nitrogen. Non-harmful materials, such as dry ice or
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58 303 technical ice packs are the best option to meet standard luggage regulations (e.g., < 2.5 kg of dry ice
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2 304 per person) and safety (e.g. the package permitting the release of carbon dioxide gas when
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4 305 transporting dry ice).

6 306 Transporting freeze-dried samples kept in a hermetically closed bag (or a thermos filled with ice)
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9 307 is likely the safest and cheapest option. Upon arrival, samples need to be stored in a freezer
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11 308 (preferentially at -80°C) or immediately put in a solvent solution for lipid extraction (see **section 1**
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13 309 for more details on storage duration). Dried lipid extract should be transported as frozen samples and
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15
16 310 be returned to solvent as soon as possible (see section 2.1). Note that preservation methods requiring
17
18 311 flammable solvents (e.g. ethanol, methanol) are not permitted on aircraft within passenger luggage.
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20 312 Recently, the emerging technology of drones opens new opportunities for a rapid and customisable
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23 313 aerial transport of sensitive samples from remote areas to laboratory facilities (e.g., Amukele *et al.*,
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25 314 2017).

29 315 2.3 Regulations and permits

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33 316 Prior to initiating sample collection and considering transporting them, it is necessary to be well
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35 317 informed on import and export regulations that differ widely across countries and studied organisms'
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37 318 status. It can be difficult to obtain the necessary information within custom departments and the
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40 319 paperwork involved. Some research institutions may have specialised services available to ensure
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42 320 proper paperwork so that samples are shipped and received safely. To transport samples out of a
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44 321 country, it is often necessary to follow The Nagoya Protocol on Access and Benefit Sharing (United
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46 322 Nations Convention on Biological Diversity, 2011). Although it was initially stated that this only
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49 323 applies to samples for molecular research, all transports of tissue for biochemical screening are now
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51 324 included. This applies only for countries that have signed and ratified the Nagoya Protocol.

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53 325 When samples contain species listed in the Convention on International Trade in Endangered
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55 326 Species of wild fauna and flora (CITES), it is necessary to request an import/export CITES permit
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58 327 from each of the countries involved. This needs to be organised well ahead of sample collection, as
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328 it can take up to 3 years to obtain these permits. Additional research permits may be required for the
329 import and export of samples depending on national regulations and target species.

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3 Lipid extraction

Because of their diverse structures, lipids range from non-polar to highly polar, which makes the selection of a solvent with matching polarity challenging. Methods based on chloroform and methanol (Folch *et al.*, 1957; Bligh and Dyer, 1959) have long been used across a wide range of matrix types with great success. Current alterations in environmental regulations have brought restrictions on the type and amount of solvent usage in laboratories worldwide, encouraging the exploitation of alternative organic solvents and novel eco-friendly methodologies for extraction (e.g., accelerated solvent extraction). Here, we review both the well-established techniques and the newly developed methods; we caution readers that full validation must be carried out with a given biological material before adopting any modified or adapted method.

3.1 Handling cautions

Contamination is an issue that needs to be carefully addressed at every stage of sample handling. It is recommended to; i) use material that have been cleaned using acetone or extraction solvent mixture to remove any potential lipids (e.g. Teflon-coated needles used for evaporation), ii) avoid plastic containers, tools or tips, iii) combust at 450 °C for 6 h or solvent-rinse all glassware and glass fibre filters, iv) use glass tubes fitted with Teflon-lined screw caps, v) prefer amber glassware (or aluminium foil protected) to limit solvent photo-degradation, vi) use the highest quality solvents for material/tool cleaning, extraction, or elution, vii) perform blank and reference samples for each batch of samples, and viii) wear laboratory gloves to limit exposure to toxic solvents and cross-contamination.

3.2 Sample preparation

Regardless of the technique used, extraction of lipids from aquatic samples requires that the solvent penetrates the sample particles so that it can make contact and solubilise the lipids present. The most efficient extractions ensure that the particle size is as small as possible. The gold standard is to homogenise samples with a ball mill homogenizer (at $-196\text{ }^{\circ}\text{C}$ using liquid nitrogen with wet samples). With wet plant or animal tissue samples at macro-scales, this may be as simple as chopping or blending. For wet microalgae, grinding of samples after immersion in solvent works well to break open cells. Small organisms (roughly between $1\text{ }\mu\text{m}$ and 1 mm ; on a filter or without) can also be directly extracted by direct immersion in the solvent mixture. The use of ultrasound-assisted extraction with a probe or bath may aid in breaking cell walls (Pernet and Tremblay, 2003). When samples have been freeze-dried (**section 1**) for preservation, they may be most conveniently ground with mortar and pestle and it may be necessary to rehydrate prior to carrying out the extraction. Any of these techniques, when applied prior to subsampling for analysis, should also serve to homogenise samples effectively, to ensure representative sampling. These diverse techniques also illustrate the need to adapt the preparatory steps and approach to the sample matrix.

3.3 Traditional extraction methods

While many researchers consider Folch et al. (1957) and Bligh and Dyer (1959) extraction methods to be similar, based on their use of chloroform and methanol, the two are quite different and were developed for different objectives. Prior to using those methods, one would do well to read the original publications. Both methods rely on the formation of a biphasic mixture, with a lower layer principally consisting of chloroform, and containing the isolated lipids, and an upper phase consisting of methanol and water and containing the non-lipids. The Folch et al. procedure, using CHCl_3 :MeOH (2:1, v:v) and a solvent to sample ratio of at least 20:1, was designed as a general and robust method for recovery of lipids from animal tissues (i.e., brain, liver, muscle), now applicable to several tissues

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2 375 and species with a range of lipid content (Iverson et al. 2001). The Bligh and Dyer method uses a
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4 376 more polar solvent mixture of CHCl₃:MeOH: H₂O) (1:2:0.8, v:v:v) with a 4:1 solvent to sample ratio,
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7 377 and was originally developed to rapidly extract lipids, principally polar lipids, from lean fish tissues.

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9 378 While the Folch et al. method is more complicated to perform, it is more reliable and applicable
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11 379 to almost all sample types. A key aspect of the original Folch et al. method that is often overlooked,
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13 380 is the washing of the bottom phase that contains lipids with a mixture of solvents of the same
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15 proportions as originally found in the upper phases. As originally described by Folch et al. (1957),
16 381 the upper phase consists of CHCl₃:MeOH:H₂O at 3:48:47 and while some groups do use a 50:50
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18 382 MeOH: H₂O wash as recommended by Christie (1982), it is exceedingly rare that the correct mixture
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20 383 is used. Thus, almost all Folch et al. procedures are best described as ‘modified’. The Bligh and Dyer
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22 384 method is easier to carry out, but requires greater consideration of sample type and potential
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24 385 modification of the procedure. For instance, Christie (1982) notes that with high fat samples, an initial
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26 386 pre-extraction with chloroform or diethyl ether is necessary to extract TAG that would otherwise
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28 387 remain unrecovered. The use of the correct proportions of solvents and water are critical for
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30 388 quantitative lipid recovery with both methods. One must take care to adjust the volume of water added
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32 389 when samples with varying moisture contents are analysed (i.e., freeze-dried material vs fresh tissue)
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34 390 and it is often necessary to determine moisture content before beginning extraction.
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39 392 These basic procedures may require some adjustments depending on lipid matrix before and/or
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41 393 during extraction to increase the yield or specificity of lipids to be analysed. For example, it may
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43 394 include inactivation of lipase, changes of solvent proportions, or cell disruption (e.g. Kumari *et al.*,
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45 395 2011; Ryckebosch *et al.*, 2012). In a general manner, authors must thus refer to the available literature
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47 396 on their model organisms or matrices. Given the numerous modified extraction procedures that reflect
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49 397 the diversity of aquatic biological matrices, it is also essential that authors detail their protocol in
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51 398 publications when it differs from original methods.
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399 3.4 Alternatives to chloroform: toward less toxic solvents

400 3.4.1 Dichloromethane

401 Many laboratories have become increasingly conscious of a range of occupational health and safety
402 areas in recent decades. Lipid studies historically used chloroform as a major solvent in many steps
403 including extraction. The less toxic dichloromethane (CH_2Cl_2) has regularly been used instead of
404 chloroform for lipid extraction in many aquatic labs. This replacement originated from food chemistry
405 research (e.g. Chen *et al.*, 1981) and was later formally reported for aquatic samples (e.g., Cequier-
406 Sánchez *et al.*, 2008). Regardless of solvent choice, both dichloromethane and chloroform may
407 degrade on storage to produce HCl and phosgene (Noweir *et al.*, 1972). Phosgene is a much more
408 acutely toxic chemical than either chloroform or dichloromethane, and inhalation can lead to
409 pulmonary oedema (e.g., Snyder *et al.*, 1992). Chloroform degradation or insufficiently stabilised
410 may lead to lipid degradation (i.e., empty chromatograms, including disappearance of the internal
411 standard) (Le Grand and Pernet, pers. obs). To limit phosgene production, chloroform is stabilised
412 with either ethanol or amylene, with ethanol being the more effective stabiliser. Because
413 dichloromethane is less susceptible to degradation than chloroform, it is typically stabilised with
414 alkenes only. Safe lab practices, including restricting solvent use to fume hoods, can effectively avoid
415 risks associated with chlorinated solvents and phosgene formation. In the field, when samples are
416 preserved by direct immersion in solvent, caution is advised.

417 3.4.2 Methyl-tert-butyl ether

418 Methyl-tert-butyl ether (MTBE) was recently used as a less toxic solvent for the extraction of lipids,
419 but had lower recovery rates than chlorinated solvents (Sheng *et al.*, 2011; Ryckebosch *et al.*, 2012).
420 However, the MTBE based extraction protocol appeared especially well adapted to high-throughput
421 lipidomics, as the lipid extract recovered in the upper phase was cleaner with non-extractible material
422 remaining in the lower phase (Matyash *et al.*, 2008).

3.5 The special case of ethanol preserved samples

The availability of historically preserved samples can often occur for a range of lipid studies (see section 1.3.3.1). However, because ethanol extracts some lipids, the sample and all added ethanol must be used in the lipid extraction protocol (Phleger *et al.*, 2001). When using the Bligh and Dyer (1959) method, the extraction should be performed with chloroform (or dichloromethane), ethanol and water, where ethanol replaces methanol. Care is needed when selecting historic ethanol-preserved samples for lipid analysis and in which the ethanol preservative may have been replaced during storage.

3.6 Other methods

Accelerated Solvent Extraction (ASE, Dionex), also known as Pressurised Fluid Extraction (PFE), is widely used method based on a controlled increase in solvent temperature and pressure during extraction. This process maintains the liquid state of solvents near their supercritical region where they have high extraction properties. Several ASE parameters can be adjusted to improve the extraction, such as temperature, pressure, extraction duration (including pre-heating) and number of extraction cycles. All of these parameters affect the final solvent volume and can be adjusted individually to each sample type to optimise the extraction yields and quality (Schäfer, 1998). This sample type-specific adjustment enables high extraction efficiency with a low solvent volume and a short extraction time which is beneficial from a cost and environmental standpoint. Microscale lipid extraction of fish tissues (100 mg) using ASE was shown to be as quantitatively and qualitatively efficient as classical lipid extraction methods (Dodds *et al.*, 2004).

3.7 Concluding remarks

This section did not aim to be exhaustive in describing existing extraction methods. When comparing data from different studies, it is important to consider the lipid extraction techniques used as well as

1
2 446 handling and storage methods. When working on new matrices and developing new or optimised
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4 447 extraction protocols, we recommend to compare them with Folch et al. (1957) or to Bligh & Dyer
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6 448 (1959) methods, which generally remain the gold standards for lipid recovery. Ensuring complete
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9 449 lipid extraction is critical when applying new protocols. Performing successive lipid extractions on a
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11 450 same sample and measuring its lipid content at each step is a suitable approach to establish at which
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13 451 stage the sample is totally lipid-depleted. We recommend monitoring extraction efficiency values and
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15
16 452 ideally reporting them in publications. Alternatively to successive extractions, the fatty acid methyl
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18 453 ester (FAME) content of the lipid extract can be compared to FAME recovered after direct
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20 454 transesterification (**section 6**). A number of studies have compared the effect of extraction techniques
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23 455 (ASE, Soxhlet, manual) on lipid recovery and FA content (e.g. Ewald *et al.*, 1998; Mulbry *et al.*,
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25 456 2009; Balasubramanian *et al.*, 2013). While all report differences, there is little agreement as to which
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27 457 may be an optimal method, likely due to substantial differences in the followed protocols.
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4 Indicators of sample degradation

Sample lipids may degrade through hydrolysis of ester bonds or through oxidation of carbon-carbon double bonds. These are two, largely independent processes leading to different compounds or groups of compounds, which may be used as indicators of sample degradation. Therefore, samples showing indicators of hydrolysis may not have been oxidised and *vice versa*. Unless necessary for a short time period (e.g. shipping), purified lipid extracts are susceptible to oxidation and should be kept under N₂ atmosphere with solvent (Folch mixture is generally preferred).

4.1 Hydrolysis

The most frequently encountered aquatic lipids are acylglycerols (TAG, PL, and glycolipids) in which FA are esterified to a glycerol backbone. There are also ether lipids that replace one ester bond with an ether linkage or a vinyl ether linkage. Esters of carboxylic acids undergo hydrolysis in a reversible reaction which is catalysed by hydrolase enzymes called esterases present in biological samples. Lipases, a subclass of esterases, are found in animals, plants, fungi, bacteria and even in certain viruses (Beisson *et al.*, 2000). Lipid hydrolysis can be assessed from the FFA content, and from the lysophospholipid content when samples are dominated by PL. Standard measurements of oil quality are the acid value (AV) and FFA content, determined in the oil industry by titration and chromatography, respectively. However, both these measurements are rarely used outside of the industrial context.

Many chromatographic systems have been used over the past years for quantitative analysis of major lipid classes including FFA. Free FA can be easily separated from extracted lipids by means of well-known methods based on either liquid (LC) or thin layer chromatography (TLC) (reviewed by Fuchs *et al.*, 2011). For example, HPLC with light-scattering detection (HPLC-ELSD) has been successfully used to detect and quantify FFA in fish with a relatively short processing time and highly reproducible measurements (Christie, 1985; Silversand and Haux, 1997). As for LC, a number of

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2 483 improvements to classic TLC have led to more sophisticated forms, providing better separation
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4 484 efficiencies and quantifications. High-performance thin-layer chromatography (HPTLC) coupled
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6 485 with densitometry or TLC coupled with flame ionisation detection (TLC-FID, Iatroscan, Shantha,
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9 486 1992) have also been used to quantify lipid classes and FFA in samples from different origins
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11 487 including marine (Lochmann *et al.*, 1996; Parrish *et al.*, 2009), terrestrial (Zoumpoulakis *et al.*, 2012)
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13 488 and freshwater species (Litz *et al.*, 2017). In food studies, FFA determined by TLC-FID is generally
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16 489 equivalent to AV (Nishiba *et al.*, 2000). TLC is normally less expensive and less time-consuming
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18 490 than HPLC-based methods that can, of course, also be used to provide similar results.
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20 491 The hydrolysis index of non-polar acyl lipids (HI) and the lipolysis index of all acyl lipids (LI) can
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23 492 be used as degradation indicators (Week *et al.*, 1993; Parrish *et al.* 1995) and are calculated as:

$$25 \quad HI = \frac{FFA + ALC}{TAG + WE + FFA + ALC} * 100$$

$$29 \quad 494 \quad LI = \frac{FFA + ALC}{\text{total acyl lipids} + ALC} * 100$$

32 495 LI values are always lower than HI values for any sample because all acyl lipids are considered.
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36 496 4.2 Oxidation

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40 497 Lipid oxidation mainly occurs through autoxidation when lipids react spontaneously with
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42 498 atmospheric oxygen. The process is usually autocatalytic, with oxidation products accelerating the
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45 499 reaction so that it increases over time. Other catalysts such as light, heat, enzymes and metals lead to
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47 500 photo-oxidation, thermal oxidation and enzymatic oxidation. Fatty acid oxidation is a process in
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49 501 which free radicals cause unsaturated FA to react with molecular oxygen forming acyl
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51 502 hydroperoxides, which can then breakdown into a variety of secondary products. A decrease in PUFA
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54 503 content may indicate oxidation as they are particularly susceptible to degradation (Sasaki and
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56 504 Capuzzo, 1984).

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59 505 Lipid hydrolysis may accelerate lipid oxidation due to the formation of FFA that can be substrates
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506 for oxidation reactions, as found *in vivo* (Saponaro *et al.*, 2015). High levels of PUFA in lipids make

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2 507 them susceptible to oxidation (Koshio *et al.*, 1994). Oxidation of *n*-3 and *n*-6 PUFA leads to the
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4 508 formation of highly chemically reactive compounds such as malondialdehyde (MDA), 4-hydroxy-2-
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6 509 hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE). HHE is an aldehyde derived from *n*-3 PUFA,
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9 510 and HNE from *n*-6 PUFA.

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11 511 A number of physical and chemical tests, including instrumental analyses, are available to
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13 512 determine primary and secondary products of oxidation (Shahidi and Zhong, 2005). Oil supplement,
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15 513 food and other industries have a range of standard measurements for determining oil oxidation. These
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18 514 include the internationally recommended peroxide value (PV) and *p*-anisidine value (pAV) (Nichols
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20 515 *et al.*, 2016). For example, PV can be measured by titration and 2-thiobarbituric acid reactive
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22 516 substances (TBARS) by spectrophotometry, and both can be determined using highly precise Fourier
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24 517 transform infrared spectroscopy (FTIR). MDA is commonly monitored using the direct TBARS test
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27 518 (Fernández *et al.*, 1997; Shahidi and Zhong, 2005) and with HPLC (Mendes *et al.*, 2009), while HHE
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29 519 and HNE can be quantified by GC-MS (using chemical ionisation) (Kenmogne-Domguia *et al.*,
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31 520 2014). Recent analytical developments allowed simultaneous quantification of MDA, HHE and HNE
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33 521 by LC/APCI-MS and LC-MS/MS (Douny *et al.*, 2016; Tullberg *et al.*, 2016). Together with pAV,
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35 522 MDA, HHE and HNE are secondary markers of oxidation while PV, hydroperoxides and conjugated
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37 523 dienes are primary markers. However, it has to be noted that MDA, HHE and HNE quantifications
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39 524 do not necessary reflect PV and anisidine values (Viau *et al.*, 2016).
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43 525 Sterols are also a source of oxygenated lipids named oxysterols which are sterols bearing a second
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45 526 oxygen functionality. Cholesterol oxidation likely proceeds in conjunction with oxidative
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47 527 decomposition of coexisting PUFA (Ohshima *et al.*, 1993). Cholesterol oxidation products (COP)
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49 528 can be determined by cold saponification of lipids (Ohshima *et al.*, 1993), followed by enrichment of
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51 529 COP by solid-phase extraction (SPE), and then quantification and identification by GC and GC-MS
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55 530 (Pickova and Dutta, 2003).
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531 4.3 Concluding remarks

532 To conclude, we encourage as a minimum the assessing and reporting of hydrolysis indicators as a
533 quality control criterion of sample handling and extraction procedures. The threshold above which
534 sample/data quality should be questioned depends on the sample matrix, the logistical context and
535 the study objective. In any case, the extent of either form of degradation should ideally be evaluated
536 and compared with baseline values from fresh and properly extracted samples.

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5 Separation of neutral and polar lipids

Fatty acids are incorporated almost unaltered into the storage lipids such as WE, TAG, and ALC, reflecting the FA profiles of the consumed food (Dalsgaard *et al.*, 2003). In contrast, polar lipids are primarily involved in cell membranes which regulate and adjust their FA composition in response to environmental factors like temperature, salinity and hydrostatic pressure (Hazel and Williams, 1990). As such, inference of an organism's diet based on total lipids can be biased by the ratio of FA originating from storage (mostly neutral lipids) and membrane lipids (mostly polar lipids). This ratio varies widely across species, ontogenetic stages, reproduction, nutritional status and tissue types. For example, in small pelagic fish from the Mediterranean, the contribution of TAG stored in the muscle in winter was 1.5%, 5.2% and 47.6% of total lipids for sardine, anchovy and sprat, respectively, the remainder being mostly polar lipids (Pethybridge *et al.*, 2014). This demonstrates that bulk FA composition variations among species can be confounded with variations in lipid class composition. However, many studies in trophic ecology have based their trophic interpretation on the FA composition of bulk lipids without considering the FA distribution between storage and structural lipids.

5.1 When should the lipid fraction be separated?

Lipid class composition should be assessed alongside the FA composition to decide whether lipid class separation is necessary for robust dietary inference. Calculating the ratio of storage to structural lipids provides a comprehensive assessment of the cost/benefit of separating neutral and polar lipid fractions. For samples dominated by storage lipids, the FA composition of bulk lipids will mostly mirror the diet, and lipid class fractionation is not mandatory (e.g., fatty fish species, marine mammal blubber, and digestive gland and liver of marine organisms) (e.g. Sardenne *et al.*, 2020). However, for samples rich in structural lipids, fractionation of lipid classes will provide more reliable dietary indicators. The analysis of FA in polar lipids can inform on nutritional requirements of the study

1
2 562 organism and on environmental conditions prevailing at the time of sampling. Comparing the relative
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4 563 FA proportions in polar and neutral lipids allows the identification of the dietary FA that were
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6 564 selectively retained/eliminated to form cell membranes (e.g. Delaunay *et al.*, 1993; Plante *et al.*,
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8
9 565 2007). The unsaturation level of FA and PUFA incorporated in polar lipids is linked to the
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11 566 temperature in ectothermic organisms (Hazel and Williams, 1990; Hall *et al.*, 2002; Pernet *et al.*,
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13 567 2007) and can be associated with the basal metabolic rate (Hulbert and Else, 1999; Pernet *et al.*,
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15
16 568 2008). Consequently, the separate analysis of neutral and polar lipids is a practical approach to
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18 569 address questions on trophic ecology as well as on nutrition and comparative physiology.
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20 570 To establish trophic relationships of species for which all potential food sources are known, a good
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23 571 strategy is to analyse the FA composition of total lipids in food sources (e.g., prey) and of neutral
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25 572 lipids in consumers to characterise their diet (e.g., predators). Such approach was implemented by the
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27 573 pioneers of the use of FA as trophic indicators (reviewed in Dalsgaard *et al.*, 2003). Alternatively,
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30 574 when study organisms are both prey and predator, or when predator-prey relationships are not well-
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32 575 known, we suggest at a minimum analysing FA from both neutral and total lipids. Beyond trophic
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34 576 considerations, quantification of lipid classes provides useful information on the physiological
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36 577 condition of organisms (Fraser *et al.*, 1989) and the quality of sample preservation through FFA and
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39 578 lysophospholipids levels (see **section 4**).

40 41 42 579 5.2 Commonly used separation methods

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46 580 Storage lipids (SE, WE, TAG, DAG, ALC) and FFA are defined as ‘neutral’ because they are
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49 581 hydrophobic molecules lacking charged groups, whereas PL are defined as ‘polar’ because they have
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51 582 a charged hydrophilic head. Neutral and polar lipids can be separated from each other by TLC or by
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54 583 adsorption chromatography using either laboratory packed or commercial prepared silica columns. A
55
56 584 simple and widely used protocol for separation of neutral and polar lipids consists of a short column
57
58 585 of silica prepared in a glass disposable Pasteur pipette plugged with cotton wool (Christie, 1982b).
59
60 586 The sample is loaded on the top of the column and washed at low pressure with chloroform (or

1
2 587 dichloromethane) often mixed with methanol, (e.g., CHCl₃:MeOH, 98:2 v/v) to elute neutral lipids.
3
4 588 It can then be washed with acetone to obtain acetone mobile polar lipids, generally dominated by
5
6 589 pigments and glycolipids. Finally, a last wash is performed with methanol to yield polar lipids
7
8
9 590 dominated by PL. Some PL with lower polarity may elute with acetone, while some glycolipids with
10
11 591 higher polarity may elute with methanol. Including an elution step with acetone may not be necessary
12
13 592 in non-plant samples as they contain negligible amounts of glycolipids (Christie, 1982b). Yet, the
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15
16 593 latter step (elution step with acetone) can be useful in deciphering trophic links in deep-sea fishes
17
18 594 (Şen Özdemir *et al.*, 2019). Diethyl-ether:acetic acid (98:2, v/v) can be used to recover pigments,
19
20 595 prior to elution of glycolipids and PL (Da Costa *et al.*, 2017). Eluent and sample volumes must be
21
22
23 596 adjusted to optimise separation. It is also important to note that hydration of silica changes the
24
25 597 efficiency of retention through a partition effect between bound water and polar lipids (Christie,
26
27 598 1982b). The use of silica hydrated with 6% water (m/m) can improve the recovery of polar lipids
28
29
30 599 from marine bivalves (e.g., Marty *et al.*, 1992; Pernet *et al.*, 2006).

31
32 600 Commercially pre-packed columns connected to a vacuum manifold are widely used instead of
33
34 601 the manually packed Pasteur pipette (Christie, 1992; Ruiz-Gutiérrez and Pérez-Camino, 2000). This
35
36 602 procedure, often referred to as SPE, is time effective and provides similar results as classic column
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38
39 603 chromatography (Juaneda and Rocquelin, 1985). However, it is difficult to achieve quantitative
40
41 604 recovery of PL with this approach. Only ~62% of total lipids were recovered from clam tissue using
42
43 605 SPE due to the selective retention of phosphatidylcholine as silica is not rehydrated in these pre-
44
45
46 606 packed columns (Pernet *et al.*, 2006). Commercial SPE columns are available with a wide range of
47
48 607 chemically-bonded stationary phases. These have great potential for the isolation of specific lipid
49
50 608 classes, yet surprisingly few applications have been described to date. For example, an alternative
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52
53 609 method developed by Kaluzny *et al.* (1985) using aminopropyl-bonded silica is suitable for separating
54
55 610 lipid classes in oyster tissues, though particular care is required to recover the acidic lipids
56
57 611 quantitatively (Kim and Salem, 1990; Pernet *et al.*, 2006). Aminopropyl bonded phases have also
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59
60 612 been used to separate groups of lipid classes, including neutral lipids such as cholesterol, and non-

1
2 613 acidic PL, and they are especially useful for the isolation of the FFA fraction from lipid extracts
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4 614 (Kaluzny *et al.*, 1985; Kim and Salem, 1990).
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8 615 5.3 Practical recommendations 9

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11
12 616 Before starting to use a separation protocol, we recommend reading handbooks describing the use
13
14 617 and applications of SPE for lipid separation including the choice of eluting solvents (Christie, 1982b;
15
16
17 618 Perkins, 1991), and to consult technical resources provided by the SPE manufacturers. Specifically,
18
19 619 care must be taken to avoid sample overload and subsequent cross-contamination. This problem can
20
21 620 be avoided by knowing the lipid composition of the sample and the carrying capacity of the column
22
23
24 621 prior to SPE. The amount of sample that can be loaded onto a column is governed by the proportion
25
26 622 of the retained lipids. For example, 2 to 5 mg of PL is considered as the maximum load for SPE of
27
28 623 neutral and polar lipids on a 100 mg silica column (Christie, 1982b). Although sample overload leads
29
30
31 624 to cross-contamination, an excess of sorbent can also reduce recovery of the retained analytes. It is
32
33 625 therefore suggested to experimentally determine the sample load (mass ratio of lipid to sorbent) to
34
35 626 optimise purity and recovery (Pernet *et al.*, 2006). In order to use the SPE system, we suggest
36
37 627 replacing the silica originally contained in the pre-conditioned column with rehydrated silica
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39
40 628 (generally 6% m/m).
41

42 629 Purity and recovery analyses after SPE are crucial, particularly when the sample matrix is new to
43
44 630 the laboratory and when the stationary phase changes. Different brands or batches of silica gel may
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46
47 631 vary in their properties and cross-contamination of fractions may occur (Christie, 1982b). Purity can
48
49 632 be evaluated by TLC of lipid classes of each eluted fraction. The simplest way to evaluate recovery
50
51 633 is to quantify the lipid class content of samples before and after SPE by TLC and proceed with
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53
54 634 recovery calculation (i.e., the amount of collected lipid after SPE / the amount of lipids before SPE,
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56 635 as a percent). Alternatively, the recovery purity and efficiency can be estimated by adding an internal
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58 636 standard of known mass before SPE. As such, lyso-phosphatidylcholine 12:0 (22.8 µg of FA) was
59
60 637 added to the lipid extract before SPE to assess PL recovery in oyster samples (Pernet *et al.*, 2006).

1
2 638 Occurrence of 12:0 was exclusively associated to LPC as this FA is absent in oysters. A full recovery
3
4 639 of LPC following SPE on silica indicated that other PL were fully recovered as LPC is the most
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6 640 strongly retained phospholipid on silica (Pernet *et al.*, 2006). To evaluate the purity of each lipid
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9 641 fraction and the absence of cross-contamination, it is necessary to add one standard based on the least
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11 642 neutral lipid which must remain in the neutral lipid fraction, and another one which corresponds to
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13 643 the least polar lipid which must be retained in the polar lipid fraction. For example, commercially
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16 644 available 12:0 diacylglycerol (DG) and 10:0 phosphatidic acid (PA) can be added to the lipid extracts
17
18 645 of oysters (F. Pernet unpub. data). Because these two shorter chain FA are naturally absent in oysters,
19
20 646 their occurrence was exclusively associated with DG and PA standards. The FA 12:0 and 10:0 were
21
22
23 647 expected in neutral and polar lipid fractions, respectively. Note that these standards do not replace
24
25 648 the traditional 23:0 used for mass quantification of FA (see section 7). In the case of SPE, 23:0 is
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27 649 generally added directly in the elution tubes to avoid passage in the column as this standard would
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30 650 only be distributed in the neutral fraction.
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652 **6 Transesterification**

653 Except in highly degraded samples, where FFA proportion can reach high values, FA are generally
654 part of more complex lipid molecules, and are linked to glycerol by an ester linkage (e.g., TAG, PL,
655 glycolipids). Therefore, one approach to analyse FA by gas chromatography (GC) is to hydrolyse the
656 ester linkage and to convert the released non-volatile FA into their volatile derivatives, usually FA
657 methyl esters (FAME). Saponification followed by methylation is the classical method for preparing
658 FAME, and while laborious, it produces FAME without contamination with unsaponifiable lipid
659 compounds (e.g., non-volatiles, sterols, alcohols, hydrocarbons, phthalates and pigments). However,
660 most researchers use transesterification. A number of transesterification methods exist, all based on
661 the presence of excess methanol, a catalyst (acid or base) and heat (see Schlechtriem *et al.*, 2008). To
662 be analysed by GC, FAME are then extracted in a GC compatible solvent (often hexane), and the
663 organic phase is washed several times by water to remove acid and glycerol. Produced FAME are
664 stable for months at 4 °C in the dark under nitrogen. Transesterification using ethanol instead of
665 methanol is also possible, but less used, leading to the formation of FA ethyl esters. Butanol can also
666 be used as a substitute to methanol to quantify very short chain FA (down to *iso5:0*) by increasing
667 aliphatic chain length (Koopman *et al.*, 1996, 2003). Although the use of the boron trifluoride (BF₃)
668 has been recommended by the AOCS (1989), many catalysts can be used in both acid- and base-
669 catalysed transesterification, with each method having their own advantages and limits.

670 The efficiency of these transesterification methods differs as a function of the bond between the
671 aliphatic chains and the glycerol or sphingosine backbones (Christie and Han, 2010). This is
672 particularly important in aquatic samples because, depending on the matrix, a considerable part of
673 these aliphatic chains can be linked to the entire lipid molecule not by an ester linkage, but by a vinyl-
674 ether (e.g., plasmalogens), an ether (e.g., alkyl lipids) or an amide (e.g., sphingolipids) linkage. It is
675 thus important to choose the most pertinent transesterification method with respect to the sample
676 nature and the scientific question. For example, the acid-catalysed transesterification of plasmalogens

1
2 677 leads to the formation of FAME (from the acyl lipids) and dimethyl acetals (DMA) (from vinyl-ether
3
4 678 lipids). DMA are mainly detected in polar lipids but are marginally present in neutral lipids, reflecting
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6 679 the contribution of glyceride ether also named “neutral plasmalogens” (TAG containing at least one
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9 680 vinyl ether bound aliphatic chain). Base-catalysed transesterification of these molecules only leads to
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11 681 the formation of FAME, leaving vinyl ether bound aliphatic chains unreacted (Crackel *et al.*, 1988;
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13 682 Cruz-Hernandez *et al.*, 2006). However, attention needs to be paid to the particular procedure
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15
16 683 followed, as base-catalysed transesterification based on ISO standard methodology incorporates an
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18 684 acid wash to neutralise the catalyst. This acidic wash may lead to DMA formation (Gomez-Cortes et
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20 685 al. 2019). Moreover, neither acid- nor base-transesterifications have any effect on ether linkages
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22
23 686 (Christie 1989). This point is important to consider when analysing samples containing large amounts
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25 687 of alkyl ether lipids, such as alkylglyceryl ether lipids in shark liver oil. Transesterification of FA
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27 688 sphingolipids components is acid-catalysed and is achieved by increasing the reaction time (Eder,
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29
30 689 1995; Le Grand *et al.*, 2011). Finally, esterification of FFA is only possible by acid-catalysed
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32 690 transesterification (Christie, 2003).
33

34 691 Depending on the scientific question (e.g. nutritional value, trophic relationships, physiological
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36 692 responses), FA transesterification can be realised directly on the biomass (direct transesterification),
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39 693 on a total lipid extract or on lipid fractions, such as polar or neutral lipids, lipid classes (e.g., TAG,
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41 694 phosphatidylcholine) or lipid subclasses (e.g., phosphatidylethanolamine plasmalogen) isolated
42
43 695 beforehand. It can be useful to purify FAME by HPLC or TLC before GC injection (Marty *et al.*,
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45
46 696 1992). This prevents the saturation and the degradation of the column through the injection of non-
47
48 697 FAME molecules (e.g. non-volatiles, sterols, hydrocarbons, phthalates, pigments) and thus increases
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50 698 its lifespan. Obtained chromatograms are also “cleaner” (no contaminants) and easier to identify.
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54 699 6.1 Boron trifluoride (BF₃) 55 56 57

58 700 The Lewis acid boron trifluoride (BF₃) in methanol (12-14%, w/v) is a powerful acidic catalyst for
59
60 701 the transesterification of FA. In general, 800 µL to 1 mL of BF₃-MeOH (12-14%, w/v) is added to a

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2 702 dry lipid extract containing a maximum of 1-4 mg of lipids (Liu, 1994; Carrapiso and García, 2000;
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4 703 Le Grand *et al.*, 2011). This method is fast and efficient (Morrison and Smith, 1964; Carrapiso and
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6 704 García, 2000; Cavonius *et al.*, 2014), and it has been successfully used on marine lipid samples where
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8
9 705 almost all the lipid classes, including FFA and plasmalogens, were transesterified in 10 min at 100 °C
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11 706 (Le Grand *et al.*, 2011; Cavonius *et al.*, 2014). For the sphingolipids, such as sphingomyelin and
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13 707 ceramide aminoethylphosphonate, the transesterification should be prolonged and last for 1.5 h to 5
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15
16 708 h at 100 °C (Eder, 1995; Le Grand *et al.*, 2011).

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18 709 Heating is generally conducted in a dry bath, water bath or heat block. The higher the temperature
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20 710 used, the shorter the reaction time. An increase in reaction time has no negative effect, provided that
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22
23 711 no evaporation occurs (Morrison and Smith, 1964). Presence of residual water in the sample limits
24
25 712 the acid-catalysed esterification by BF₃ (Sattler *et al.*, 1991). It is thus necessary to dry the sample by
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27 713 either nitrogen evaporation or by addition of anhydrous sodium sulphate (Molnar-Perl and Pinter-
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29
30 714 Szakacs, 1986).

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32 715 Because evaporation during the transesterification leads to acid concentration, which degrades FA
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34 716 and especially PUFA (Lough, 1964), it is crucial to prevent vial caps from unscrewing when heating
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36 717 samples (Morrison and Smith, 1964). Poor storage conditions and quality of BF₃ cause PUFA
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38
39 718 degradation (Morrison and Smith, 1964; Christie, 1993). It is recommended to use fresh reagent and
40
41 719 to store it at 4 °C or colder (-20 °C). Despite these issues, the use of BF₃-MeOH for transesterification
42
43 720 was referenced as an official method (AOCS, 1973; IUPAC, 1979) and Ackman (1998) showed that
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45
46 721 issues could be avoided following simple rules. Yet, the potential PUFA degradation induced by using
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48 722 BF₃ as a transesterification catalyst, the difficulty in purchasing guaranteed anhydrous BF₃-MeOH,
49
50 723 its high cost and its high toxicity have led to a decreasing use of this reagent (Liu, 1994; Carrapiso
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52 724 and García, 2000; Cavonius *et al.*, 2014). In samples containing very high levels of storage lipids,
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54
55 725 such as salmon flesh, copepod oil and cod liver, the derivatisation efficiency can be quite low, leading
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57 726 to small but significant differences in some FA proportions (Schlechtriem *et al.*, 2008). For these
58
59
60 727 reasons, BF₃ is no longer recommended as an acidic catalyst in the preparation of FAME.

6.2 Acidic alternatives to BF_3

A number of alternatives to Lewis acids have been employed (Christie, 1982a). Methanolic HCl (3 N) appears to be the most commonly used reagent for transesterification of lipid-bound FA to FAME in lipids from freshwater plankton (von Elert, 2002; Martin-Creuzburg *et al.*, 2012; Windisch and Fink, 2018), but also from marine plankton (e.g., Troedsson *et al.*, 2005). It can easily be produced by adding 24 ml acetyl chloride to 100 ml of methanol, which leads to the formation of 10% (approx. equivalent to 3 N) methanolic HCl. H_2SO_4 is often recommended (Christie, 1982a; Budge *et al.*, 2006), as it can easily be prepared by mixing a small amount of concentrated H_2SO_4 (~98%) in methanol to achieve a 1% solution (Schlechtriem *et al.*, 2008).

The most commonly used acid catalysed reagents, such as HCl or H_2SO_4 , are not associated with the issues described for BF_3 . A number of comparisons of acid catalysts for esterification/transesterification have been made (e.g. Medina *et al.*, 1992; Budge and Parrish, 2003; Thiemann *et al.*, 2004; Carvalho and Malcata, 2005), with no clear consensus on optimal methods. The best advice is likely to select a method and consistently apply it. As with BF_3 , caution is needed during heating to prevent evaporation.

6.3 Basic alternatives

Several basic catalysts are available (e.g., NaOH, KOH) to transesterify acyl lipids. While a range of procedures have been described (e.g. Christie, 1982a; Medina *et al.*, 1992; Velasco *et al.*, 2002), most are very similar to the acid catalysed method described above with the substitution of the catalyst and the introduction of an additional step after the reaction is quenched to neutralise the remaining base. All base-catalysed transesterifications proceed very rapidly at room temperature (Christie, 1982a), thereby preserving labile structures (i.e., epoxides, conjugated double bonds) that are often destroyed with harsher acid catalysts. This 'gentler' transesterification is the primary motivation for use of basic catalysts. As noted above, basic-catalysts are unable to esterify FFA so these approaches should only

1
2 752 be used when one can be confident that FFA are present in very low amounts, or else when the analyst
3
4 753 is targeting esterified FA exclusively (e.g., studies on membrane lipids or storage lipids).

5
6 754 Finally, there are a number of official and approved methods that employ combined approaches,
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8
9 755 using both basic and acidic catalysts (e.g., AOCS, 1989). While at least one of these methods (i.e.,
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11 756 AOAC 969.33-1969, 1997) describes the procedure as first saponification with NaOH, followed by
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13 757 methylation with BF₃ in methanol, the short reaction time with NaOH in methanol is actually an
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16 758 alkali-catalysed transesterification, followed by acid-catalysed methylation of any FFA that remain
17
18 759 (Ackman, 1998). Although direct acid-catalysed transesterification is sufficient for a typical aquatic
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20 760 lipid if performed with sufficient time and heat, the alkali-catalysed step may persist because of its
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23 761 adoption by official bodies (Ackman, 1998).

24 25 26 762 6.4 Direct transesterification

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30 763 The standard procedure for determining FA profiles (e.g. extraction to isolate lipids, followed by
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33 764 transesterification to yield FAME), can be labour-intensive and increases analytical cost. In some
34
35 765 cases, studies are only interested into the FA composition of total lipids in samples and therefore
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37 766 perform a direct one-step transesterification that considerably reduces the analytical time (Lewis *et*
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39
40 767 *al.*, 2000). This method is realised directly on the sample, and differs from the classical
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42 768 transesterification by the elimination of the lipid extraction step (Lewis *et al.*, 2000; Castro-Gómez
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44 769 *et al.*, 2014; Parrish *et al.*, 2015a).

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46 770 Different methods have been tested and provided reproducible and similar results to the classical
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49 771 extraction-transesterification method (Castro-Gómez *et al.*, 2014; Parrish *et al.*, 2015a). Careful
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51 772 consideration is needed when using this approach as it may not be appropriate to the study goal and/or
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54 773 the matrices analysed. It may also be preferentially applied to small and homogenous (representative
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56 774 of the whole) samples. For example, while it is possible to carry out direct analyses on a small amount
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58 775 of tissue, accurate determination of total blubber FA composition in marine mammals was not
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60 776 possible with this approach due to significant variation in FA composition within the layers of the

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2 777 blubber (Thiemann *et al.*, 2008). Although this rapid direct transesterification approach is less time-
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4 778 consuming and cheaper, once the entire sample is transesterified, it is not possible to redo the analysis,
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6 779 or to analyse the FA composition of different lipid fractions.
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10 780 6.5 Alternatives to hexane

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14 781 Hexane, iso-hexane (2-methyl-pentane), or iso-octane are most frequently used to recover FAME
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16 782 after transesterification and before injection. However, hexane is carcinogenic, mutagenic and
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18 783 reprotoxic, and is a hazardous airborne pollutant. Less toxic solvents such as pentane and heptane can
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20 784 be used as alternatives to hexane (Alfonsi *et al.*, 2008). However, recovery of FA from macroalgae
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22 785 is lower with heptane than with hexane, with PUFA tending to be more affected than SFA and MUFA
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24 786 (Le Grand unpub. data). This preliminary result needs to be verified on other matrices. Pentane rather
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26 787 than hexane may also be used as the solvent in the BF_3/MeOH procedure to reduce the reaction
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28 788 temperature from 85-100 °C to 80 °C to decrease artefact risk (Parrish, 1999).
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789 7 Analysis of fatty acids

790 The full process of FAME analysis consists of injection, separation, identification and quantification
791 of FAME and each of these steps has to be optimised to achieve high accuracy and precision (see
792 Eder, 1995; Christie, 2003 for review). Gas chromatography (GC) with flame ionisation detection
793 (FID) is generally the preferred method for the quantification of FAME in aquatic samples. GC-MS
794 methods for FAME quantification can be advantageous on a routine basis when spectrometric
795 confirmation of compound identity is required. This is especially useful for complex samples
796 containing contaminants, artifacts or co-eluting compounds. Although these methods compared
797 satisfactorily with GC-FID, they are more complex and tedious to implement and calibrate. Such
798 approaches also require stronger chemistry / spectrometry backgrounds of the user and greater routine
799 maintenance of the instrumentation.

800 7.1 Injection

801 Sample injection is a critical step to achieve high accuracy in FAME analysis and we encourage
802 readers to refer to seminal papers of Konrad Grob for a thorough overview of available techniques
803 (e.g., Grob, 2007). There are currently three injection techniques. Before being transferred into the
804 capillary column, the sample can be (i) evaporated in a permanently hot vaporising chamber (classical
805 vaporising injection), (ii) injected into a cool chamber which is subsequently heated to vaporise the
806 sample (programmed temperature vaporising injection, referred to as PTV), or (iii) directly injected
807 into the column inlet (direct injection or on-column).

808 Once vaporised, the sample can be split (i.e., split injection) with only a small part of the vapour
809 entering the column while the rest is vented. This is the most appropriate technique for concentrated
810 samples (e.g., Shantha and Ackman, 1990). Alternatively, the whole vaporised sample is transferred
811 from the injector to the column without splitting of the sample (i.e., splitless injection). This technique
812 is particularly appropriate for trace analysis. The major drawback of the split or splitless injection

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2 813 techniques is the possible discrimination between high- and low-boiling compounds in the sample.
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4 814 Fatty acids in aquatic samples have a wide range of boiling points and quantification may require
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6 815 application of relative response factors (Grob, 2007). On-column injection avoids discrimination of
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9 816 FA and therefore provides the best results. Yet, a large number of aquatic scientists use the vaporising
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11 817 injection method for suitability and flexibility reasons. High-speed injection, high injector
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13 818 temperature ($> 220\text{ }^{\circ}\text{C}$) and proper injector inserts all contribute to decreasing discrimination
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16 819 problems. However, we recommend regular checks of FAME discrimination factors using certified
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18 820 reference mixtures (e.g. Supelco 37).
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22 821 7.2 Separation

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26 822 Upon injection, FAME are transferred into a wall-coated open tubular (WCOT) capillary column
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28 823 allowing separation according to their carbon number, the number and location of the double bonds,
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30 824 and the cis-trans configuration. The column is located in an oven and exposed to temperature ramps
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33 825 ranging from $60\text{ }^{\circ}\text{C}$ to $300\text{ }^{\circ}\text{C}$ (e.g. Tang and Row, 2013 for examples of temperature ramping). The
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35 826 carrier gas is generally helium or hydrogen. The resolution capability of the column depends on
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37 827 several factors such as polarity of the stationary phase, column length, internal diameter and film
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40 828 thickness. In view of its importance, this information should be reported in detail in the method
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42 829 description of any study.
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44 830 Based on the type of FA being investigated, one of the most important steps is the selection of the
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46 831 stationary phase of the column which influences elution times of FAME, especially PUFA. The
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49 832 polarity of the column ranges from non-polar to extremely polar. On non-polar columns, the elution
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51 833 time generally increases with increasing boiling points of analytes. Therefore, the elution time of
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53 834 FAME increases with chain length and with decreasing unsaturation (e.g., $18:2n-6$ elutes first,
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56 835 followed by $18:1n-7$, and then by $18:0$). The major drawback with non-polar columns is that some
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58 836 FA isomers (*cis-trans*) or FA pairs may co-elute. Conversely, non-polar columns exhibit low bleed,
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60 837 long lifetime, retention time stability and high temperature resistance (to $360\text{ }^{\circ}\text{C}$), which makes their

1
2 838 use suitable for a large range of compounds. These columns are well-suited for separating neutral
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4 839 compounds such as sterols, alkenones, hydrocarbons. Commonly used non-polar columns are made
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6 840 of 100% dimethylpolysiloxane or 5% phenyl-95% dimethylpolysiloxane.
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9 841 Many researchers use polar columns which allow for a better separation of aquatic FA than non-
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11 842 polar phases, with only some rare and minor co-elutions. Ackman (1986) was a strong advocate of
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13 843 the polyethylene glycol (PEG) phase. Columns coated with PEG are easily recognisable because of
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15 844 their commercial name with the suffix "wax". With this polar phase, FAME continue to elute in order
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17 845 of increasing boiling point, but also roughly in order of increasing numbers of double bonds.
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19 846 However, polar columns are highly sensitive to temperature (max 250 °C), water and oxygen, and
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21 847 are thus less stable over time than non-polar columns. Retention times may also vary (decrease) over
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23 848 time, especially for highly unsaturated compounds. Consequently, peak identification (based on
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25 849 elution time) needs to be checked regularly and rigorously.
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30 850 An alternative phase consists of substituting 50% dimethylpolysiloxane (non-polar) with the polar
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32 851 cyanopropylmethyl (e.g., Iverson *et al.*, 2004; Pedro *et al.*, 2019). This phase provides similar
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34 852 resolution as PEG phases, although it is much less sensitive to oxygen, resulting in longer column
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36 853 lifetimes. The chemistry of this phase is more influenced by temperature than other polar columns.
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38 854 Separation of co-eluting peaks can sometimes easily be achieved simply by subtle adjustments to
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40 855 oven temperature ramps.
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45 856 7.3 Identification

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49 857 The most common way to identify FAME by GC-FID analysis is to compare their retention times
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51 858 with those of commercially-available individual purified standards. Among the most used standards
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53 859 for the FA identification in aquatic samples, there are PUFA No. 3, a complex qualitative standard
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55 860 mixture extracted from Menhaden oil, and Supelco® 37 Component FAME Mix, a comprehensive
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57 861 mixture of 37 FAME ranging from 4:0 to 24:1*n*-9. Several long-chain and (highly) unsaturated
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59 862 FAME occurring in some samples are not commercially available, but can be identified using fully
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2 863 characterised natural products (e.g., the non-methylene interrupted fatty acids typical of marine
3
4 864 molluscs in Zhukova and Svetashev, 1986; Ackman, 1989; Kraffe *et al.*, 2004).

6 865 In addition to these protocols, confirmation of FA identification is best achieved using GC-mass
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9 866 spectrometry (GC-MS). Peak identification is achieved using commercial spectral libraries (e.g. The
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11 867 LipidWeb <<http://lipidhome.co.uk>>), or via personal/laboratory FA mass spectral libraries to assist
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13 868 in identification of often complex environmental FAME samples. Further details on various GC-MS
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15
16 869 protocols together with specific MS information are available in Christie (1989). Briefly, the base
17
18 870 peak at m/z 74 is termed the McLafferty rearrangement ion, and is formed by cleavage of the parent
19
20 871 FAME molecule beta to the carboxyl group. Where chemical ionisation is used, the mass spectra
21
22
23 872 have a more prominent quasi-molecular ion (MH^+), and minor ions only at $[MH - 32]^+$ and $[MH -$
24
25 873 $32 - 18]^+$. Because all FA will have the carboxylic acid (or ester) functionality in common,
26
27 874 knowledge of the molecular mass is particularly valuable in determining basic structure with respect
28
29
30 875 to numbers of carbon atoms and double bonds. There are unfortunately generally no characteristic
31
32 876 ions that indicate the position or geometry (i.e., *cis-trans*) of double bonds in positional isomers of
33
34 877 monounsaturated and polyunsaturated FAME (Christie, 1989).

36 878 Where hydroxyl groups are present on the FAME, conversion of the hydroxyl group to a trimethyl
37
38
39 879 silyl (O-TMSi) ether or other derivative is generally used. Bistrimethylsilyltrifluoroacetamide
40
41 880 (BSTFA) is a useful reagent to accomplish this. The high polarity of the free hydroxyl group is
42
43 881 reduced by formation of the O-TMSi ether, thereby enhancing the chromatography of the derivative.
44
45
46 882 Fragmentation of the OTMSi derivative occurs alpha to the carbon containing the hydroxyl group,
47
48 883 with the main cleavage on the side adjacent to the carboxyl group.

50 884 Derivatisation protocols have been developed to provide more detailed position of double bonds
51
52
53 885 or geometry information. These include, for example, use of pyrrolidine, 3-pyridyl carbinol (picolinyl
54
55 886 ester), dimethyl disulphide (DMDS) and 4,4-dimethyloxazolazine (DMOX) derivatives. An
56
57 887 advantage of the use of the DMOX protocol is that the FAME and their corresponding DMOX
58
59 888 derivatives have the same elution order when run on the same GC column. PUFA DMOX derivatives

1
2 889 can be readily formed using the protocols described in Svetashev, 2011 and Lee Chang *et al.*, 2016.
3
4 890 Interesting examples of application of the DMOX protocol have been the characterisation of a novel
5
6 891 series of C₁₈ to C₂₂ *trans* n-3 PUFA from a marine haptophyte (Lee Chang *et al.*, 2016) and the 22:4n-
8
9 892 9t in scallop (Marty *et al.*, 1999). MUFA double bond position and geometry is readily determined
10
11 893 by capillary GC-MS of their DMDS derivatives (Nichols *et al.*, 1986). An example of application of
12
13 894 the DMDS protocol has been the characterisation of a range of novel C₁₆ and C₁₈ MUFA, including
15
16 895 both *cis* and *trans* isomers, from microbial monocultures and complex soil consortia (Nichols *et al.*,
17
18 896 1986). Other derivatisation procedures are also available, although they are not covered here, and are
19
20 897 as detailed in Christie (1989).

24 898 7.4 Quantification

28 899 In GC-FID analyses, the response intensity is based on the number of oxidisable carbon atoms in the
29
30 900 analyte; equivalent masses of FA having different carbon chain lengths and/or numbers of double
32
33 901 bonds will not produce the same response in the FID (Ackman, 1972). FAME are usually quantified
34
35 902 by peak areas, and absolute concentrations are determined by adding an internal standard.
36
37 903 Discrimination of FAME may be associated with injection technique and it is often necessary to
39
40 904 introduce empirical response factors, determined through careful use of commercially available
41
42 905 authentic mixed standards (see section 7.1). A way to estimate the importance of bias from different
43
44 906 origins (such as injector, or detector) is for example to analyse a FA mixture of known theoretical
46
47 907 mass composition such as the Supelco® 37 Component FAME Mix. It is also possible to analyse a
48
49 908 phospholipid with two different aliphatic chains, of different length and saturation (e.g.,
50
51 909 phosphatidylcholine 16:0/22:6n-3) and confirm that the molar percentage of each FA is 50/50.

53
54 910 The internal standard must not coelute with the FAME, its chemical structure must be close to the
55
56 911 majority of the FAME and it must be absent from the sample. A known mass of internal standard
57
58 912 should be added either after SPE or before transesterification to account for derivatisation yield and
59
60 913 recovery of FAME (see section 5). The most widely used standards are the carboxylic form of

1
2 914 nonadecanoic acid (19:0), heneicosanoic (21:0) and tricosanoic acid (23:0). Data are generally
3
4 915 expressed as relative (area %, mass % or mole %) or absolute contents (e.g., mg FA g⁻¹ sample dry
5
6 916 mass).

8
9 917 With GC-MS analyses, one approach is to use the total ion chromatogram (TIC) in a similar
10
11 918 fashion as peak area is used with GC-FID, by comparing the peak area of an internal standard with
12
13 919 peak areas of compounds of interest, and relative response factors determined individually using
14
15 920 authentic standards. Note here that response factors vary with structure (carbon and unsaturation
16
17 921 numbers, mostly notably), ionisation methods (electronic vs chemical) and mass detector (e.g.
18
19 922 Quadrupole vs ion trap) (Dodds *et al.*, 2005; Quehenberger *et al.*, 2011). Consequently, the range of
20
21 923 response factors is greater in MS than with FID. Response factors for FA, where standards are not
22
23 924 available, are assumed the same as those of structurally similar and available compounds but this is
24
25 925 arguable.

26
27 926 For greater sensitivity, calibration can be based on prominent ion(s) using selected ion
28
29 927 monitoring (SIM) as a single ion can be scanned many more times in a cycle than the full mass
30
31 928 spectrum, giving a higher signal to noise ratio (Dodds *et al.*, 2005; Thurnhofer and Vetter, 2005;
32
33 929 Quehenberger *et al.*, 2011). It involves the exclusive acquisition of a group of selected ions during a
34
35 930 given period if the identity and retention time of a given analyte have been established through full
36
37 931 scan mode. Since fragmentation can vary depending on chain length and degree of unsaturation,
38
39 932 careful assignments of selected ions group to analytes and appropriate adjustments of SIM parameters
40
41 933 are necessary to achieve accurate quantification and complete discrimination when some FA are co-
42
43 934 eluting.

44
45 935 Quantification of FA can be further improved by using mixtures of isotopically-labelled FA
46
47 936 internal standards (e.g. deuterated FA). These offer the advantage of being identical to the analyte of
48
49 937 interest in all aspects except mass, so that response, as well as matrix effects, of the labelled and
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51 938 unlabelled component are the same (Quehenberger *et al.*, 2011). The main limitation to this approach
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939 is the commercial availability of isotope-labelled fatty acids that could “match” all fatty acids present
940 in aquatic samples.

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8 Data treatment

As emphasised by the previous sections, obtaining reliable FA data can be a long and complex process. The purpose of this section is therefore to offer guidance in how to valorise these hard-earned data in the best possible way, and to use them to shape robust answers to a wide range of scientific questions. As environmental phenomena are inherently complex, a single variable rarely depicts them in a satisfactory way (Buttigieg and Ramette, 2014). Moreover, FA studies typically deal with datasets containing many variables (i.e., individual FA). This section will therefore mostly focus on multivariate analysis. Readers looking for guidance about univariate tests and procedures are encouraged to refer to other publications (e.g. McDonald, 2014).

After adequate standardisation and/or transformation (section 8.1), FA data can be analysed in different, non-mutually exclusive ways. Unconstrained ordination methods (section 8.2) can help visualising the data, and finding out which samples group together according to their similarity in FA composition. In doing so, they allow determining how similar to one another entities such as sampling locations (Hughes *et al.*, 2005; Parrish *et al.*, 2015b), habitats (Hixson *et al.*, 2015) or species (Sardenne *et al.*, 2016) are. These exploratory methods are adequate to identify patterns between samples, as well as FA markers responsible for these patterns. Conversely, investigators can be interested in assessing the influence of one or several previously identified factors on FA composition, e.g. to explicitly test whether FA composition is different across multiple experimental treatments, ecological conditions, or species. This can be done through hypothesis testing and/or constrained ordination (section 8.3).

Regardless of the data analysis approach, FA studies commonly rely on "biomarker compounds", i.e., FA (or combinations of FA) whose origin can be assigned to a biogeochemical process, an ecosystem compartment and/or an organism group. When doing so, it is important to remember that there are no ubiquitous FA biomarkers, and that their applicability will depend on the studied system or organism (e.g., Feiner *et al.*, 2018). To avoid interpretation biases, biomarker compounds should

1
2 966 be chosen carefully with respect to the ecological context and scientific question of interest. When
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4 967 used sensibly, biomarker compounds can be very useful, as proven by their routine use in ecological
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6 968 literature (see Dalsgaard *et al.*, 2003; Kelly and Scheibling, 2012 for review).
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10 969 8.1 Standardisation and transformation

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14 970 Choosing standardisation or a prior transformation method for FA data needs to be based on the
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16
17 971 research questions, as it can have important impacts on the final results and interpretations (Mocking
18
19 972 *et al.*, 2012). FA can be expressed as either an absolute content or mass fraction, such as $\text{mg}\cdot\text{g}^{-1}$ of
20
21 973 lipids or of tissue, or as relative proportion (%) of total FA mass or molar concentration.
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23
24 974 Concentrations are commonly used in nutrition studies, and can be useful in controlled feeding
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26 975 experiments (Elsdon, 2010). Proportions of total FA mass is the commonly used standardisation
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28 976 method in trophic studies. It avoids occultation of compositional differences in FA profiles by
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31 977 removing the influence of non-FA components from both the lipid fraction (e.g., polar vs neutral
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33 978 lipids) and the total lipid contents of tissues. Yet, proportion data are generally calculated in relation
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35 979 to identified FA in a sample, which vary across studies and FA analysis methodologies, limiting inter-
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37
38 980 study comparison.

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40 981 Choice of data standardisation is interdependent with the choice of data transformation method (if
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42 982 needed) and of applied statistical test (univariate vs. multivariate tests). FA data transformation can
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44 983 be recommended in some cases (e.g., Kelly and Scheibling, 2012), but the overall effect of the
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46
47 984 procedure is highly variable (Happel *et al.*, 2017). Deciding on a transformation method depends on
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49 985 the test used and its assumptions (e.g., normal distribution of data, homoscedasticity). Although these
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51 986 assumptions can be met for univariate analyses (that test the effect of one or several factors on
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53
54 987 individual FA), this is rarely the case for multivariate analyses using large FA datasets, even after
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56 988 transformation (Budge *et al.*, 2006). However, multivariate methods such as unconstrained
57
58 989 ordinations (e.g., principal component analysis, see below) give useful and adequate results when the
59
60 990 data are properly transformed (e.g., Legendre and Gallagher, 2001). Moreover, proper testing

1
2 991 procedures through permutations allows one to circumvent the violation of the multivariate normality
3
4 992 assumption in constrained ordination methods such as redundancy analysis (RDA, Legendre and
5
6 993 Legendre, 2012; see subsection 8.3).

8
9 994 Kelly and Scheibling (2012) reviewed some of the most common data transformation methods
10
11 995 used in FA studies, including log-ratio (Budge *et al.*, 2006), arcsine and logit transformations. Arcsine
12
13 996 transformation has been widely applied to transform proportional FA data, yet its use in biological
14
15 997 analysis has been challenged in some studies. Warton and Hui (2011) suggested that applying logistic
16
17 998 regression is more appropriate to transform proportion data. It is also important to note that some
18
19 999 transformations such as $\log(x)$ or $\log(x+1)$ will give more weight to rare (i.e., found in small amounts)
20
21 1000 FA, while reducing the influence of the abundant FA (Happel *et al.*, 2017). Depending on the study
22
23 1001 objectives, it is essential to identify clearly whether the scientific question requires emphasising the
24
25 1002 presence and the role of rare FA, or if focusing on the dominant FA will give a more global pattern
26
27 1003 within and across organisms.
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31 32 33 1004 8.2 Exploring fatty acid data through ordination 34 35 36

37 1005 Ordination methods are essential to describe and visualise sample dispersion in a complex
38
39 1006 multidimensional dataset, and to identify groups of co-varying variables. Those are computed based
40
41 1007 on a matrix of resemblances (distance, dissimilarities, similarities), or derived ranks, rather than raw
42
43 1008 (or transformed) data. In FA literature, the most commonly used resemblance measure between
44
45 1009 samples are either the Euclidean distance or Percentage difference (also called Bray-Curtis
46
47 1010 dissimilarity; Legendre and Legendre, 2012). Euclidean distance is generally preferred over Bray-
48
49 1011 Curtis for FA analysis (Happel *et al.*, 2017). However, just like data transformations, the choice of
50
51 1012 the resemblance measure can have important impacts on the final results. The choice of matrix will
52
53 1013 depend on whether a dataset includes null/absent values and if they need to be included in the analysis
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55 1014 according to the study question. The Percentage difference coefficient is double-zero asymmetric, i.e.
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57 1015 the absence of one FA from two individuals is not considered as contributing to their similarity. The
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1
2 1016 Euclidean distance, on the contrary, is double-zero symmetric, i.e. will be affected by double null
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4 1017 values (Legendre and Legendre, 2012). Other properties of resemblance measures should also be
5
6 1018 considered. Choice of the resemblance coefficient has received extensive attention in multivariate
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8
9 1019 community ecology (e.g., Legendre and Legendre, 2012).

10
11 1020 Commonly used methods to plot ordinations include principal components analysis (PCA),
12
13 1021 principal coordinates analysis (PCoA) and non-metric multidimensional scaling (nMDS). PCA can
14
15
16 1022 only use Euclidean distance, while PCoA and nMDS can use any resemblance measure. PCoA and
17
18 1023 nMDS use the ranks of similarities or dissimilarities matrix to find a spatial arrangement of
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20 1024 individuals and represent relationships among them (Kelly and Scheibling, 2012). NMDS is
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22
23 1025 conceptually simple, generally applicable, and well adapted to ecological data (Clarke and Warwick,
24
25 1026 2001). However, it is crucial to take into account the stress coefficient, which is measured from the
26
27 1027 relationship between ranks of dissimilarities and ranks of sample distances on the plot. A high stress
28
29
30 1028 score (> 0.05) indicates that data have been highly distorted to fit in the visualisation, which may not
31
32 1029 be representative and should be considered with caution (Clarke and Warwick, 2001; Buttigieg and
33
34 1030 Ramette, 2014). PCA also describes relationships among samples, reducing large numbers of
35
36 1031 variables to a few components that represent most of the variance and that are built by combining
37
38
39 1032 correlated variables (Budge *et al.*, 2006; Kelly and Scheibling, 2012). It also helps to visualise
40
41 1033 variables that contribute the most to the dissimilarity among observed groups and FA that are highly
42
43 1034 correlated (Budge *et al.*, 2006). After ordination, several procedures (similarity percentage, also
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45
46 1035 called SIMPER, analysis; comparison of PCA loadings) can be applied to identify FA driving
47
48 1036 resemblance or dissimilarity among groups (Clarke and Warwick, 2001).

51 52 1037 8.3 Multivariate hypothesis tests and modelling

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55
56 1038 Multivariate analyses are commonly applied in ecological studies to discriminate the influence of
57
58 1039 different factors on FA composition, such as physiological, spatial or even temporal variations
59
60 1040 (Anderson, 2001). Statistical comparison of groups based on FA composition is generally performed

1
2 1041 through non-parametric or randomisation procedures, as FA datasets rarely match the prerequisites
3
4 1042 of parametric statistical tests (Anderson and Walsh, 2013). Analysis of similarity (ANOSIM; Clarke,
5
6 1043 1993) or permutational analysis of variance (PERMANOVA; Anderson, 2001) are among the most
8
9 1044 commonly used statistical tests (e.g. Hughes *et al.*, 2005; Hixson *et al.*, 2015; Parrish *et al.*, 2015;
10
11 1045 Sardenne *et al.*, 2016; Mathieu-Resuge *et al.*, 2019). ANOSIM uses ranks of pairwise dissimilarities
12
13 1046 and generates its own statistic by randomisation of rank dissimilarities (Clarke and Warwick, 2001).
14
15
16 1047 PERMANOVA tests for differences among group centroids (Anderson, 2001). Both methods are
17
18 1048 affected by dispersion heterogeneity, with ANOSIM being more sensitive than PERMANOVA
19
20 1049 (Anderson and Walsh, 2013). PERMANOVA is unaffected by differences in correlation structure
21
22
23 1050 and seems more powerful to detect changes in FA composition. As such, applying ANOSIM is
24
25 1051 appropriate for a 1-way test (e.g. to compare one species submitted to different diets), but not for
26
27 1052 more complex study designs (Kelly and Scheibling, 2012).

29
30 1053 Besides hypothesis testing, constrained ordination (e.g., redundancy analysis, RDA) offers a way
31
32 1054 to extract and summarise variability from a multivariate dataset (FA data) that can be explained by
33
34 1055 one or a set of explanatory variables (e.g., locations, species, diets, etc.). In other words, it allows
35
36 1056 regression of multiple response variables (FA) on multiple explanatory variables (chosen according
37
38
39 1057 to the scientific question). More details on this technique can be found elsewhere (Legendre and
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41 1058 Legendre, 2012).

45 1059 8.4 Coupling fatty acids with other ecological markers

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49 1060 When tackling elaborate ecological questions, combining FA with other tracers has proven to be very
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51 1061 powerful. Tracers commonly combined with FA include stable isotope ratios of light biogenic
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53
54 1062 elements (Kharlamenko *et al.*, 1995, 2001; Nyssen *et al.*, 2005), as well as contaminants (Kainz and
55
56 1063 Fisk, 2009; Le Croizier *et al.*, 2016). In many cases, those combinations are qualitative. However,
57
58 1064 modern data analysis frameworks allow quantitative coupling of stable isotope and fatty acids in
59
60 1065 mixing models (Stock *et al.*, 2018) or ecological niche models (Jackson *et al.*, 2011; Sardenne *et al.*,

1
2 1066 2016). Such developments hold great promise for ecological research, due to the complementarity of
3
4 1067 these two methods. More generally, multiple factor analysis (Pagès, 2014) or co-inertia analysis (Dray
5
6 1068 *et al.*, 2003) could provide flexible, widely applicable and quantitative ways to couple data from
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8
9 1069 different methods by allowing exploration of the links between multiple multivariate datasets.
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Conclusion

The use of FA analysis in trophic ecology, aquatic nutrition and aquaculture research and development holds considerable promise to improve our understanding of the natural world from organism taxonomy and physiology to ecosystem functioning. However, the application of this approach needs to be carefully considered when initiating a new study to ensure that interpretations proposed for FA data are relevant and appropriate. Although protocols for lipid analysis are well-described, their application to aquatic sciences often requires modifications to adapt to field conditions and to sample types. Here, we presented the current state of knowledge on methods dedicated to aquatic lipid analyses, from sampling to data treatment. We reviewed sample preservation, storage and transport protocols and their effects on lipids, lipid extraction methods, separation of polar and neutral lipids, transesterification and quantification techniques, and available tools for treatment and statistical analysis of FA data. A simplified flowchart of all these steps is represented in Figure 1.

Lipids can be degraded by hydrolysis or oxidation at each stage of the lipid analysis process. We thus encourage processing samples at low temperature under nitrogen atmosphere, and reporting hydrolysis indicators. To limit possible deleterious effects, samples should be handled at the lowest suitable temperature before direct extraction, or be immediately frozen for storage, ideally at $-20\text{ }^{\circ}\text{C}$ for less than one month, $-80\text{ }^{\circ}\text{C}$ for less than 6 months or under liquid nitrogen with no time limit. Long-distance transport of samples must be planned well ahead of the start of the study to obtain relevant import/export permits and to choose appropriate transportation to maintain sample viability and respect transport regulations and national laws. When possible, we recommend transporting samples freshly frozen under a nitrogen atmosphere. Depending on the objectives of the study, samples can be either directly transesterified or undergo extraction to isolate lipids. Lipid extraction is usually performed following well-established methods using either chloroform or dichloromethane (less toxic), mixed with methanol. We strongly encourage scientists to describe in their publication any modifications from original extraction methods.

1
2 1097 The lipid extract is either separated into classes or directly transesterified. Separation of polar and
3
4 1098 neutral lipids prior to transesterification allows for a better interpretation of FA profiles when seeking
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6 1099 information on nutritional status and/or physiology of organisms. Special attention should be paid to
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8
9 1100 control for the purity and recovery of each fraction. Non-volatile FA are then transesterified into their
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11 1101 volatile derivatives, usually FAME, before being analysed by GC. Acid catalysed reagents, such as
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13 1102 HCl or H₂SO₄ are the most commonly used in aquatic science as they present fewer analytical issues
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15
16 1103 than BF₃. Hexane is the most widely used solvent to recover FAME after transesterification. Less
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18 1104 toxic alternatives are emerging, but are not applicable to all sample types. The analysis and
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20 1105 quantification of FAME is usually conducted by GC-FID. FAME are identified by comparing their
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23 1106 retention times with those of individual purified standards. They are quantified as relative proportions
24
25 1107 using peak areas or by absolute concentration when an internal standard was added before
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27 1108 transesterification. When necessary, peak identification can be confirmed using GC-MS, but this
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29
30 1109 process may require additional derivatisation steps. Data treatment and statistical analysis of FA
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32 1110 should be carefully applied according to the investigated scientific question. We presented common
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34 1111 multivariate statistical procedures that we consider widely relevant for FA-related problems. This list
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36 1112 is by no means exhaustive. We encourage analysts to explore their data in multiple ways. A good
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39 1113 starting point is usually a simple preliminary visualisation of data. This will not only help to check
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41 1114 for data quality, but also to make informed choices about subsequent procedures. When interpreting
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43 1115 data and statistical results, it is crucial to take into account the studied organisms and/or ecosystems
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45
46 1116 and their specificities.

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48 1117 We argue that methodological consistency, controlled experimentation, and interlaboratory
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50 1118 comparison and calibration can lead to enhanced progress in FA studies and are particularly important
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52 1119 for comparative studies. Standardisation and optimisation of FA methodologies will ultimately
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54
55 1120 improve our understanding on the role of lipids in biological mechanisms, and will enable us to use
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57 1121 them as ecological or physiological tracers as efficiently as possible.
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1
2 1122 **Figure caption**
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5 1123 **Figure 1.** Workflow for the analysis of fatty acids in aquatic samples. The star symbol indicates
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7 recommended methods. Dashed arrows point to suggested storage precautions after each analytical
8 1124 step. Transport requirements are color-coded, and the coldest transport temperature is always the best
9
10 1125 option regardless of the step. The grey syringe symbol represents the step at which the internal
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12 1126 standard for recovery and purity should be added. The black syringe symbol represents the step at
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14 1127 which the internal standard for quantification should be added.
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1
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37 1143 **Authors’ contribution:**

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39
40 1144 LC; LM; FP; PS equally contributed to the conception of the paper and its Figure, and led the writing
41
42 1145 of the paper. All other authors contributed to writing and editing the manuscript and approve the final
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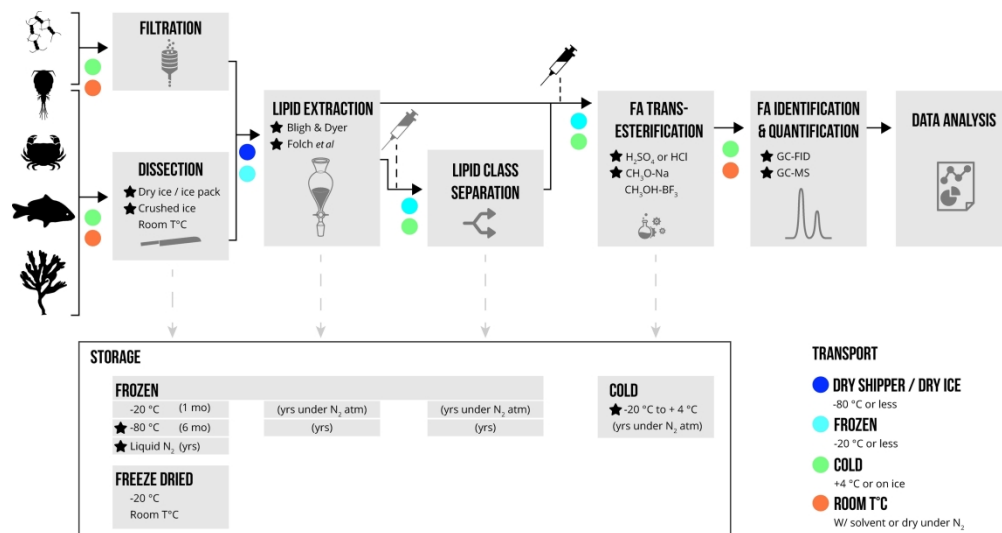


Figure 1. Workflow for the analysis of fatty acids in aquatic samples. The star symbol indicates recommended methods. Dashed arrows point to suggested storage precautions after each analytical step. Transport requirements are color-coded, and the coldest transport temperature is always the best option regardless of the step. The grey syringe symbol represents the step at which the internal standard for recovery and purity should be added. The black syringe symbol represents the step at which the internal standard for quantification should be added.

301x158mm (300 x 300 DPI)

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2 1 **State of art and best practices for fatty acid analysis in aquatic sciences FATTY**
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5 2 **ACID ANALYSES IN AQUATIC TROPHIC ECOLOGY, AQUACULTURE**
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7 3 **AND NUTRITION: STATE OF THE ART AND BEST PRACTICES**
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1
2 36 **Abstract**
3

4 37 Determining the lipid content and fatty acid composition of aquatic organisms has been of major
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6 38 interest in trophic ecology, aquaculture and nutrition for over half a century. Although protocols for
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8 39 lipid analysis are well-described, their application to aquatic sciences often requires modifications to
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10 40 adapt to field conditions and to sample type. Here, we present the current state of knowledge of
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12 41 methods dedicated to both marine and freshwater lipid analyses, from sampling to data treatment. We
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14 42 review: (i) sample preservation, storage and transport protocols and their effects on lipids, (ii) lipid
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16 43 extraction, separation of polar and neutral lipids, derivatisation and detection methods, and (iii)
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18 44 available tools for statistical analysis of fatty acid data. We provide recommendations for best-
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20 45 practices in field situations and advocate for protocol standardisation and inter-laboratory calibration.
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25 46 **Keywords:** Marine and freshwater lipids, fatty acids, methods, recommended practices,
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27 47 biomarkers, aquatic nutrition
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49 **Abbreviations:**

4-HHE	4-Hydroxy-2-hexenal	HPLC-	High performance liquid
4-HNE	4-Hydroxy-2-nonenal	ELSD	chromatography-
ALC	Fatty alcohols		Electrospray light scattering
AV	Acid value		detector
BCl ₃	Boron trichloride	HPTLC	High performance thin layer
BF ₃	Boron trifluoride		chromatography
BHT	Butylated hydroxytoluene	LC	Liquid chromatography
BSTFA	Bistrimethylsilyl	LC-MS/MS	Liquid chromatography-mass
	trifluoroacetamide		spectrometry/mass
CH ₂ Cl ₂	Dichloromethane		spectrometry
CHCl ₃	Chloroform	LI	Lipolysis index
	(Trichloromethane)	MAG	Monoacylglycerols
CI	Chemical ionisation	MeOH	Methanol
CITES	Convention on International	MDA	Malondialdehyde
	Trade in Endangered Species	MUFA	Monounsaturated fatty acid
COP	Cholesterol oxidation	NCI	Negative chemical ionisation
	products	O-TMSi	O-Trimethylsilyl
DAG	Diacylglycerol	pAV	Para-Anisidine value
DHA	Docosahexaenoic acid	PEG	Polyethyleneglycol
	(22:6n-3)	PFE	Pressurised fluid extraction
DMA	Dimethyl acetal	PL	Phospholipids
DMDS	Dimethyl disulphide	PUFA	Polyunsaturated fatty acid
DMOX	4,4-Dimethyloxazolazine	PTV	Programmed temperature
FA	Fatty acid		vaporising injection
FAME	Fatty acid methyl ester	PV	Peroxide value
FATM	Fatty acid trophic markers	SCOT	Support coated open tubular
FFA	Free fatty acids	SE	Steryl ester
FID	Flame ionisation detector	SFA	Saturated fatty acid
FTIR	Fourier transform infra-red	<u>SIM</u>	<u>Selected ion monitoring</u>
GC-FID	Gas chromatography-flame	SPE	Solid phase extraction
	ionisation detection	TAG	Triacylglycerol
GC-MS	Gas chromatography-mass	TBARS	Thiobarbituric acid reactive
	spectrometry		substances
GF/C GF/F	Glass microfiber filters	<u>TIC</u>	<u>Total ion chromatogram</u>
H ₂ SO ₄	Sulfuric acid	TLC	Thin layer chromatography
HCl	Hydrochloric acid	WCOT	Wall coated open tubular
HI	Hydrolysis index	WE	Wax ester
HPLC	High performance liquid		
	chromatography		

51

52 Introduction

53 Lipids and their ~~derived~~ fatty acids (FA) are ubiquitous components of all living organisms. They are
54 essential compounds for the formation of cell and tissue membranes, act as major sources of
55 metabolic energy, provide thermal insulation and serve as chemical messengers. ~~Fatty acids~~
56 generally consist of a linear hydrocarbon chain with a carboxyl group (-COOH) at its end. They are
57 characterised by the length of their carbon chain, generally from 14 to 24 (up to 36) atoms, as well as
58 by the position and the number of double bonds (usually from 0 to 6) that determine their degree of
59 unsaturation. Fatty acids ~~FA~~ are constituents of lipids such as wax and sterol esters (WE, SE),
60 triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), glycolipids and
61 phospholipids (PL), which show different levels of regulation from ingestion to incorporation. One
62 or several FA are linked either with an ester (or ether / vinyl-ether) bond to an alcohol, mainly
63 glycerol, or with an amide linkage to a long-chain nitrogen base, mainly sphingosine. All of these
64 molecules are categorised as either neutral lipids or polar lipids, with reference to chromatographic
65 methods of lipid class separation but also to their metabolic roles (Christie, 1984). Among neutral
66 lipids, FA-containing compounds include TAG and WE, which are the main storage lipids, whereas
67 DAG, free FA (FFA), and MAG are generally minor constituents. Sterols (both in their free and
68 esterified form) and fatty alcohols (ALC) are also found in neutral lipids. Free FA are rarely present
69 in living organisms, as they may be toxic in high concentrations (Jüttner, 2001)-. Polar lipids include
70 PL, glycolipids, and sphingolipids. Polar lipids are characterised by a hydrophilic polar head group
71 which, in contrast to their hydrophobic aliphatic chains, allows for the formation of lipid bilayers
72 found in cell membranes. Polar lipid properties are determined by the nature of the polar head groups,
73 the nature of the linkage between the polar head group and aliphatic chain, the length of the carbon
74 chain, as well as the position and the number of double bonds within the FA. These different
75 properties influence the functioning of membrane receptors, the fluidity of membranes, and the
76 stability of the bilayer structure (Hazel and Williams, 1990).

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2 77 Fatty acids have been analysed in aquatic sciences for over 50 years (Ackman *et al.*, 1968) with
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4 78 the first evidence that FA composition of consumers resembles that of its food (Lee *et al.*, 1971).
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6 79 Over the next 30 years, the concept of FA trophic markers (FATM) gained in popularity in aquatic
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9 80 trophic ecology (Dalsgaard *et al.*, 2003). ~~The According to this concept of FATM, is based on the~~
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11 81 ~~fact that the~~ FA composition of some primary producers is characterised by specific FA compounds
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13 82 that can be transferred to upper trophic levels (e.g. (Kelly and Scheibling, 2012). The FA composition
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15 83 of a consumer is therefore expected to reflect the FA composition of its food sources ('you are what
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17 84 you eat' principle). FA have accordingly been used to study energy flow in food webs in freshwater,
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19 85 estuarine, coastal and deep-sea, environments in all latitudes, and to examine functional responses to
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21 86 stressors (Kelly and Scheibling, 2012). More recently, the FATM concept has culminated in
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23 87 quantitative FA-based diet modelling (Iverson *et al.*, 2004; Galloway *et al.*, 2014; Bromaghin *et al.*,
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25 88 2017), and recent methodological developments allow for accurate estimation of prey contribution to
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27 89 consumer diet (Litmanen *et al.*, 2020). However, diet modeling is based on the fact that aquatic
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29 90 consumers generally lack the ability to synthesise long-chain polyunsaturated FA (LC-PUFA, $\geq C_{20}$),
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31 91 and have limited capacity for bioconversion of short-chain PUFA into long-chain PUFA (Castell *et*
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33 92 *al.*, 1972; Langdon and Waldock, 1981; Sargent *et al.*, 1999; Taipale *et al.*, 2011). This view has
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35 93 recently been challenged (Kabeya *et al.*, 2018).
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41 94 Diet is not the only determinant of the FA composition of aquatic consumers. FA composition
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43 95 varies with intrinsic factors such as phylogeny and developmental/reproductive stages (e.g. Galloway
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45 96 and Winder, 2015), and extrinsic factors such as temperature, salinity and hydrostatic pressure (e.g.
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47 97 Wodtke, 1981; Cossins and Macdonald, 1989; Hazel and Williams, 1990), thus providing information
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49 98 about physiological condition and habitat type of aquatic organisms (e.g. Meyer *et al.*, 2019).
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52 99 The analysis of the FA composition of aquatic organisms is based on procedures originally
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54 100 developed for applications in food science and medicine. Briefly, once a sample is collected, it is
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56 101 immediately stored in the dark at the lowest possible temperature for the shortest period of time with
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58 102 added antioxidants, and under a nitrogen atmosphere or vacuum. This is to avoid alteration caused by
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2 103 enzymes, heat, oxygen and light. Lipids are then extracted, and lipid classes may be separated using
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4 104 chromatography. Next, derivatisation of FA is performed (methylation in most cases) and FA
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6 105 composition is determined by gas chromatography. Finally, data are treated to obtain the quantitative
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9 106 and qualitative FA profiles of the sample (Figure 1).

11 107 There are likely to be as many variations of procedures as there are laboratories (Parrish, 1999).
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13 108 Yet, not all may be applicable to aquatic samples. Procedures involved in sample collection, handling
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16 109 and storage, as well as extraction and transesterification steps, can cause biochemical modifications
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18 110 of FA and differential loss, leading to false or misleading interpretations. Identifying the most
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20 111 appropriate protocols and the best-practices in FA analysis of aquatic organisms may prove to be a
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23 112 difficult task for novices, as available literature is widespread and highly diverse, and many studies
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25 113 do not fully describe their methodology (e.g., “modified Folch extraction”).

27 114 Our objective is to provide an up-to-date review of the procedures used for a large number and
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30 115 variety of aquatic samples and laboratories around the world. This review emerged from technical
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32 116 workshops of the conference “*Lipids in the oceans*” held in Brest (France) in November 2018. We
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34 117 answer practical questions that are not necessarily formulated in papers and textbooks, but essential
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36 118 to obtain a reliable FA profile. For example, which organ/tissue of particular specimens should be
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39 119 analysed to be representative in the context of trophic ecology? How are samples properly collected,
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41 120 transported or shipped? What are the most appropriate extraction/transesterification methods or
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43 121 solvent systems? How can one ensure reliable FA identification and quantification? How are FA data
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46 122 analysed? This paper aims to guide both novice and advanced scientists in identifying the most
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48 123 appropriate protocols and best-practices for FA analysis of aquatic samples. We hope that this
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50 124 comparative synthesis will stimulate further international collaborations and facilitate standardisation
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53 125 of lipid analyses.

1 Sample handling and storage before lipid extraction

The choice of biological material, its collection and handling techniques, as well as storage methods, are among the most important steps in lipid analysis (Fig. 1). Lipid degradation can occur by hydrolysis (release of FFA from acyl lipids through the enzymatic activity of lipases) or oxidation of carbon-carbon double bonds. In view of the labile nature of lipids, these processes often occur very rapidly, leading to analytical errors and increased sample variability (Christie, 1984). Handling and storage conditions and methods can help minimise sample degradation and analytical bias. Ideally, lipids should be extracted immediately after collection. The second-best option is to **deepflash**-freeze samples in liquid nitrogen immediately after collection. As sampling of aquatic organisms is often performed in the field and in remote areas, optimal conditions can rarely be achieved, but samples need to be stored adequately. A broad set of procedures exists, including: (i) sample maintenance on ice, (ii) frozen sample storage from $-18\text{ }^{\circ}\text{C}$ (standard freezer), to $-80\text{ }^{\circ}\text{C}$ (laboratory freezers) and/or to $-196\text{ }^{\circ}\text{C}$ (liquid nitrogen), (iii) freeze-drying of samples, or (iv) sample storage in organic solvents for varying storage periods, from a few hours to years prior to analysis. Adequate storage conditions will prevent (or at least limit) the degradation of lipids through hydrolysis and oxidation processes induced by oxygen, temperature, light and/or enzymatic processes (Rudy *et al.*, 2016). The rate of oxidation and hydrolysis processes (hereafter referred to as “lipid degradation”) is likely to vary greatly across species and tissue types. These processes can be revealed by an increase in FFA and a decrease in glycerolipids and polyunsaturated FA (PUFA) in samples (Christie, 1984, see **section 4**). Here, we provide an overview of different methods used in the literature and discuss their strengths and limitations.

1.1 Sample type/ sampling strategy

Aquatic samples are highly diverse, ranging from bacteria to whales, and sampling procedures need to be adapted to the nature of the studied organisms/functional groups. Samples of seston and

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2 150 phytoplankton are generally collected through water filtration on pre-combusted glass microfiber
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4 151 filters (GF/C or GF/F), while zooplankton can be sorted manually. Such small organisms or functional
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6 152 groups are often directly extracted as whole organisms and stored in solvent. For other organisms
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9 153 such as larger invertebrates, fish, and aquatic mammals, tissue dissection or biopsy is usually
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11 154 required. In the case of multi-purpose samples, it is possible to grind them under liquid nitrogen and
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13 155 subsample the powder (Soudant *et al.*, 1996). In the case of a quantitative study where the mass of
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15 lipid or FA is reported per gram of sample, the sample will have to be weighed before or after storage.
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17 Sample mass can be measured from fresh or dry tissue. In the latter case, the dry mass may be
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19 measured on a dedicated subsample. All material should be lipid-cleaned prior any sampling to avoid
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21 cross contamination (see section 3.1). Because it is not possible to describe all sampling strategies
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23 159 here, we highly recommend that any study involving lipid and FA analysis must start with a literature
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25 160 review of available information on targeted organisms or on species that are phylogenetically
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27 161 comparable.
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33 163 1.2 Sample handling

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37 164 Maintaining samples on ice is often the first and only practical option available to limit lipid
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39 degradation between collection and further processing. Whenever possible, long-lasting dry ice is
40 165 preferable to water ice. Ideally, samples should be immediately flash-frozen in liquid N₂ in the field
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42 156 and transported to a freezer using a dry shipper. Crushed ice is useful for transportation between
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44 157 locations such as sample transport from the ocean (e.g., small and large vessels) to land, or transport
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46 168 between laboratories. Crushed ice should preferably be used only for a few hours, as this method only
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48 159 slows, but does not stop, lipid and FA degradation (Wood and Hintz, 1971; Chaijan *et al.*, 2006), and
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50 170 samples should therefore be frozen as soon as possible. As an alternative, deep-frozen, long-lasting
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52 171 ice packs are a convenient option for short-term storage in the field (e.g., TechniIce™). These ice
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54 172 packs maintain temperatures around 0 °C for a few hours in a well-insulated container (L. Couturier
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56 173 pers. obs.). In the particular case of studies on thermal acclimation or adaptation, it may be preferable
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2 175 to maintain samples at environment temperature rather than to cool them before freezing as it may
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4 176 induce a short-term cold-response. Freezing / thawing samples more than once should be avoided.
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8 177 1.3 Storage 9

12 178 1.3.1 Frozen storage (−20 °C to −196 °C) 13 14 15

16 179 Storing samples at cryogenic temperature ~~Frozen storage~~ is likely the most common method of
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18 180 preservation before performing FA analyses. Yet, depending on the temperature, storage duration and
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21 181 type of biological material, this may not be sufficient to limit lipid degradation. It can lead to increased
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23 182 FFA and products of oxidation (e.g., Christie, 1984; Gullian-Klanian *et al.*, 2017; Tenyang *et al.*,
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25 183 2019). Lower freezing temperatures and short-term storage are generally associated with lower lipid
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28 184 degradation (e.g., Baron *et al.*, 2007; Rudy *et al.*, 2016; Gullian-Klanian *et al.*, 2017). The level of
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30 185 lipid degradation in samples is highly dependent on initial freezing temperature and temperature
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32 186 variation during storage, as well as storage duration prior to analyses (Han and Liston, 1987;
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35 187 Ramalhosa *et al.*, 2012; Romotowska *et al.*, 2017). When long-term storage is required, the safest
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37 188 approach is to freeze samples at −80 °C immediately after collection. Although not ideal, long-term
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39 189 storage at −20 °C can be suitable for some species and sample/tissue types, in particular when samples
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42 190 are kept frozen within the organic extraction solvent. Levels of *n*-3 and *n*-6 PUFA in fish muscle,
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44 191 stored at −30 °C and −80 °C, did not change for up to 12 months in both TAG and phospholipid
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46 192 fractions (Passi *et al.*, 2005). Shark muscle samples archived for up to 16 years at −20 °C retained
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48 193 their FA profiles, while lipid class composition was not analysed (Meyer *et al.* 2017). Impacts of
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51 194 frozen storage on FA content and composition are species-specific and can depend on the total lipid
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53 195 content of the organism and tissue (Rudy *et al.*, 2016). Species with high total lipid content (>10%
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55 196 wet weight) must be treated with increased care (low temperature, and short storage period) to avoid
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58 197 changes in FA content during freezing (Rudy *et al.*, 2016; Sardenne *et al.*, 2019).
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2 198 Based on the literature cited, samples should be stored for less than 6 months prior to analysis at
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4 199 temperature lower than $-20\text{ }^{\circ}\text{C}$ when no information is available on the collected biological material.
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6 200 Note that lipid analysis is also possible for samples stored using brine immersion freezing technique
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9 201 (e.g., fisheries-issued material) (Bodin *et al.*, 2014). The sensitivity of lipids to storage varies
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11 202 according to species and tissues. We therefore recommend evaluating the sensitivity of the samples
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13 203 for which there is no information available before the start of the study. For example, a temporal
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15 204 monitoring of the lipid composition of the biological material of interest at the available storage
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17 205 temperatures will make it possible to define the optimal conservation strategy.
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21 206 1.3.2 Freeze-drying

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25 207 Freeze-drying (or lyophilisation) is a low temperature dehydration process of frozen material that is
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27 208 used to reduce enzymatic activity and thus lipid degradation in samples. Freeze-drying samples
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29 209 immediately prior to extraction does not affect lipid class or FA compositions of aquatic animal
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31 210 tissues (e.g., Dunstan *et al.*, 1993; Murphy *et al.*, 2003; Sardenne *et al.*, 2019). This can be a good
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33 211 alternative to frozen storage, as freeze-dried tissues can be easier to grind and to transport than frozen
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35 212 tissues. Rehydration may be required to improve the lipid extraction of TAG (e.g., Dunstan *et al.*,
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37 213 1993) and to comply with extraction procedures that rely on a particular proportion of water in the
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39 214 sample. Nonetheless, freeze-dried tissues still need to be carefully stored to prevent lipid degradation.
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41 215 Storage duration can also have detrimental effects on lipid composition of samples. In fish, crustacean
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43 216 and molluscs, the total duration between freeze-drying and lipid extraction should not exceed one
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45 217 month at $-20\text{ }^{\circ}\text{C}$ for quantitative analysis (Sardenne *et al.*, 2019). Similarly, freeze-dried microalgae
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47 218 samples should not be stored for extended period of time [\(but see Rudy *et al.* 2015\)](#). The PUFA
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49 219 proportion of filtered *Isochrysis galbana* biomass decreased from 36% to 26% after 3 months storage
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51 220 at $-76\text{ }^{\circ}\text{C}$ (Babarro *et al.*, 2001). Proportions of FFA in freeze-dried *Phaeodactylum tricorutum*
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53 221 increased from 4 mg/g to 6 mg/g DW after 35 days at $-20\text{ }^{\circ}\text{C}$ while the degree of oxidation was
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55 222 unaffected (Ryckebosch *et al.*, 2011). Variability in freeze-dried sample stability/viability for lipid
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analysis is likely to reflect differences of biological material and length/duration of the freeze-drying process.

Overall, the quality of the freeze-dried storage depends on: (i) the storage temperature (lower temperatures are the best), (ii) the storage duration (shorter durations are better) and (iii) the lipid content of samples (lean samples are conserved better than lipid-rich samples). Lipid oxidation in food products occurs more ~~rapidely~~rapidly when water activity is high (Labuza *et al.*, 1972). Conversely, lowering water activity below 0.2 through freeze-drying process ~~Deep freeze drying~~ may be a factor promoting lipid degradation through oxidation (Labuza *et al.*, 1972), and specific testing should be considered for aquatic samples. Freeze-drying may offer some benefits when used as a short-term preservation method (e.g., during transport), but it can be expensive (necessary equipment) and time-consuming, while potentially shortening the viability of samples for lipid analyses.

1.3.3 Solvent and fixative storage

1.3.3.1 Organic solvent

One common storage strategy is to directly immerse small organisms such as phytoplankton collected on filters or small-bodied zooplankton into the lipid extraction solvent (e.g., Marty *et al.*, 1992; Soudant *et al.*, 1998; Windisch and Fink, 2018). If samples in the extraction solvent (generally chloroform CHCl₃ and methanol MeOH) are kept in the dark and at low (preferably freezing) temperatures, lipid degradation is minimal. To our knowledge, maximum storage duration of extracted lipids has not been formally investigated. However, samples are likely to remain useable for months to years. For microalgae samples, it is advised to deactivate lipases by a rapid addition of boiling water or isopropanol prior to extraction (Berge *et al.*, 1995; Budge and Parrish, 1999; Ryckebosch *et al.*, 2012).

Synthetic antioxidants (0.01% butylated hydroxytoluene, BHT or 0.1% tertiary butylhydroquinone, TBHQ) can be added to solvent-stored samples to reduce radical formation and oxidative losses of unsaturated lipids (Christie, 2003; Ryckebosch *et al.*, 2012). However, addition

1
2 248 of synthetic antioxidant is unnecessary in samples containing large amount of natural antioxidants
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4 249 such as microalgae (Ryckebosch *et al.*, 2012). Note that BHT elute with short-chain FA and when
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6 250 present in large quantities, it can overlap with FA of interest.
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10 251 1.3.3.2 *Fixatives*

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12 252 Fixatives such as ethanol or formalin are commonly used for biodiversity, taxonomy, morphological,
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15 253 or DNA analyses. The use of fixed samples opens new research opportunities, by making it possible
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17 254 to analyse historical samples collected for purposes other than lipid analysis. However, fixatives
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19 255 influence the chemical composition of the samples and should therefore not be used as a routine
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22 256 procedure for lipid analyses. For example, FA composition of crustacean tissues is altered in
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24 257 formalin-preserved samples, but not in ethanol preserved samples (Phleger *et al.*, 2001). Direct
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26 258 evaluation of fixative effects on lipids are scarce and preliminary tests on different sample types are
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29 259 highly recommended.
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2 Transport and shipping

The packaging, transport and shipping of samples for lipid analysis is a complex process, driven by safety laws, financial costs, stringent storage conditions, and limited duration of storage. Specific legal regulations, including quarantine, must be followed and they can vary, depending on whether the samples are shipped domestically or internationally, and whether there are stopovers during long distance travels. Shipping recommendations to ensure that samples arrive at their destination in good condition include triple packaging with a rigid outer layer and absorbent material, appropriate cooling, accurate labelling (i.e., name, address, phone number, e-mail address, size and weight of the package), and complete legal documentation (e.g., itemised list of content, permits) (IATA, 2010). This section reviews different options of packaging for shipping and traveling, and gives an overview of the main flying regulations and key legal information.

2.1 Shipping samples

Long-distance shipping from the sampling site to the laboratory is challenging. Multiple issues need consideration, such as the conservation state of the samples prior to transport (cooled, frozen, deep-frozen, freeze-dried), the distance, the duration and the type of transport. As samples are generally prone to degradation, temperatures must be kept low and oxidative conditions reduced as much as possible. Regardless of the method used, we recommend only to ship a part of the original sample and keep a backup sample when possible, and to take great care in the packaging (minimum empty space, adapted container to accommodate samples and cooling methods).

When possible, frozen samples should be shipped using dry shippers, which are aluminium containers flooded with liquid nitrogen. They contain adsorbent material that prevents spills of liquid nitrogen during shipment and have attached temperature loggers. Samples can be stored in these conditions for several weeks. However, dry shippers may not be readily available at sampling locations or in laboratories. Restrictions for the transport of liquid nitrogen also need to be considered

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2 284 (e.g. in airplanes or closed vehicles). With transporter authorisation, frozen samples can also be
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4 285 shipped on dry ice depending on transport duration (about 24 h for 2.5 kg of dry ice). Alternatively,
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6 286 deep frozen (-80 °C) aqueous ice packs or reusable technical ice packs (e.g. TechniIce™, 2019) can
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9 287 be used. A cost-effective option is to freeze-dry samples prior to shipping, as it reduces both the
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11 288 volume to be shipped and the risk of lipid hydrolysis by removing water (see section 1). When dry
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13 289 ice packaging or deep-frozen ice packs are not available at the sampling location, freeze-dried
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16 290 samples should be kept cool and in an oxygen-free atmosphere by flushing containers with nitrogen
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18 291 gas and then sealing prior to packaging and shipping. It is also possible to ship samples with small
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20 292 amounts of solvents with respect to international regulations. Note however that depressurisation in
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23 293 aircrafts promotes solvent leakage (F. Pernet pers. obs). To limit this phenomenon, tubes and caps
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25 294 can be coated with Teflon. Alternatively, preserved and/or extracted samples in solvent may be
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27 295 evaporated and flushed with nitrogen so that dried extracts can be air-transported as frozen biological
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30 296 material. Dried lipid extracts will have to be re-immersed in solvent as soon as possible upon arrival.
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32 297 We however recommend to perform preliminary testing before proceeding with this method.
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34 298 The gold standard for shipping samples are specialised transport companies that maintain samples
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36 299 on dry ice and guarantee express delivery to the final destination. However, these options may not be
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39 300 practical for some laboratories and/or available for all sampling sites and destinations. Care should
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41 301 be taken when selecting the shipping company, as several unfortunate experiences have shown that
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43 302 some may not be reliable in maintaining the cold transportation chain.
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47 303 2.2 Flying with samples

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51 304 Although courier is preferable, one might consider putting samples in checked luggage. One of the
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53 305 safest options to transport samples as checked luggage is to use a dry shipper. However, airlines may
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56 306 have different regulations in transporting liquid nitrogen. Non-harmful materials, such as dry ice or
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58 307 technical ice packs are the best option to meet standard luggage regulations (e.g., < 2.5 kg of dry ice
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2 308 per person) and safety (e.g. the package permitting the release of carbon dioxide gas when
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4 309 transporting dry ice).

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6 310 Transporting freeze-dried samples kept in a hermetically closed bag (or a thermos filled with ice)
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9 311 is likely the safest and cheapest option. Upon arrival, samples need to be stored in a freezer
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11 312 (preferentially at -80°C) or immediately put in a solvent solution for lipid extraction (see **section 1**
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13 313 for more details on storage duration). Dried lipid extract should be transported as frozen samples and
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16 314 be returned to solvent as soon as possible (see section 2.1). Note that preservation methods requiring
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18 315 flammable solvents (e.g. ethanol, methanol) are not permitted on aircraft within passenger luggage.
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20 316 Recently, the emerging technology of drones opens new opportunities for a rapid and customisable
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23 317 aerial transport of sensitive samples from remote areas to laboratory facilities (e.g., Amukele *et al.*,
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25 318 2017).

26 27 28 29 319 2.3 Regulations and permits

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33 320 Prior to initiating sample collection and considering transporting them, it is necessary to be well
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35 321 informed on import and export regulations that differ widely across countries and studied organisms'
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37 322 status. It can be difficult to obtain the necessary information within custom departments and the
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40 323 paperwork involved. Some research institutions may have specialised services available to ensure
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42 324 proper paperwork so that samples are shipped and received safely. To transport samples out of a
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44 325 country, it is often necessary to follow The Nagoya Protocol on Access and Benefit Sharing (United
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46 326 Nations Convention on Biological Diversity, 2011). Although it was initially stated that this only
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49 327 applies to samples for molecular research, all transports of tissue for biochemical screening are now
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51 328 included. This applies only for countries that have signed and ratified the Nagoya Protocol.

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53 329 When samples contain species listed in the Convention on International Trade in Endangered
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56 330 Species of wild fauna and flora (CITES), it is necessary to request an import/export CITES permit
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58 331 from each of the countries involved. This needs to be organised well ahead of sample collection, as
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332 it can take up to 3 years to obtain these permits. Additional research permits may be required for the
333 import and export of samples depending on national regulations and target species.

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For Review Only

3 Lipid extraction

Because of their diverse structures, lipids range from non-polar to highly polar, which makes the selection of a solvent with matching polarity challenging. Methods based on chloroform and methanol (Folch *et al.*, 1957; Bligh and Dyer, 1959) have long been used across a wide range of matrix types with great success. Current alterations in environmental regulations have brought restrictions on the type and amount of solvent usage in laboratories worldwide, encouraging the exploitation of alternative organic solvents and novel eco-friendly methodologies for extraction (e.g., accelerated solvent extraction). Here, we ~~will~~ review both the well-established techniques and the newly developed methods; we caution readers that full validation must be carried out with a given biological material before adopting any modified or adapted method.

3.1 Handling cautions

Contamination is an issue that needs to be carefully addressed at every stage of sample handling. It is recommended to: i) use material that have been cleaned using acetone or extraction solvent mixture to remove any potential lipids (e.g. Teflon-coated needles used for evaporation), ii) avoid plastic containers, tools or tips, iii) combust at 450 °C for 6 h or solvent-rinse all glassware and glass fibre filters, iiii) use glass tubes fitted with a-Teflon-lined screw caps, iv) prefer amber glassware (or aluminium foil protected) to limit solvent photo-degradation, vi) use the highest quality solvents for material/tool cleaning, extraction, or elution, vii) perform blank and reference samples for each batch of samples, and viii) wear laboratory gloves to limit exposure to toxic solvents and cross-contamination.

3.2 Sample preparation

Regardless of the technique used, extraction of lipids from aquatic samples requires that the solvent penetrates the sample particles so that it can make contact and solubilise the lipids present. The most efficient extractions ensure that the particle size is as small as possible. The gold standard is to homogenise samples with a ball mill homogenizer (at $-196\text{ }^{\circ}\text{C}$ using liquid nitrogen with wet samples). With wet plant or animal tissue samples at macro-scales, this may be as simple as chopping or blending. For wet microalgae, grinding of samples after immersion in solvent works well to break open cells. Small organisms (roughly between $1\text{ }\mu\text{m}$ and 1 mm ; on a filter or ~~not~~without) can also be directly extracted by direct immersion in the solvent mixture. The use of ultrasound-assisted extraction with a probe or bath may aid in breaking cell walls (Pernet and Tremblay, 2003). When samples have been freeze-dried (**section 1**) for preservation, they may be most conveniently ground with mortar and pestle and it may be necessary to rehydrate prior to carrying out the extraction. Any of these techniques, when applied prior to subsampling for analysis, should also serve to homogenise samples effectively, to ensure representative sampling. These diverse techniques also illustrate the need to adapt the preparatory steps and approach to the sample matrix.

3.3 Traditional extraction methods

While many researchers consider Folch et al. (1957) and Bligh and Dyer (1959) extraction methods to be similar, based on their use of chloroform and methanol, the two are quite different and were developed for different objectives. Prior to using those methods, one would do well to read the original publications. Both methods rely on the formation of a biphasic mixture, with a lower layer principally consisting of chloroform, and containing the isolated lipids, and an upper phase consisting of methanol and water and containing the non-lipids. The Folch et al. procedure, using CHCl_3 :MeOH (2:1, v:v) and a solvent to sample ratio of at least 20:1, was designed as a general and robust method for recovery of lipids from animal tissues (i.e., brain, liver, muscle), now applicable to several tissues

1
2 379 and species with a range of lipid ~~concentration~~ content (Iverson et al. 2001). The Bligh and Dyer
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4 380 method uses a more polar solvent mixture of CHCl₃:MeOH: H₂O) (1:2:0.8, v:v:v) with a 4:1 solvent
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7 381 to sample ratio, and was originally developed to rapidly extract lipids, principally polar lipids, from
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9 382 lean fish tissues.

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11 383 While the Folch et al. method is more complicated to perform, it is more reliable and applicable
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13 384 to almost all sample types. A key aspect of the original Folch et al. method that is often overlooked,
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16 385 is the washing of the bottom phase that contains lipids with a mixture of solvents of the same
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18 386 proportions as originally found in the upper phases. As originally described by Folch et al. (1957),
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20 387 the upper phase consists of CHCl₃:MeOH:H₂O at 3:48:47 and while some groups do use a 50:50
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23 388 MeOH: H₂O wash as recommended by Christie (1982), it is exceedingly rare that the correct mixture
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25 389 is used. Thus, almost all Folch et al. procedures are best described as ‘modified’. The Bligh and Dyer
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27 390 method is easier to carry out, but requires greater consideration of sample type and potential
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30 391 modification of the procedure. For instance, Christie (1982) notes that with high fat samples, an initial
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32 392 pre-extraction with chloroform or diethyl ether is necessary to extract TAG that would otherwise
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34 393 remain unrecovered. The use of the correct proportions of solvents and water are critical for
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37 394 quantitative lipid recovery with both methods. One must take care to adjust the volume of water added
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39 395 when samples with varying moisture contents are analysed (i.e., freeze-dried material vs fresh tissue)
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41 396 and it is often necessary to determine moisture content before beginning extraction.

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43 397 These basic procedures may require some adjustments depending on lipid matrix before and/or
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46 398 during extraction to increase the yield or specificity of lipids to be analysed. For example, it may
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48 399 include inactivation of lipase, changes of solvent proportions, or cell disruption (e.g. Kumari *et al.*,
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50 400 2011; Ryckebosch *et al.*, 2012). In a general manner, authors must thus refer to the available literature
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53 401 on their model organisms or matrices. Given the numerous modified extraction procedures that reflect
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55 402 the diversity of aquatic biological matrices, it is also essential that authors detail their protocol in
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57 403 publications when it differs from original methods.
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404 3.4 Alternatives to chloroform: toward less toxic solvents

405 3.4.1 Dichloromethane

406 Many laboratories have become increasingly conscious of a range of occupational health and safety
407 areas in recent decades. Lipid studies historically used chloroform as a major solvent in many steps
408 including extraction. The less toxic dichloromethane (CH_2Cl_2) has regularly been used instead of
409 chloroform for lipid extraction in many aquatic labs. This replacement originated from food chemistry
410 research (e.g. Chen *et al.*, 1981) and was later formally reported for aquatic samples (e.g., Cequier-
411 Sánchez *et al.*, 2008). Regardless of solvent choice, both dichloromethane and chloroform may
412 degrade on storage to produce HCl and phosgene (Noweir *et al.*, 1972). Phosgene is a much more
413 acutely toxic chemical than either chloroform or dichloromethane, and inhalation can lead to
414 pulmonary oedema (e.g., Snyder *et al.*, 1992). Chloroform degradation or insufficiently stabilised
415 may lead to lipid degradation (i.e., empty chromatograms, including disappearance of the internal
416 standard) (Le Grand and Pernet, pers. obs). To limit phosgene production, chloroform is stabilised
417 with either ethanol or amylene, with ethanol being the more effective stabiliser. Because
418 dichloromethane is less susceptible to degradation than chloroform, it is typically stabilised with
419 alkenes only. Safe lab practices, including restricting solvent use to fume hoods, can effectively avoid
420 risks associated with chlorinated solvents and phosgene formation. In the field, when samples are
421 preserved by direct immersion in solvent, caution is advised.

422 3.4.2 Methyl-tert-butyl ether

423 Methyl-tert-butyl ether (MTBE) was recently used as a less toxic solvent for the extraction of lipids,
424 but had lower recovery rates than chlorinated solvents (Sheng *et al.*, 2011; Ryckebosch *et al.*, 2012).
425 However, the MTBE based extraction protocol appeared especially well adapted to high-throughput
426 lipidomics, as the lipid extract recovered in the upper phase was cleaner with non-extractible material
427 remaining in the lower phase (Matyash *et al.*, 2008).

3.5 The special case of ethanol preserved samples

The availability of historically preserved samples can often occur for a range of lipid studies (see section 1.3.3.1). However, because ethanol extracts some lipids, the sample and all added ethanol must be used in the lipid extraction protocol (Phleger *et al.*, 2001). When using the Bligh and Dyer (1959) method, the extraction should be performed with chloroform (or dichloromethane), ethanol and water, where ethanol replaces methanol. Care is needed when selecting historic ethanol-preserved samples for lipid analysis and in which the ethanol preservative may have been replaced during storage.

3.6 Other methods

Accelerated Solvent Extraction (ASE, Dionex), also known as Pressurised Fluid Extraction (PFE), is widely used method based on a controlled increase in solvent temperature and pressure during extraction. This process maintains the liquid state of solvents near their supercritical region where they have high extraction properties. Several ASE parameters can be adjusted to improve the extraction, such as temperature, pressure, extraction duration (including pre-heating) and number of extraction cycles. All of these parameters affect the final solvent volume and can be adjusted individually to each sample type to optimise the extraction yields and quality (Schäfer, 1998). This sample type-specific adjustment enables high extraction efficiency with a low solvent volume and a short extraction time which is beneficial from a cost and environmental standpoint. Microscale lipid extraction of fish tissues (100 mg) using ASE was shown to be as quantitatively and qualitatively efficient as classical lipid extraction methods (Dodds *et al.*, 2004).

3.7 Concluding remarks

This section did not aim to be exhaustive in describing existing extraction methods. When comparing data from different studies, it is important to consider the lipid extraction techniques used as well as

1
2 451 handling and storage methods. When working on new matrices and developing new or optimised
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4 452 extraction protocols, we recommend to compare them with Folch et al. (1957) or to Bligh & Dyer
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6 453 (1959) methods, which generally remain the gold standards for lipid recovery. Ensuring complete
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9 454 lipid extraction is critical when applying new protocols. Performing successive lipid extractions on a
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11 455 same sample and measuring its lipid content at each step is a suitable approach to establish at which
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13 456 stage the sample is totally lipid-depleted. We recommend monitoring extraction efficiency values and
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16 457 ideally reporting them in publications. Alternatively to successive extractions, the fatty acid methyl
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18 458 ester (FAME) content of the lipid extract can be compared to FAME recovered after direct
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20 459 transesterification (**section 6**). A number of studies have compared the effect of extraction techniques
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23 460 (ASE, Soxhlet, manual) on lipid recovery and FA ~~concentration-content~~ (e.g. Ewald *et al.*, 1998;
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25 461 Mulbry *et al.*, 2009; Balasubramanian *et al.*, 2013). While all report differences, there is little
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27 462 agreement as to which may be an optimal method, likely due to substantial differences in the followed
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30 463 protocols.
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4 Indicators of sample degradation

Sample lipids may degrade through hydrolysis of ester bonds or through oxidation of carbon-carbon double bonds. These are two, largely independent processes leading to different compounds or groups of compounds, which may be used as indicators of sample degradation. Therefore, samples showing indicators of hydrolysis may not have been oxidised and *vice versa*. Unless necessary for a short time period (e.g. shipping), purified lipid extracts are susceptible to oxidation and should be kept under N₂ atmosphere with solvent (Folch mixture is generally preferred).

4.1 Hydrolysis

The most frequently encountered aquatic lipids are acylglycerols (TAG, PL, and glycolipids) in which FA are esterified to a glycerol backbone. There are also ether lipids that replace one ester bond with an ether linkage or a vinyl ether linkage. Esters of carboxylic acids undergo hydrolysis in a reversible reaction which is catalysed by hydrolase enzymes called esterases present in biological samples. Lipases, a subclass of esterases, are found in animals, plants, fungi, bacteria and even in certain viruses (Beisson *et al.*, 2000). Lipid hydrolysis can be assessed from the FFA content, and from the lysophospholipid content when samples are dominated by PL. Standard measurements of oil quality are the acid value (AV) and FFA content, determined in the oil industry by titration and chromatography, respectively. However, both these measurements are rarely used outside of the industrial context.

Many chromatographic systems have been used over the past years for quantitative analysis of major lipid classes including FFA. Free FA can be easily separated from extracted lipids by means of well-known methods based on either liquid (LC) or thin layer chromatography (TLC) (reviewed by Fuchs *et al.*, 2011). For example, HPLC with light-scattering detection (HPLC-ELSD) has been successfully used to detect and quantify FFA in fish with a relatively short processing time and highly reproducible measurements (Christie, 1985; Silversand and Haux, 1997). As for LC, a number of

improvements to classic TLC have led to more sophisticated forms, providing better separation efficiencies and quantifications. High-performance thin-layer chromatography (HPTLC) coupled with densitometry or TLC coupled with flame ionisation detection (TLC-FID, Iatroscan, Shantha, 1992) have also been used to quantify lipid classes and FFA in samples from different origins including marine (Lochmann *et al.*, 1996; Parrish *et al.*, 2009), terrestrial (Zoumpoulakis *et al.*, 2012) and freshwater species (Litz *et al.*, 2017). In food studies, FFA determined by TLC-FID is generally equivalent to AV (Nishiba *et al.*, 2000). TLC is normally less expensive and less time-consuming than HPLC-based methods that can, of course, also be used to provide similar results.

The hydrolysis index of non-polar acyl lipids (HI) and the lipolysis index of all acyl lipids (LI) can be used as degradation indicators (Week *et al.*, 1993; Parrish *et al.* 1995) and are calculated as:

$$HI = \frac{FFA + ALC}{TAG + WE + FFA + ALC} * 100$$

$$LI = \frac{FFA + ALC}{\text{total acyl lipids} + ALC} * 100$$

LI values are always lower than HI values for any sample because all acyl lipids are considered.

4.2 Oxidation

Lipid oxidation mainly occurs through autoxidation when lipids react spontaneously with atmospheric oxygen. The process is usually autocatalytic, with oxidation products accelerating the reaction so that it increases over time. Other catalysts such as light, heat, enzymes and metals lead to photo-oxidation, thermal oxidation and enzymatic oxidation. Fatty acid oxidation is a process in which free radicals cause unsaturated FA to react with molecular oxygen forming acyl hydroperoxides, which can then breakdown into a variety of secondary products. A decrease in PUFA concentration-content may indicate oxidation as they are particularly susceptible to degradation (Sasaki and Capuzzo, 1984).

Lipid hydrolysis may accelerate lipid oxidation due to the formation of FFA that can be substrates for oxidation reactions, as found *in vivo* (Saponaro *et al.*, 2015). High levels of PUFA in lipids make

1
2 514 them susceptible to oxidation (Koshio *et al.*, 1994). Oxidation of *n*-3 and *n*-6 PUFA leads to the
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4 515 formation of highly chemically reactive compounds such as malondialdehyde (MDA), 4-hydroxy-2-
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6 516 hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE). HHE is an aldehyde derived from *n*-3 PUFA,
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9 517 and HNE from *n*-6 PUFA.

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11 518 A number of physical and chemical tests, including instrumental analyses, are available to
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13 519 determine primary and secondary products of oxidation (Shahidi and Zhong, 2005). Oil supplement,
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15 520 food and other industries have a range of standard measurements for determining oil oxidation. These
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18 521 include the internationally recommended peroxide value (PV) and *p*-anisidine value (pAV) (Nichols
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20 522 *et al.*, 2016). For example, PV can be measured by titration and 2-thiobarbituric acid reactive
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22 523 substances (TBARS) by spectrophotometry, and both can be determined using highly precise Fourier
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24 524 transform infrared spectroscopy (FTIR). MDA is commonly monitored using the direct TBARS test
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27 525 (Fernández *et al.*, 1997; Shahidi and Zhong, 2005) and with HPLC (Mendes *et al.*, 2009), while HHE
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29 526 and HNE can be quantified by GC-MS (using chemical ionisation) (Kenmogne-Domguia *et al.*,
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31 527 2014). Recent analytical developments allowed simultaneous quantification of MDA, HHE and HNE
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33 528 by LC/APCI-MS and LC-MS/MS (Douny *et al.*, 2016; Tullberg *et al.*, 2016). Together with pAV,
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35 529 MDA, HHE and HNE are secondary markers of oxidation while PV, hydroperoxides and conjugated
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37 530 dienes are primary markers. However, it has to be noted that MDA, HHE and HNE quantifications
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39 531 do not necessary reflect PV and anisidine values (Viau *et al.*, 2016).
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43 532 Sterols are also a source of oxygenated lipids named oxysterols which are sterols bearing a second
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45 533 oxygen functionality. Cholesterol oxidation likely proceeds in conjunction with oxidative
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47 534 decomposition of coexisting PUFA (Ohshima *et al.*, 1993). Cholesterol oxidation products (COP)
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49 535 can be determined by cold saponification of lipids (Ohshima *et al.*, 1993), followed by enrichment of
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51 536 COP by solid-phase extraction (SPE), and then quantification and identification by GC and GC-MS
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55 537 (Pickova and Dutta, 2003).
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538 4.3 Concluding remarks

539 To conclude, we encourage as a minimum the assessing and reporting of hydrolysis indicators as a
540 quality control criterion of sample handling and extraction procedures. The threshold above which
541 sample/data quality should be questioned depends on the sample matrix, the logistical context and
542 the study objective. In any case, the extent of either form of degradation should ideally be evaluated
543 and compared with baseline values from fresh and properly extracted samples.

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5 Separation of neutral and polar lipids

Fatty acids are incorporated almost unaltered into the storage lipids such as WE, TAG, and ALC, reflecting the FA profiles of the consumed food (Dalsgaard *et al.*, 2003). In contrast, polar lipids are primarily involved in cell membranes which regulate and adjust their FA composition in response to environmental factors like temperature, salinity and hydrostatic pressure (Hazel and Williams, 1990). As such, inference of an organism's diet based on total lipids can be biased by the ratio of FA originating from storage (mostly neutral lipids) and membrane lipids (mostly polar lipids). This ratio varies widely across species, ontogenetic stages, reproduction, nutritional status and tissue types. For example, in small pelagic fish from the Mediterranean, the contribution of TAG stored in the muscle in winter was 1.5%, 5.2% and 47.6% of total lipids for sardine, anchovy and sprat, respectively, the remainder being mostly polar lipids (Pethybridge *et al.*, 2014). This demonstrates that bulk FA composition variations among species can be confounded with variations in lipid class composition. However, many studies in trophic ecology have based their trophic interpretation on the FA composition of bulk lipids without considering the FA distribution between storage and structural lipids.

5.1 When should the lipid fraction be separated?

Lipid class composition should be assessed alongside the FA composition to decide whether lipid class separation is necessary for robust dietary inference. Calculating the ratio of storage to structural lipids provides a comprehensive assessment of the cost/benefit of separating neutral and polar lipid fractions. For samples dominated by storage lipids, the FA composition of bulk lipids will mostly mirror the diet, and lipid class fractionation is not mandatory (e.g., fatty fish species, marine mammal blubber, and digestive gland and liver of marine organisms) (e.g. Sardenne *et al.*, 2020). However, for samples rich in structural lipids, fractionation of lipid classes will provide more reliable dietary indicators. The analysis of FA in polar lipids can inform on nutritional requirements of the study

1
2 569 organism and on environmental conditions prevailing at the time of sampling. Comparing the relative
3
4 570 FA proportions in polar and neutral lipids allows the identification of the dietary FA that were
5
6 571 selectively retained/eliminated to form cell membranes (e.g. Delaunay *et al.*, 1993; Plante *et al.*,
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8
9 572 2007). The unsaturation level of FA and PUFA incorporated in polar lipids is linked to the
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11 573 temperature in ectothermic organisms (Hazel and Williams, 1990; Hall *et al.*, 2002; Pernet *et al.*,
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13 574 2007) and can be associated with the basal metabolic rate (Hulbert and Else, 1999; Pernet *et al.*,
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15 575 2008). Consequently, the separate analysis of neutral and polar lipids is a practical approach to
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18 576 address questions on trophic ecology as well as on nutrition and comparative physiology.
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20 577 To establish trophic relationships of species for which all potential food sources are known, a good
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23 578 strategy is to analyse the FA composition of total lipids in food sources (e.g., prey) and of neutral
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25 579 lipids in consumers to characterise their diet (e.g., predators). Such approach was implemented by the
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27 580 pioneers of the use of FA as trophic indicators (reviewed in Dalsgaard *et al.*, 2003). Alternatively,
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30 581 when study organisms are both prey and predator, or when predator-prey relationships are not well-
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32 582 known, we suggest at a minimum analysing FA from both neutral and total lipids. Beyond trophic
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34 583 considerations, quantification of lipid classes provides useful information on the physiological
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36 584 condition of organisms (Fraser *et al.*, 1989) and the quality of sample preservation through FFA and
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39 585 lysophospholipids levels (see **section 4**).
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43 586 5.2 Commonly used separation methods

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47 587 Storage lipids (SE, WE, TAG, DAG, ALC) and FFA are defined as ‘neutral’ because they are
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49 588 hydrophobic molecules lacking charged groups, whereas PL are defined as ‘polar’ because they have
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51 589 a charged hydrophilic head. Neutral and polar lipids can be separated from each other by TLC or by
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54 590 adsorption chromatography using either laboratory packed or commercial prepared silica columns. A
55
56 591 simple and widely used protocol for separation of neutral and polar lipids consists of a short column
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58 592 of silica prepared in a glass disposable Pasteur pipette plugged with cotton wool (Christie, 1982b).
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60 593 The sample is loaded on the top of the column and washed at low pressure with chloroform (or

1
2 594 dichloromethane) often mixed with methanol, (e.g., CHCl₃:MeOH, 98:2 v/v) to elute neutral lipids.
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4 595 It can then be washed with acetone to obtain acetone mobile polar lipids, generally dominated by
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6 596 pigments and glycolipids. Finally, a last wash is performed with methanol to yield polar lipids
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8
9 597 dominated by PL. Some PL with lower polarity may elute with acetone, while some glycolipids with
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11 598 higher polarity may elute with methanol. Including an elution step with acetone may not be necessary
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13 599 in non-plant samples as they contain negligible amounts of glycolipids (Christie, 1982b). Yet, the
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16 600 latter step (elution step with acetone) can be useful in deciphering trophic links in deep-sea fishes
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18 601 (Şen Özdemir *et al.*, 2019). Diethyl-ether:acetic acid (98:2, v/v) can be used to recover pigments,
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20 602 prior to elution of glycolipids and PL (Da Costa *et al.*, 2017). Eluent and sample volumes must be
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22
23 603 adjusted to optimise separation. It is also important to note that hydration of silica changes the
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25 604 efficiency of retention through a partition effect between bound water and polar lipids (Christie,
26
27 605 1982b). The use of silica hydrated with 6% water (m/m) can improve the recovery of polar lipids
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30 606 from marine bivalves (e.g., Marty *et al.*, 1992; Pernet *et al.*, 2006).

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32 607 Commercially pre-packed columns connected to a vacuum manifold are widely used instead of
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34 608 the manually packed Pasteur pipette (Christie, 1992; Ruiz-Gutiérrez and Pérez-Camino, 2000). This
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36 609 procedure, often referred to as SPE, is time effective and provides similar results as classic column
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39 610 chromatography (Juaneda and Rocquelin, 1985). However, it is difficult to achieve quantitative
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41 611 recovery of PL with this approach. Only ~62% of total lipids were recovered from clam tissue using
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43 612 SPE due to the selective retention of phosphatidylcholine as silica is not rehydrated in these pre-
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46 613 packed columns (Pernet *et al.*, 2006). Commercial SPE columns are available with a wide range of
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48 614 chemically-bonded stationary phases. These have great potential for the isolation of specific lipid
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50 615 classes, yet surprisingly few applications have been described to date. For example, an alternative
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52 616 method developed by Kaluzny *et al.* (1985) using aminopropyl-bonded silica is suitable for separating
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55 617 lipid classes in oyster tissues, though particular care is required to recover the acidic lipids
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57 618 quantitatively (Kim and Salem, 1990; Pernet *et al.*, 2006). Aminopropyl bonded phases have also
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60 619 been used to separate groups of lipid classes, including neutral lipids such as cholesterol, and non-

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2 620 acidic PL, and they are especially useful for the isolation of the FFA fraction from lipid extracts
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4 621 (Kaluzny *et al.*, 1985; Kim and Salem, 1990).
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8 622 5.3 Practical recommendations 9

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12 623 Before starting to use a separation protocol, we recommend reading handbooks describing the use
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14 624 and applications of SPE for lipid separation including the choice of eluting solvents (Christie, 1982b;
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16 625 Perkins, 1991), and to consult technical resources provided by the SPE manufacturers. Specifically,
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18 626 care must be taken to avoid sample overload and subsequent cross-contamination. This problem can
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21 627 be avoided by knowing the lipid composition of the sample and the carrying capacity of the column
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23 628 prior to SPE. The amount of sample that can be loaded onto a column is governed by the proportion
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26 629 of the retained lipids. For example, 2 to 5 mg of PL is considered as the maximum load for SPE of
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28 630 neutral and polar lipids on a 100 mg silica column (Christie, 1982b). Although sample overload leads
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31 631 to cross-contamination, an excess of sorbent can also reduce recovery of the retained analytes. It is
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33 632 therefore suggested to experimentally determine the sample load (mass ratio of lipid to sorbent) to
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35 633 optimise purity and recovery (Pernet *et al.*, 2006). In order to use the SPE system, we suggest
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37 634 replacing the silica originally contained in the pre-conditioned column with rehydrated silica
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40 635 (generally 6% m/m).
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42 636 Purity and recovery analyses after SPE are crucial, particularly when the sample matrix is new to
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44 637 the laboratory and when the stationary phase changes. Different brands or batches of silica gel may
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46 638 vary in their properties and cross-contamination of fractions may occur (Christie, 1982b). Purity can
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49 639 be evaluated by TLC of lipid classes of each eluted fraction. The simplest way to evaluate recovery
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51 640 is to quantify the lipid class content of samples before and after SPE by TLC and proceed with
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53 641 recovery calculation (i.e., the amount of collected lipid after SPE / the amount of lipids before SPE,
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55 642 as a percent). Alternatively, the recovery purity and efficiency can be estimated by adding an internal
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58 643 standard of known mass before SPE. As such, lyso-phosphatidylcholine 12:0 (22.8 µg of FA) was
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60 644 added to the lipid extract before SPE to assess PL recovery in oyster samples (Pernet *et al.*, 2006).

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2 645 Occurrence of 12:0 was exclusively associated to LPC as this FA is absent in oysters. A full recovery
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4 646 of LPC following SPE on silica indicated that other PL were fully recovered as LPC is the most
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6 647 strongly retained phospholipid on silica (Pernet *et al.*, 2006). To evaluate the purity of each lipid
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9 648 fraction and the absence of cross-contamination, it is necessary to add one standard based on the least
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11 649 neutral lipid which must remain in the neutral lipid fraction, and another one which corresponds to
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13 650 the least polar lipid which must be retained in the polar lipid fraction. For example, commercially
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16 651 available 12:0 diacylglycerol (DG) and 10:0 phosphatidic acid (PA) can be added to the lipid extracts
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18 652 of oysters (F. Pernet unpub. data). Because these two shorter chain FA are naturally absent in oysters,
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20 653 their occurrence was exclusively associated with DG and PA standards. The FA 12:0 and 10:0 were
21
22 654 expected in neutral and polar lipid fractions, respectively. Note that these standards do not replace
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25 655 the traditional 23:0 used for mass quantification of FA (see section 7). In the case of SPE, 23:0 is
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27 656 generally added directly in the elution tubes to avoid passage in the column as this standard would
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29 657 only be distributed in the neutral fraction.
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659 6 Transesterification

660 Except in highly degraded samples, where FFA proportion can reach high values, FA are generally
661 part of more complex lipid molecules, and are linked to glycerol by an ester linkage (e.g., TAG, PL,
662 glycolipids). Therefore, one approach to analyse FA by gas chromatography (GC) is to hydrolyse the
663 ester linkage and to convert the released non-volatile FA into their volatile derivatives, usually FA
664 methyl esters (FAME). Saponification followed by methylation is the classical method for preparing
665 FAME, and while laborious, it produces FAME without contamination with unsaponifiable lipid
666 compounds (e.g., non-volatiles, sterols, alcohols, hydrocarbons, phthalates and pigments). However,
667 most researchers use transesterification. A number of transesterification methods exist, all based on
668 the presence of excess methanol, a catalyst (acid or base) and heat (see Schlechtriem *et al.*, 2008). To
669 be analysed by GC, FAME are then extracted in a GC compatible solvent (often hexane), and the
670 organic phase is washed several times by water to remove acid and glycerol. Produced FAME are
671 stable for months at 4 °C in the dark under nitrogen. Transesterification using ethanol instead of
672 methanol is also possible, leading to the formation of FA ethyl esters. Butanol can also
673 be used as a substitute to methanol to quantify very short chain FA (down to *iso5:0*) by increasing
674 aliphatic chain length (Koopman *et al.*, 1996, 2003). Although the use of the boron trifluoride (BF₃)
675 has been recommended by the AOCS (1989), many catalysts can be used in both acid- and base-
676 catalysed transesterification, with each method having their own advantages and limits.

677 The efficiency of these transesterification methods differs as a function of the bound between the
678 aliphatic chains and the glycerol or sphingosine backbones (Christie and Han, 2010)~~The efficiency~~
679 ~~of these transesterification methods differs as a function of the nature of the aliphatic chains.~~ This is
680 particularly important in aquatic samples because, depending on the matrix, a considerable part of
681 these aliphatic chains can be linked to the entire lipid molecule not by an ester linkage, but by a vinyl-
682 ether (e.g., plasmalogens), an ether (e.g., alkyl lipids) or an amide (e.g., sphingolipids) linkage. It is
683 thus important to choose the most pertinent transesterification method with respect to the sample

1
2 684 nature and the scientific question. For example, the acid-catalysed transesterification of plasmalogens
3
4 685 leads to the formation of FAME (from the acyl lipids) and dimethyl acetals (DMA) (from vinyl-ether
5
6 686 lipids). DMA are mainly detected in polar lipids but are marginally present in neutral lipids, reflecting
7
8
9 687 the contribution of glyceride ether also named “neutral plasmalogens” (TAG containing at least one
10
11 688 vinyl ether bound aliphatic chain). Base-catalysed transesterification of these molecules only leads to
12
13 689 the formation of FAME, leaving vinyl ether bound aliphatic chains unreacted (Crackel *et al.*, 1988;
14
15
16 690 Cruz-Hernandez *et al.*, 2006). However, attention needs to be paid to the particular procedure
17
18 691 followed, as base-catalysed transesterification based on ISO standard methodology incorporates an
19
20 692 acid wash to neutralise the catalyst. This acidic wash may lead to DMA formation (Gomez-Cortes et
21
22
23 693 al. 2019). Moreover, neither acid- nor base-transesterifications have any effect on ether linkages
24
25 694 (Christie 1989). This point is important to consider when analysing samples containing large amounts
26
27 695 of alkyl ether lipids, such as alkylglyceryl ether lipids in shark liver oil. Transesterification of FA
28
29
30 696 sphingolipids components is acid-catalysed and is achieved by increasing the reaction time (Eder,
31
32 697 1995; Le Grand *et al.*, 2011). Finally, esterification of FFA is only possible by acid-catalysed
33
34 698 transesterification (Christie, 2003).

36 699 Depending on the scientific question (e.g. nutritional value, trophic relationships, physiological
37
38
39 700 responses), FA transesterification can be realised directly on the biomass (direct transesterification),
40
41 701 on a total lipid extract or on lipid fractions, such as polar or neutral lipids, lipid classes (e.g., TAG,
42
43 702 phosphatidylcholine) or lipid subclasses (e.g., phosphatidylethanolamine plasmalogen) isolated
44
45
46 703 beforehand. It can be useful to purify FAME by HPLC or TLC before GC injection (Marty *et al.*,
47
48 704 1992). This prevents the saturation and the degradation of the column through the injection of non-
49
50 705 FAME molecules (e.g. non-volatiles, sterols, hydrocarbons, phthalates, pigments) and thus increases
51
52
53 706 its lifespan. Obtained chromatograms are also “cleaner” (no contaminants) and easier to identify.

6.1 Boron trifluoride (BF₃)

The Lewis acid boron trifluoride (BF₃) in methanol (12-14%, w/v) is a powerful acidic catalyst for the transesterification of FA. In general, 800 µL to 1 mL of BF₃-MeOH (12-14%, w/v) is added to a dry lipid extract containing a maximum of 1-4 mg of lipids (Liu, 1994; Carrapiso and García, 2000; Le Grand *et al.*, 2011). This method is fast and efficient (Morrison and Smith, 1964; Carrapiso and García, 2000; Cavonius *et al.*, 2014), and it has been successfully used on marine lipid samples where almost all the lipid classes, including FFA and plasmalogens, were transesterified in 10 min at 100 °C (Le Grand *et al.*, 2011; Cavonius *et al.*, 2014). For the sphingolipids, such as sphingomyelin and ceramide aminoethylphosphonate, the transesterification should be prolonged and last for 1.5 h to 5 h at 100 °C (Eder, 1995; Le Grand *et al.*, 2011).

Heating is generally conducted in a dry bath, water bath or heat block. The higher the temperature used, the shorter the reaction time. An increase in reaction time has no negative effect, provided that no evaporation occurs (Morrison and Smith, 1964). Presence of residual water in the sample limits the acid-catalysed esterification by BF₃ (Sattler *et al.*, 1991). It is thus necessary to dry the sample by either nitrogen evaporation or by addition of anhydrous sodium sulphate (Molnar-Perl and Pinter-Szakacs, 1986).

Because evaporation during the transesterification leads to acid concentration, which degrades FA and especially PUFA (Lough, 1964), it is crucial to prevent vial caps from unscrewing when heating samples (Morrison and Smith, 1964). Poor storage conditions and quality of BF₃ cause PUFA degradation (Morrison and Smith, 1964; Christie, 1993). It is recommended to use fresh reagent and to store it at 4 °C or colder (-20 °C). Despite these issues, the use of BF₃-MeOH for transesterification was referenced as an official method (AOCS, 1973; IUPAC, 1979) and Ackman (1998) showed that issues could be avoided following simple rules. Yet, the potential PUFA degradation induced by using BF₃ as a transesterification catalyst, the difficulty in purchasing guaranteed anhydrous BF₃-MeOH, its high cost and its high toxicity have led to a decreasing use of this reagent (Liu, 1994; Carrapiso

1
2 732 and García, 2000; Cavonius *et al.*, 2014). In samples containing very high levels of storage lipids,
3
4 733 such as salmon flesh, copepod oil and cod liver, the derivatisation efficiency can be quite low, leading
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6 734 to small but significant differences in some FA proportions (Schlechtriem *et al.*, 2008). For these
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9 735 reasons, BF_3 is no longer recommended as an acidic catalyst in the preparation of FAME.
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12 736 6.2 Acidic alternatives to BF_3

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17 737 A number of alternatives to Lewis acids have been employed (Christie, 1982a). Methanolic HCl (3
18
19 738 N) appears to be the most commonly used reagent for transesterification of lipid-bound FA to FAME
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21 739 in lipids from freshwater plankton (von Elert, 2002; Martin-Creuzburg *et al.*, 2012; Windisch and
22
23 740 Fink, 2018), but also from marine plankton (e.g., Troedsson *et al.*, 2005). It can easily be produced
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25
26 741 by adding 24 ml acetyl chloride to 100 ml of methanol, which leads to the formation of 10% (approx.
27
28 742 equivalent to 3 N) methanolic HCl. H_2SO_4 is often recommended (Christie, 1982a; Budge *et al.*,
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30 743 2006), as it can easily be prepared by mixing a small amount of concentrated H_2SO_4 (~98%) in
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32 744 methanol to achieve a 1% solution (Schlechtriem *et al.*, 2008).
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35 745 The most commonly used acid catalysed reagents, such as HCl or H_2SO_4 , are not associated with
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37 746 the issues described for BF_3 . A number of comparisons of acid catalysts for
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40 747 esterification/transesterification have been made (e.g. Medina *et al.*, 1992; Budge and Parrish, 2003;
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42 748 Thiemann *et al.*, 2004; Carvalho and Malcata, 2005), with no clear consensus on optimal methods.
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44 749 The best advice is likely to select a method and consistently apply it. As with BF_3 , caution is needed
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46 during heating to prevent evaporation.
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50 751 6.3 Basic alternatives

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55 752 Several basic catalysts are available (e.g., NaOH, KOH) to transesterify acyl lipids. While a range of
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57 753 procedures have been described (e.g. Christie, 1982a; Medina *et al.*, 1992; Velasco *et al.*, 2002), most
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59 754 are very similar to the acid catalysed method described above with the substitution of the catalyst and
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1
2 755 the introduction of an additional step after the reaction is quenched to neutralise the remaining base.
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4 756 All base-catalysed transesterifications proceed very rapidly at room temperature (Christie, 1982a),
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6 757 thereby preserving labile structures (i.e., epoxides, conjugated double bonds) that are often destroyed
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9 758 with harsher acid catalysts. This ‘gentler’ transesterification is the primary motivation for use of basic
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11 759 catalysts. As noted above, basic-catalysts are unable to esterify FFA so these approaches should only
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13 760 be used when one can be confident that FFA are present in very low amounts, or else when the analyst
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16 761 is targeting esterified FA exclusively (e.g., studies on membrane lipids or storage lipids).

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18 762 Finally, there are a number of official and approved methods that employ combined approaches,
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20 763 using both basic and acidic catalysts (e.g., AOCS, 1989). While at least one of these methods (i.e.,
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22 764 AOAC 969.33-1969, 1997) describes the procedure as first saponification with NaOH, followed by
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25 765 methylation with BF_3 in methanol, the short reaction time with NaOH in methanol is actually an
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27 766 alkali-catalysed transesterification, followed by acid-catalysed methylation of any FFA that remain
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30 767 (Ackman, 1998). Although direct acid-catalysed transesterification is sufficient for a typical aquatic
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32 768 lipid if performed with sufficient time and heat, the alkali-catalysed step may persist because of its
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34 769 adoption by official bodies (Ackman, 1998).

37 38 770 6.4 Direct transesterification

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42 771 The standard procedure for determining FA profiles (e.g. extraction to isolate lipids, followed by
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44 772 transesterification to yield FAME), can be labour-intensive and increases analytical cost. In some
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47 773 cases, studies are only interested into the FA composition of total lipids in samples and therefore
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49 774 perform a direct one-step transesterification that considerably reduces the analytical time (Lewis *et*
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51 775 *al.*, 2000). This method is realised directly on the sample, and differs from the classical
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53 776 transesterification by the elimination of the lipid extraction step (Lewis *et al.*, 2000; Castro-Gómez
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55
56 777 *et al.*, 2014; Parrish *et al.*, 2015a).

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58 778 Different methods have been tested and provided reproducible and similar results to the classical
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60 779 extraction-transesterification method (Castro-Gómez *et al.*, 2014; Parrish *et al.*, 2015a). Careful

1
2 780 consideration is needed when using this approach as it may not be appropriate to the study goal and/or
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4 781 the matrices analysed. It may also be preferentially applied to small and homogenous (representative
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6 782 of the whole) samples. For example, while it is possible to carry out direct analyses on a small amount
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9 783 of tissue, accurate determination of total blubber FA composition in marine mammals was not
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11 784 possible with this approach due to significant variation in FA composition within the layers of the
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13 785 blubber (Thiemann *et al.*, 2008). Although this rapid direct transesterification approach is less time-
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15 786 consuming and cheaper, once the entire sample is transesterified, it is not possible to redo the analysis,
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18 787 or to analyse the FA composition of different lipid fractions.
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22 788 6.5 Alternatives to hexane

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26 789 Hexane, iso-hexane (2-methyl-pentane), or iso-octane are most frequently used to recover FAME
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28 790 after transesterification and before injection. However, hexane is carcinogenic, mutagenic and
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30 791 reprotoxic, and is a hazardous airborne pollutant. Less toxic solvents such as pentane and heptane can
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32
33 792 be used as alternatives to hexane (Alfonsi *et al.*, 2008). However, recovery of FA from macroalgae
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35 793 is lower with heptane than with hexane, with PUFA tending to be more affected than SFA and MUFA
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37 794 (Le Grand unpub. data). This preliminary result needs to be verified on other matrices. Pentane rather
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40 795 than hexane may also be used as the solvent in the BF_3/MeOH procedure to reduce the reaction
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42 796 temperature from 85-100 °C to 80 °C to decrease artefact risk (Parrish, 1999).
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7 Analysis of fatty acids

The full process of FAME analysis consists of injection, separation, identification and quantification of FAME and each of these steps has to be optimised to achieve high accuracy and precision (see Eder, 1995; Christie, 2003 for review). Gas chromatography (GC) with flame ionisation detection (FID) is generally the preferred method for the quantification of FAME in aquatic samples. GC-MS methods for FAME quantification can be advantageous on a routine basis when spectrometric confirmation of compound identity is required. This is especially useful for complex samples containing contaminants, artifacts or co-eluting compounds. Although these methods compared satisfactorily with GC-FID, they are more complex and tedious to implement and calibrate. Such approaches also require stronger chemistry / spectrometry backgrounds of the user and greater routine maintenance of the instrumentation.

7.1 Injection

Sample injection is a critical step to achieve high accuracy in FAME analysis and we encourage readers to refer to seminal papers of Konrad Grob for a thorough overview of available techniques (e.g., Grob, 2007). There are currently three injection techniques. Before being transferred into the capillary column, the sample can be (i) evaporated in a permanently hot vaporising chamber (classical vaporising injection), (ii) injected into a cool chamber which is subsequently heated to vaporise the sample (programmed temperature vaporising injection, referred to as PTV), or (iii) directly injected into the column inlet (direct injection or on-column).

Once vaporised, the sample can be split (i.e., split injection) with only a small part of the vapour entering the column while the rest is vented. This is the most appropriate technique for concentrated samples (e.g., Shantha and Ackman, 1990). Alternatively, the whole vaporised sample is transferred from the injector to the column without splitting of the sample (i.e., splitless injection). This technique is particularly appropriate for trace analysis. The major drawback of the split or splitless injection

1
2 821 techniques is the possible discrimination between high- and low-boiling compounds in the sample.
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4 822 Fatty acids in aquatic samples have a wide range of boiling points and quantification may require
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6 823 application of relative response factors (Grob, 2007). On-column injection avoids discrimination of
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8
9 824 FA and therefore provides the best results. Yet, a large number of aquatic scientists use the vaporising
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11 825 injection method for suitability and flexibility reasons. High-speed injection, high injector
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13 826 temperature ($> 220\text{ }^{\circ}\text{C}$) and proper injector inserts all contribute to decreasing discrimination
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15 827 problems. However, we recommend regular checks of FAME discrimination factors using certified
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18 828 reference mixtures (e.g. Supelco 37).

22 829 7.2 Separation

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26 830 Upon injection, FAME are transferred into a wall-coated open tubular (WCOT) capillary column
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28 831 allowing separation according to their carbon number, the number and location of the double bonds,
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30 832 and the cis-trans configuration. The column is located in an oven and exposed to temperature ramps
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33 833 ranging from $60\text{ }^{\circ}\text{C}$ to $300\text{ }^{\circ}\text{C}$ (e.g. Tang and Row, 2013 for examples of temperature ramping). The
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35 834 carrier gas is generally helium or hydrogen. The resolution capability of the column depends on
36
37 835 several factors such as polarity of the stationary phase, column length, internal diameter and film
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39
40 836 thickness. In view of its importance, this information should be reported in detail in the method
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42 837 description of any study.

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44 838 Based on the type of FA being investigated, one of the most important steps is the selection of the
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46 839 stationary phase of the column which influences elution times of FAME, especially PUFA. The
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48 840 polarity of the column ranges from non-polar to extremely polar. On non-polar columns, the elution
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51 841 time generally increases with increasing boiling points of analytes. Therefore, the elution time of
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53 842 FAME increases with chain length and with decreasing unsaturation (e.g., $18:2n-6$ elutes first,
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55 843 followed by $18:1n-7$, and then by $18:0$). The major drawback with non-polar columns is that some
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57
58 844 FA isomers (*cis-trans*) or FA pairs may co-elute. Conversely, non-polar columns exhibit low bleed,
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60 845 long lifetime, retention time stability and high temperature resistance (to $360\text{ }^{\circ}\text{C}$), which makes their

1
2 846 use suitable for a large range of compounds. These columns are well-suited for separating neutral
3
4 847 compounds such as sterols, alkenones, hydrocarbons. Commonly used non-polar columns are made
5
6 848 of 100% dimethylpolysiloxane or 5% phenyl-95% dimethylpolysiloxane.
7

8
9 849 Many researchers use polar columns which allow for a better separation of aquatic FA than non-
10
11 850 polar phases, with only some rare and minor co-elutions. Ackman (1986) was a strong advocate of
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13 851 the polyethylene glycol (PEG) phase. Columns coated with PEG are easily recognisable because of
14
15 852 their commercial name with the suffix "wax". With this polar phase, FAME continue to elute in order
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17 853 of increasing boiling point, but also roughly in order of increasing numbers of double bonds.
18
19 854 However, polar columns are highly sensitive to temperature (max 250 °C), water and oxygen, and
20
21 855 are thus less stable over time than non-polar columns. Retention times may also vary (decrease) over
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23 856 time, especially for highly unsaturated compounds. Consequently, peak identification (based on
24
25 857 elution time) needs to be checked regularly and rigorously.
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30 858 An alternative phase consists of substituting 50% dimethylpolysiloxane (non-polar) with the polar
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32 859 cyanopropylmethyl (e.g., Iverson *et al.*, 2004; Pedro *et al.*, 2019). This phase provides similar
33
34 860 resolution as PEG phases, although it is much less sensitive to oxygen, resulting in longer column
35
36 861 lifetimes. The chemistry of this phase is more influenced by temperature than other polar columns.
37
38 862 Separation of co-eluting peaks can sometimes easily be achieved simply by subtle adjustments to
39
40 863 oven temperature ramps.
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45 864 7.3 Identification

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49 865 The most common way to identify FAME by GC-FID analysis is to compare their retention times
50
51 866 with those of commercially-available individual purified standards. Among the most used standards
52
53 867 for the FA identification in aquatic samples, there are PUFA No. 3, a complex qualitative standard
54
55 868 mixture extracted from Menhaden oil, and Supelco® 37 Component FAME Mix, a comprehensive
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57 869 mixture of 37 FAME ranging from 4:0 to 24:1*n*-9. Several long-chain and (highly) unsaturated
58
59 870 FAME occurring in some samples are not commercially available, but can be identified using fully
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1
2 871 characterised natural products (e.g., the non-methylene interrupted fatty acids typical of marine
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4 872 molluscs in Zhukova and Svetashev, 1986; Ackman, 1989; Kraffe *et al.*, 2004).

6 873 In addition to these protocols, confirmation of FA identification is best achieved using GC-mass
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8
9 874 spectrometry (GC-MS). Peak identification is achieved using commercial spectral libraries (e.g. The
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11 875 [LipidWeb <http://lipidhome.co.uk>](http://lipidhome.co.uk)), or via personal/laboratory FA mass spectral libraries to assist
12
13 876 in identification of often complex environmental FAME samples. Further details on various GC-MS
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15
16 877 protocols together with specific MS information are available in Christie (1989). Briefly, the base
17
18 878 peak at m/z 74 is termed the McLafferty rearrangement ion, and is formed by cleavage of the parent
19
20 879 FAME molecule beta to the carboxyl group. Where chemical ionisation is used, the mass spectra
21
22
23 880 have a more prominent quasi-molecular ion (MH^+), and minor ions only at $[MH - 32]^+$ and $[MH -$
24
25 881 $32 - 18]^+$. Because all FA will have the carboxylic acid (or ester) functionality in common,
26
27 882 knowledge of the molecular mass is particularly valuable in determining basic structure with respect
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29
30 883 to numbers of carbon atoms and double bonds. There are unfortunately generally no characteristic
31
32 884 ions that indicate the position or geometry (i.e., *cis-trans*) of double bonds in positional isomers of
33
34 885 monounsaturated and polyunsaturated FAME (Christie, 1989).

36 886 Where hydroxyl groups are present on the FAME, conversion of the hydroxyl group to a trimethyl
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38
39 887 silyl (O-TMSi) ether or other derivative is generally used. Bistrimethylsilyltrifluoroacetamide
40
41 888 (BSTFA) is a useful reagent to accomplish this. The high polarity of the free hydroxyl group is
42
43 889 reduced by formation of the O-TMSi ether, thereby enhancing the chromatography of the derivative.
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45
46 890 Fragmentation of the OTMSi derivative occurs alpha to the carbon containing the hydroxyl group,
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48 891 with the main cleavage on the side adjacent to the carboxyl group.

50 892 Derivatisation protocols have been developed to provide more detailed position of double bonds
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52
53 893 or geometry information. These include, for example, use of pyrrolidine, 3-pyridyl carbinol (picolinyl
54
55 894 ester), dimethyl disulphide (DMDS) and 4,4-dimethyloxazolazine (DMOX) derivatives. An
56
57 895 advantage of the use of the DMOX protocol is that the FAME and their corresponding DMOX
58
59 896 derivatives have the same elution order when run on the same GC column. PUFA DMOX derivatives

1
2 897 can be readily formed using the protocols described in Svetashev, 2011 and Lee Chang *et al.*, 2016.
3
4 898 Interesting examples of application of the DMOX protocol have been the characterisation of a novel
5
6 899 series of C₁₈ to C₂₂ *trans* n-3 PUFA from a marine haptophyte (Lee Chang *et al.*, 2016) and the 22:4n-
8
9 900 9t in scallop (Marty *et al.*, 1999). MUFA double bond position and geometry is readily determined
10
11 901 by capillary GC-MS of their DMDS derivatives (Nichols *et al.*, 1986). An example of application of
12
13 902 the DMDS protocol has been the characterisation of a range of novel C₁₆ and C₁₈ MUFA, including
14
15 903 both *cis* and *trans* isomers, from microbial monocultures and complex soil consortia (Nichols *et al.*,
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17 904 1986). Other derivatisation procedures are also available, although they are not covered here, and are
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19 905 as detailed in Christie (1989).
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24 906 7.4 Quantification

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28 907 In GC-FID analyses, the response intensity is based on the number of oxidisable carbon atoms in the
29
30 908 analyte; equivalent masses of FA having different carbon chain lengths and/or numbers of double
31
32 909 bonds will not produce the same response in the FID (Ackman, 1972). FAME are usually quantified
33
34 910 by peak areas, and absolute concentrations are determined by adding an internal standard.
35
36 911 Discrimination of FAME may be associated with injection technique and it is often necessary to
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38 912 introduce empirical response factors, determined through careful use of commercially available
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40 913 authentic mixed standards (see section 7.1). A way to estimate the importance of bias from different
41
42 914 origins (such as injector, or detector) is for example to analyse a FA mixture of known theoretical
43
44 915 mass composition such as the Supelco® 37 Component FAME Mix. It is also possible to analyse a
45
46 916 phospholipid with two different aliphatic chains, of different length and saturation (e.g.,
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48 917 phosphatidylcholine 16:0/22:6n-3) and confirm that the molar percentage of each FA is 50/50.
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53 918 The internal standard must not coelute with the FAME, its chemical structure must be close to the
54
55 919 majority of the FAME and it must be absent from the sample. A known mass of internal standard
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57 920 should be added either after SPE or before transesterification to account for derivatisation yield and
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59 921 recovery of FAME (see section 5). The most widely used standards are the carboxylic form of
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2 922 nonadecanoic acid (19:0), heneicosanoic (21:0) and tricosanoic acid (23:0). Data are generally
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4 923 expressed as relative (area %, mass % or mole %) or absolute ~~concentrations~~ contents (e.g., mg FA
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6 924 g⁻¹ sample dry mass).

8
9 925 With GC-MS analyses, one approach is to use the total ion chromatogram (TIC) in a similar
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11 926 fashion as peak area is used with GC-FID, by comparing the peak area of an internal standard with
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13 927 peak areas of compounds of interest, and relative response factors determined individually using
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15 928 authentic standards. Note here that response factors vary with structure (carbon and unsaturation
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17 929 numbers, mostly notably), ionisation methods (electronic vs chemical) and mass detector (e.g.
18
19 930 Quadrupole vs ion trap) (Dodds *et al.*, 2005; Quehenberger *et al.*, 2011). Consequently, the range of
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21 931 response factors is greater in MS than with FID. Response factors for FA, where standards are not
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23 932 available, are assumed the same as those of structurally similar and available compounds but this is
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25 933 arguable.

26
27 934 ~~Response factors for analytes where standards are not available are assumed to be the same as~~
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29 935 ~~those of available compounds. Note here that response also varies with structure, likely due to~~
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31 936 ~~difference in ionisation so calibration remains important.~~ For greater sensitivity, calibration can be
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33 937 based on ~~a single (or multiple)~~ prominent ion(s) using selected ion monitoring (SIM) as a single ion
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35 938 can be scanned many more times in a cycle than the full mass spectrum, giving a higher signal to
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37 939 noise ratio (Dodds *et al.*, 2005; Thurnhofer and Vetter, 2005; Quehenberger *et al.*, 2011). It involves
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39 940 the exclusive acquisition of a group of selected ions during a given period if the identity and retention
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41 941 time of a given analyte have been established through full scan mode. Since fragmentation can vary
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43 942 depending on chain length and degree of unsaturation, careful assignments of selected ions group to
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45 943 analytes and appropriate adjustments of SIM parameters are necessary to achieve accurate
46
47 944 quantification and complete discrimination when some FA are co-eluting.

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49 945 Quantification of FA can be further improved by using mixtures of isotopically-labelled FA
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51 946 internal standards (e.g. deuterated FA). These offer the advantage of being identical to the analyte of
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53 947 interest in all aspects except mass, so that response, as well as matrix effects, of the labelled and
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1
2 948 ~~unlabelled component are the same (Quehenberger *et al.*, 2011). The main limitation to this approach~~
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4 949 ~~is the commercial availability of isotope-labelled fatty acids that could “match” all fatty acids present~~
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6 950 ~~in aquatic samples. in the spectrum of the FA of interest, typically using an internal standard~~
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8
9 951 ~~technique. Another approach with GC-MS is the use of stable isotope dilution methods, where~~
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11 952 ~~labelled internal standards are used. These offer the advantage of being identical to the analyte of~~
12
13 953 ~~interest in all aspects except mass, so that response, as well as matrix effects, of the labelled and~~
14
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16 954 ~~unlabelled component are the same (Quehenberger *et al.*, 2008).~~
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For Review Only

8 Data treatment

As emphasised by the previous sections, obtaining reliable FA data can be a long and complex process. The purpose of this section is therefore to offer guidance in how to valorise these hard-earned data in the best possible way, and to use them to shape robust answers to a wide range of scientific questions. As environmental phenomena are inherently complex, a single variable rarely depicts them in a satisfactory way (Buttigieg and Ramette, 2014). Moreover, FA studies typically deal with datasets containing many variables (i.e., individual FA). This section will therefore mostly focus on multivariate analysis. Readers looking for guidance about univariate tests and procedures are encouraged to refer to other publications (e.g. McDonald, 2014).

After adequate standardisation and/or transformation (section 8.1), FA data can be analysed in different, non-mutually exclusive ways. Unconstrained ordination methods (section 8.2) can help visualising the data, and finding out which samples group together according to their similarity in FA composition. In doing so, they allow determining how similar to one another entities such as sampling locations (Hughes *et al.*, 2005; Parrish *et al.*, 2015b), habitats (Hixson *et al.*, 2015) or species (Sardenne *et al.*, 2016) are. These exploratory methods are adequate to identify patterns between samples, as well as FA markers responsible for these patterns. Conversely, investigators can be interested in assessing the influence of one or several previously identified factors on FA composition, e.g. to explicitly test whether FA composition is different across multiple experimental treatments, ecological conditions, or species. This can be done through hypothesis testing and/or constrained ordination (section 8.3).

Regardless of the data analysis approach, FA studies commonly rely on ~~so-called~~ "biomarker compounds", i.e., FA (or combinations of FA) whose origin can be assigned to a biogeochemical process, an ecosystem compartment and/or an organism group. When doing so, While this approach has proven very useful and deserves to be routinely applied, it is important to remember that there are

1
2 980 no ubiquitous FA biomarkers, and that their applicability will depend on the studied system or
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4 981 organism (e.g., Feiner *et al.*, 2018). To avoid interpretation biases, biomarker compounds should be
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6 982 chosen carefully with respect to the ecological context and scientific question of interest. When used
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9 983 sensibly, biomarker compounds can be very useful, as proven by their routine use in ecological
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11 984 literature (see (Dalsgaard *et al.*, 2003; Kelly and Scheibling, 2012) for review). This topic has been
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13 985 extensively discussed in other reviews (Dalsgaard *et al.*, 2003; Kelly and Scheibling, 2012) and will
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15
16 986 not be further elaborated upon here.
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19 987 8.1 Standardisation and transformation

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23 988 Choosing standardisation or a prior transformation method for FA data needs to be based on the
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25
26 989 research questions, as it can have important impacts on the final results and interpretations (Mocking
27
28 990 *et al.*, 2012). FA can be expressed as either an absolute concentration-content or mass ratiofraction,
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30 991 such as mg.g⁻¹ of lipids or of tissue, or as relative proportion (%) of total FA mass or molar
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32
33 992 concentration. Concentrations are commonly used in nutrition studies, and can be useful in controlled
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35 993 feeding experiments (Elsdon, 2010). Proportions of total FA mass is the commonly used
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37 994 standardisation method in trophic studies. It avoids occultation of compositional differences in FA
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39 995 profiles by removing the influence of non-FA components from both the lipid fraction (e.g., polar vs
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41 996 neutral lipids) and the total lipid contents of tissues. Yet, proportion data are generally calculated in
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43 997 relation to identified FA in a sample, which vary across studies and FA analysis methodologies,
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45 998 limiting inter-study comparison.
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49 999 Choice of data standardisation is interdependent with the choice of data transformation method (if
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51 1000 needed) and of applied statistical test (univariate vs. multivariate tests). FA data transformation can
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54 1001 be recommended in some cases (e.g., Kelly and Scheibling, 2012), but the overall effect of the
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56 1002 procedure is highly variable (Happel *et al.*, 2017). Deciding on a transformation method depends on
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58 1003 the test used and its assumptions (e.g., normal distribution of data, homoscedasticity). Although these
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60 1004 assumptions can be met for univariate analyses (that test the effect of one or several factors on

1
2 1005 individual FA), this is rarely the case for multivariate analyses using large FA datasets, even after
3
4 1006 transformation (Budge *et al.*, 2006). However, multivariate methods such as unconstrained
5
6 1007 ordinations (e.g., principal component analysis, see below) give useful and adequate results when the
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9 1008 data are properly transformed (e.g., Legendre and Gallagher, 2001). Moreover, proper testing
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11 1009 procedures through permutations allows one to circumvent the violation of the multivariate normality
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13 1010 assumption in constrained ordination methods such as redundancy analysis (RDA, Legendre and
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15
16 1011 Legendre, 2012; see subsection 8.3).

17
18 1012 Kelly and Scheibling (2012) reviewed some of the most common data transformation methods
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20 1013 used in FA studies, including log-ratio (Budge *et al.*, 2006), arcsine and logit transformations. Arcsine
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22
23 1014 transformation has been widely applied to transform proportional FA data, yet its use in biological
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25 1015 analysis has been challenged in some studies. Warton and Hui (2011) suggested that applying logistic
26
27 1016 regression is more appropriate to transform proportion data. It is also important to note that some
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29
30 1017 transformations such as $\log(x)$ or $\log(x+1)$ will give more weight to rare (i.e., found in small amounts)
31
32 1018 FA, while reducing the influence of the abundant FA (Happel *et al.*, 2017). Depending on the study
33
34 1019 objectives, it is essential to identify clearly whether the scientific question requires emphasising the
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36 1020 presence and the role of rare FA, or if focusing on the dominant FA will give a more global pattern
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39 1021 within and across organisms.

42 1022 8.2 Exploring fatty acid data through ordination

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45
46 1023 Ordination methods are essential to describe and visualise sample dispersion in a complex
48
49 1024 multidimensional dataset, and to identify groups of co-varying variables. Those are computed based
50
51 1025 on a matrix of resemblances (distance, dissimilarities, similarities), or derived ranks, rather than raw
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53
54 1026 (or transformed) data. In FA literature, the most commonly used resemblance measure between
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56 1027 samples are either the Euclidean distance or Percentage difference (also called Bray-Curtis
57
58 1028 dissimilarity; Legendre and Legendre, 2012). Euclidean distance is generally preferred over Bray-
59
60 1029 Curtis for FA analysis (Happel *et al.*, 2017). However, just like data transformations, the choice of

1
2 1030 the resemblance measure can have important impacts on the final results. The choice of matrix will
3
4 1031 depend on whether a dataset includes null/absent values and if they need to be included in the analysis
5
6 1032 according to the study question. The Percentage difference coefficient is double-zero asymmetric, i.e.
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8
9 1033 the absence of one FA from two individuals is not considered as contributing to their similarity. The
10
11 1034 Euclidean distance, on the contrary, is double-zero symmetric, i.e. will be affected by double null
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13 1035 values (Legendre and Legendre, 2012). Other properties of resemblance measures should also be
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15
16 1036 considered. Choice of the resemblance coefficient has received extensive attention in multivariate
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18 1037 community ecology (e.g., Legendre and Legendre, 2012).
19

20
21 1038 Commonly used methods to plot ordinations include principal components analysis (PCA),
22
23 1039 principal coordinates analysis (PCoA) and non-metric multidimensional scaling (nMDS). PCA can
24
25 1040 only use Euclidean distance, while PCoA and nMDS can use any resemblance measure. PCoA and
26
27 1041 nMDS use the ranks of similarities or dissimilarities matrix to find a spatial arrangement of
28
29 1042 individuals and represent relationships among them (Kelly and Scheibling, 2012). ~~They have been
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31
32 1043 used to classify and compare FA compositions of consumers across sampling location (Hughes *et al.*,
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34 1044 2005; Parrish *et al.*, 2015b), habitats (Hixson *et al.*, 2015) or species (Sardenne *et al.*, 2016).~~ NMDS
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36 1045 is conceptually simple, generally applicable, and well adapted to ecological data (Clarke and
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38 1046 Warwick, 2001). However, it is crucial to take into account the stress coefficient, which is measured
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40
41 1047 from the relationship between ranks of dissimilarities and ranks of sample distances on the plot. A
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43 1048 high stress score (> 0.05) indicates that data have been highly distorted to fit in the visualisation,
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45
46 1049 which may not be representative and should be considered with caution (Clarke and Warwick, 2001;
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48 1050 Buttigieg and Ramette, 2014). PCA also describes relationships among samples, reducing large
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50 1051 numbers of variables to a few components that represent most of the variance and that are built by
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52 1052 combining correlated variables (Budge *et al.*, 2006; Kelly and Scheibling, 2012). It also helps to
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54
55 1053 visualise variables that contribute the most to the dissimilarity among observed groups and FA that
56
57 1054 are highly correlated (Budge *et al.*, 2006). After ordination, several procedures (similarity percentage,
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1
2 1055 also called SIMPER, analysis; comparison of PCA loadings) can be applied to identify FA driving
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4 1056 resemblance or dissimilarity among groups (Clarke and Warwick, 2001).
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8 1057 8.3 Multivariate hypothesis tests and modelling 9

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11
12 1058 Multivariate analyses are commonly applied in ecological studies to discriminate the influence of
13
14 1059 different factors on FA composition, such as physiological, spatial or even temporal variations
15
16 (Anderson, 2001). Statistical comparison of groups based on FA composition is generally performed
17 1060
18 through non-parametric or randomisation procedures, as FA datasets rarely match the prerequisites
19 1061
20 of parametric statistical tests (Anderson and Walsh, 2013). Analysis of similarity (ANOSIM; Clarke,
21 1062
22 1993) or permutational analysis of variance (PERMANOVA; Anderson, 2001) are among the most
23
24 1063 commonly used statistical tests (e.g. Hughes *et al.*, 2005; Hixson *et al.*, 2015; Parrish *et al.*, 2015;
25
26 1054
27 Sardenne *et al.*, 2016; Mathieu-Resuge *et al.*, 2019). ANOSIM uses ranks of pairwise dissimilarities
28 1065
29 and generates its own statistic by randomisation of rank dissimilarities (Clarke and Warwick, 2001).
30
31 1066 PERMANOVA tests for differences among group centroids (Anderson, 2001). Both methods are
32
33 1067 affected by dispersion heterogeneity, with ANOSIM being more sensitive than PERMANOVA
34
35 1068 (Anderson and Walsh, 2013). PERMANOVA is unaffected by differences in correlation structure
36
37 1069 and seems more powerful to detect changes in FA composition. As such, applying ANOSIM is
38
39 appropriate for a 1-way test (e.g. to compare one species submitted to different diets), but not for
40 1070
41 more complex study designs (Kelly and Scheibling, 2012).
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46 Besides hypothesis testing, constrained ordination (e.g., redundancy analysis, RDA) offers a way
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48 to extract and summarise variability from a multivariate dataset (FA data) that can be explained by
49 1074
50 one or a set of explanatory variables (e.g., locations, species, diets, etc.). In other words, it allows
51 1075
52 regression of multiple response variables (FA) on multiple explanatory variables (chosen according
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54 1076 to the scientific question). More details on this technique can be found elsewhere (Legendre and
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56 1077 Legendre, 2012).
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1079 8.4 Coupling fatty acids with other ecological markers

1080 When tackling elaborate ecological questions, combining FA with other tracers has proven to be very
1081 powerful. Tracers commonly combined with FA include stable isotope ratios of light biogenic
1082 elements (Kharlamenko *et al.*, 1995, 2001; Nyssen *et al.*, 2005), as well as contaminants
1083 ~~concentrations~~ (Kainz and Fisk, 2009; Le Croizier *et al.*, 2016). In many cases, those combinations
1084 are qualitative. However, modern data analysis frameworks allow quantitative coupling of stable
1085 isotope and fatty acids in mixing models (Stock *et al.*, 2018) or ecological niche models (Jackson *et*
1086 *al.*, 2011; Sardenne *et al.*, 2016). Such developments hold great promise for ecological research, due
1087 to the complementarity of these two methods. More generally, multiple factor analysis (Pagès, 2014)
1088 or co-inertia analysis (Dray *et al.*, 2003) could provide flexible, widely applicable and quantitative
1089 ways to couple data from different methods by allowing exploration of the links between multiple
1090 multivariate datasets.

Conclusion

The use of FA analysis in trophic ecology, aquatic nutrition and aquaculture research and development holds considerable promise to improve our understanding of the natural world from organism taxonomy and physiology to ecosystem functioning. However, the application of this approach needs to be carefully considered when initiating a new study to ensure that interpretations proposed for FA data are relevant and appropriate. Although protocols for lipid analysis are well-described, their application to aquatic sciences often requires modifications to adapt to field conditions and to sample types. Here, we presented the current state of knowledge on methods dedicated to aquatic lipid analyses, from sampling to data treatment. We reviewed sample preservation, storage and transport protocols and their effects on lipids, lipid extraction methods, separation of polar and neutral lipids, transesterification and quantification techniques, and available tools for treatment and statistical analysis of FA data. A simplified flowchart of all these steps is represented in Figure 1.

Lipids can be degraded by hydrolysis or oxidation at each stage of the lipid analysis process. We thus encourage processing samples at low temperature under nitrogen atmosphere, and reporting hydrolysis indicators. To limit possible deleterious effects, samples should be handled at the lowest suitable temperature before direct extraction, or be immediately frozen for storage, ideally at $-20\text{ }^{\circ}\text{C}$ for less than one month, $-80\text{ }^{\circ}\text{C}$ for less than 6 months or under liquid nitrogen with no time limit. Long-distance transport of samples must be planned well ahead of the start of the study to obtain relevant import/export permits and to choose appropriate transportation to maintain sample viability and respect transport regulations and national laws. When possible, we recommend transporting samples freshly -frozen under a nitrogen atmosphere. Depending on the objectives of the study, samples can be either directly transesterified or undergo extraction to isolate lipids. Lipid extraction is usually performed following well-established methods using either chloroform or dichloromethane (less toxic), mixed with methanol. We strongly encourage scientists to describe in their publication any modifications from original extraction methods.

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2 1118 The lipid extract is either separated into classes or directly transesterified. Separation of polar and
3
4 1119 neutral lipids prior to transesterification allows for a better interpretation of FA profiles when seeking
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6 1120 information on nutritional status and/or physiology of organisms. Special attention should be paid to
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9 1121 control for the purity and recovery of each fraction. Non-volatile FA are then transesterified into their
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11 1122 volatile derivatives, usually FAME, before being analysed by GC. Acid catalysed reagents, such as
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13 1123 HCl or H₂SO₄ are the most commonly used in aquatic science as they present fewer analytical issues
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16 1124 than BF₃. Hexane is the most widely used solvent to recover FAME after transesterification. Less
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18 1125 toxic alternatives are emerging, but are not applicable to all sample types. The analysis and
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20 1126 quantification of FAME is usually conducted by GC-FID. FAME are identified by comparing their
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23 1127 retention times with those of individual purified standards. They are quantified as relative proportions
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25 1128 using peak areas or by absolute concentration when an internal standard was added before
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27 1129 transesterification. When necessary, peak identification can be confirmed using GC-MS, but this
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29
30 1130 process may require additional derivatisation steps. Data treatment and statistical analysis of FA
31
32 1131 should be carefully applied according to the investigated scientific question. We presented common
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34 1132 multivariate statistical procedures that we consider widely relevant for FA-related problems. This list
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36 1133 is by no means exhaustive. We encourage analysts to explore their data in multiple ways. A good
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39 1134 starting point is usually a simple preliminary visualisation of data. This will not only help to check
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41 1135 for data quality, but also to make informed choices about subsequent procedures. When interpreting
42
43 1136 data and statistical results, it is crucial to take into account the studied organisms and/or ecosystems
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45
46 1137 and their specificities.

47
48 1138 We ~~believe~~argue that methodological consistency, controlled experimentation, and
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50 1139 interlaboratory comparison and calibration can lead to enhanced progress in FA studies and are
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52
53 1140 particularly important for comparative studies. Standardisation and optimisation of FA
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55 1141 methodologies will ultimately improve our understanding on the role of lipids in biological
56
57 1142 mechanisms, and will enable us to use them as ecological or physiological tracers as efficiently as
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59
60 1143 possible.

1
2 1144 **Figure caption**
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5 1145 **Figure 1.** Workflow for the analysis of fatty acids in aquatic samples. The star symbol indicates
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7 1146 recommended methods. Dashed arrows point to suggested storage precautions after each analytical
8
9
10 1147 step. Transport requirements are color-coded, and the coldest transport temperature is always the best
11
12 1148 option regardless of the step. The grey syringe symbol represents the step at which the internal
13
14 1149 standard for recovery and purity should be added. The black syringe symbol represents the step at
15
16 1150 which the internal standard for quantification should be added.
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For Review Only

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41
42
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45 1168 draft.

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3 The Editor, Dr.Howard Browman,
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5 ICES Journal of Marine Science
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9 Re: ICES Journal of Marine Science - Decision on Manuscript ID ICESJMS-2020-247
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12 Dear Dr Browman,
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15
16 Please find attached our revised manuscript now titled: 'State of art and best practices for fatty acid
17 analysis in aquatic sciences' which we submitted for consideration as a review in ICES Journal of Marine
18 Science
19

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21
22 We would like to thank you, and the Reviewers for providing us with valuable feedback that we believe
23 has greatly improved the quality of the manuscript.
24

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26
27 We have agreed with almost all comments and have made modifications to the manuscript as
28 suggested. Following is a detailed point-by-point response to all the comments and suggestions
29 provided.
30

31
32
33 We would like to thank you for considering the publication of our manuscript in ICES Journal of Marine
34 Science
35

36 Yours faithfully,
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40 Lydie I. E. Couturier
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6 Reviewer(s)' Comments to Author:

7 Reviewer: 1

8 Comments to the Author
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10
11 This review is very well written and very useful for the non-initiated and already established
12 'lipidologists' alike. This paper follows a clear objective and provides quite a bit of background
13 information on potential pitfalls when analyzing lipids and their fatty acids. It is also a very critical
14 review and advises the readers how and when methods may be used.
15

16 Fig. 1 is excellent and very informative and easy to follow.
17

18
19 The structure of the data analysis section may be improved. So far it reads like a collection of selected
20 multivariate analysis methods that can be used. But before such methods can be used, research
21 questions must be known and then the appropriate method(s) can follow. For the moment I read a
22 series of methods but their application may be better described.

23 Agreed _ To assist the reader in choosing which data analysis procedure is best suited to their research
24 questions, we added a section detailing potential applications of described methods at the beginning
25 of section 8 (lines 951-961). As suggested by reviewer 1 in PDF, we refrained from going into too much
26 detail, as we feel that this is beyond the scope of this review, and did not want to provide a "laundry
27 list" of example research questions and matching data analysis procedures.
28

29
30 Other comments are provided directly in the text (attached).
31

32
33 Finally, I would like to congratulate the authors for such a well written and concise manuscript that will
34 be very helpful for many readers.
35

36 We thank the Reviewer for his/her positive feedback. We have accepted all the suggested edits and
37 addressed all the comments made directly on the manuscript. We have attached the PDF with our
38 point-by-point response.
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Reviewer: 2

Comments to the Author

Your manuscript deals with important topic and gives recommendations how to prepare fatty acid analysis correctly. As a referee in different journals, I need to regularly complain about protocols used for fatty acid analysis, thus I am happy that you have written this manuscript. Since author list includes many top lipid experts, you have well describe most of things.

However, since the purpose of the manuscript is help fatty acids extraction in future, I recommend you to add some recent development in the field, e.g. performance of GCMS is highly developed in last years, and when using specific ions for quantification, identification and quantification from the low concentration of FAME can be done accurately.

Agreed_ We changed the introductive part of section 7 (lines 94-800) and developed the part concerning SIM in the paragraph dedicated to GC-MS quantification. Nevertheless, we believe that this method is not as straight forward as GC-FID.

Additionally, many places you should add exact references.

Agreed_ relevant references were added where needed

Since your goal is to suggest best practices, you should be bolder in your suggestions for practical work. You could also emphasize the purpose of study in fatty acid analysis, it is requires much more to work quantitatively than work only qualitatively (FA profiles).

Agreed _ added lines 151-154: 'In the case of a quantitative study where the mass of lipid or FA is reported per gram of sample, the sample will have to be weighed before or after storage. Sample mass can be measured from fresh or dry tissue. In the latter case, the dry mass may be measured on a dedicated subsample.'

Title

I found your title a little bit complex: could it be just "State of art and best practices for fatty acid analysis in aquatic ecosystems and (aquacultures)"?

Agreed_ the title was changed to "State of art and best practices for fatty acid analysis in aquatic sciences"

Introduction

You jump directly to the deep end (complex things) explaining the chemistry of fatty acids. I would rather start with something general which is easier to digest. You could start with the line 73...Fatty acids have been analyzed in aquatic sciences....then later on go with complex things.

Agreed _ Added a sentence at the beginning of the paragraph line 50-52 that now reads:

"Lipids and their derived fatty acids (FA) are ubiquitous components of all living organisms. They are essential compounds for the formation of cell and tissue membranes, act as major sources of metabolic energy, provide thermal insulation and serve as chemical messengers."

Lines 76-86 This includes rather long sentence with complex wording and lacks of references. Your tone here could be more confident, now you are repeating old thing that fatty acid may be used in diet modeling, recent development in diet modeling has shown that fatty acid based diet modeling can give accurate results.

Agreed _ we rephrased this whole section, added some references, and now present recent development in quantitative diet modelling (see lines 80-89).

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4 Line 77 lacks references for primary producers FA studies, you could add e.g. Kelly and Scheibling 2012
5 Mar Ecol Prog Ser, Taipale et al. 2013 AME, Jonasdottir 2019 Mar. Drugs.

6 Agreed _ added
7

8
9 Line 78 you could end sentence here after levels and then start new sentence.

10 Agreed _ done
11

12
13 Line 79-82 Earlier you have mentioned that FATM is established by Dalsgaard 2003, now you could
14 briefly inform situation now. My suggestion is something like this:

15 Fatty acid based diet modeling have been employed in some aquatic consumers (Iverson et al. 2004,
16 Galloway et al. 2014, Bromaghin 2017) and recent methodological development can result accurate
17 estimation of diet contribution (Litmanen et al. 2020 Phil. Trans. R. Soc. B 20190651.). However, FA
18 modeling is based on the fact that consumers are not able synthesize PUFA and limitedly bioconvert
19 long-chain PUFA from short-chain PUFA (Castell et al., Langdon, Sargent, von Elert 2002, Taipale et al.
20 2011). However, some consumers are able to bioconversion (Kaybeya et al. 2018), and thus usability
21 of fatty acid in diet modeling should be carefully studied for different aquatic consumers.

22
23 Agreed _ we now present recent developments in quantitative FA-based diet modelling. To do so, we
24 use a modified version of the text suggested here by reviewer 2 (lines 82-88).
25

26
27 Lines 82-90 You could list here much more things, now it seems quite random list.

28 You could add e.g. that PLFA is used for determination for microbial communities and can be used for
29 taxonomic primary production etc...

30 Disagree_ Our introduction does not aim to be exhaustive but to give a broad overview in the use of
31 FA in research. Suggestions by the Reviewer are very specific and we do not feel it is necessary to add
32 them to this paragraph. Such details are well-reviewed in cited references.
33

34
35 Line 135 after types add references

36 Disagree_ We do not feel that a reference is needed for this sentence as it is an empirical statement
37 that is backed up by the review's author consortium
38

39
40 Line 146 in liquid nitrogen? fish muscles are usually freeze-dried and then make as a powder

41 Disagree_ It is safer to work on deep-frozen fish tissue samples than freeze-dried samples for grinding
42 and for subsequent FA analyses (as shown in section 1.3)
43

44
45 Line 178 this is why quantitative work requires more care than qualitative work, fatty acid profiles of
46 freeze-dried samples remains quite similar for long time even at room temperature

47 Disagree _ In this review we do not wish to advocate solely to analyse samples qualitatively. Sardenne
48 et al. (2019) clearly shows the deleterious effect of storing freeze-dried samples for different tissue
49 types, this is why we advise for caution and do not recommend freeze-drying samples when it can be
50 avoided.
51

52
53 Line 325 handling cautions; you have not mentioned here needles (e.g. Teflon-coated) for evaporation
54 unit, according to my pilot study, that is the greatest contamination source for FA samples, especially
55 if high lipid and low lipid samples are handled in the same lab.

56 Agreed _ added line 343 '(i) use material that have been cleaned using acetone or ethanol to
57 remove any potential lipids (e.g. Teflon-coated needles used for evaporation),'
58

59
60 Line 541 references are missing

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2
3 Agreed_ added (e.g. Sardenne et al., 2020)
4

5
6 Line 590 references are missing

7 Disagree _ As we provided an example in the following sentence, we do not feel that this statement
8 needs a reference
9

10
11 Line 594-595 references are missing

12 Agreed_ added (Kaluzny et al., 1985; Kim and Salem, 1990)
13

14 Line 596 Practical recommendations: You could add here your recommendations for solvents

15 Agreed_ we changed the sentence lines 617-618 to 'Before starting to use a separation protocol,
16 we recommend reading handbooks describing the use and applications of SPE for lipid
17 separation including the choice of eluting solvents (Christie, 1982b; Perkins, 1991), and to
18 consult technical resources provided by the SPE manufacturers.'

19 Each laboratory uses different solvents after running specific tests, we cannot really recommend any
20 particular ones.
21

22
23 Line 632 see Schlechtriem et al. (2008, L&O methods 6) comparison with different transmethylation
24 procedures

25 Agreed_ reference added in the introduction paragraph of the section
26

27
28 Line 651 references are missing

29 Agreed_ added (Christie and Han 2010)
30

31
32 Line 771 GC-FID has been long used for FAME determination, due to the low price and robustness,
33 however, linearity and sensitivity of GCMS systems has been greatly improved in last decades. I
34 consider that the best (accurate and works with low concentrations) results can be achieved when
35 running samples with GCMS and using reference ions for identification and SIM (specific ion mode) for
36 quantification (Taipale et al. 2016 doi: 10.3389/fpls.2016.00212). When using specific ions for
37 quantification co-elution does not matter, since SAFA and PUFA differ in major ions. For beginners, it
38 is easy to make mis-interpretation with GC-FID, but with reference and specific ions beginner can easily
39 succeed. The price of GCMS is not so high anymore and thus many laboratories buy GC-MS instead of
40 GC-FID.
41

42 Agreed_ we have clarified the paragraph regarding fatty acid quantification using GC-MS full scan, SIM
43 and isotope labelled internal standards (lines 918-941).

44 Although the main advantage of the SIM approach is indeed quantification of co-eluting compounds,
45 it only applies to a few chromatographic scenarios. In most cases, we believe that separation of FAME
46 on polar columns and quantification by FID are sufficient and remain easier to implement than GC-MS.
47 We totally agree that mis-interpretation is not uncommon in the literature. But pitfalls also exist when
48 using GC-MS for FAME quantification as it requires some spectrometry and chemistry expertise. To
49 some extent, GC-MS is probably more time consuming for data processing than GC-FID. The number
50 of selected ions to be acquired/recorded in SIM may vary from 6 to 10 ions, gathered in group of 3
51 ions, in order to cover the structural diversity of FAME in biological samples. Whenever possible, the
52 best is certainly to have a GC equipped with both FID and MS detectors.
53

54
55 Lines 793- separation: it could be also mentioned that sometimes two separate runs are required with
56 different column, e.g. for PUFA and then separating 16 MUFA (cis-trans). Identification of 16 and 18
57 MUFA requires preparing DMDS adducts (Virtue et al. 1996; J Microbial Methods).

58 We agree, but to avoid increasing the length of the manuscript, we decided not to address specific
59 biological matrices as there are so many in aquatic ecosystems.
60

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3
4 Line 832 even though supelco mix 37 is much used, some Nu Chek prep standard include more FAME,
5 so maybe it could be mentioned. If GC-FID used for identification and quantification, then relative
6 retention (RT) time should be used/recommended.
7

8 Supelco mix 37 is mentioned as example and because it is one of the most commonly used. It is up to
9 each lab to decide which mixture better fits the biological matrix they work on.

10 Line 839 most of all FAME spectrum are in LipidWeb
11 (<https://www.lipidhome.co.uk/ms/methesters/me-arch/index.htm>)

12 Agreed_ added lines 867-868 : "Peak identification is achieved using commercial spectral libraries
13 (e.g. The LipidWeb <lipidhome.co.uk>)"
14

15
16 Line 879 Quantification is most common place to do mistakes in fatty acid analysis. Some researchers
17 used only internal standard for quantification resulting underestimation of PUFA due to the different
18 response of PUFA and SAFA. Therefore, you should clearly phrase here that using only internal
19 standard for quantification is not proper way for quantification.
20

21 Agreed_ we mention in section 7, line 815: "Fatty acids in aquatic samples have a wide range of
22 boiling points and quantification may require application of relative response factors". Also, line
23 904, we said: "Discrimination of FAME may be associated with injection technique and it is
24 often necessary to introduce empirical response factors, determined through careful use of
25 commercially available authentic mixed standards (see section 7.1). A way to estimate the
26 importance of bias from different origins (such as injector, or detector) is for example to analyse
27 a FA mixture of known theoretical mass composition such as the Supelco® 37 Component
28 FAME Mix. It is also possible to analyse a phospholipid with two different aliphatic chains, of
29 different length and saturation (e.g., phosphatidylcholine 16:0/22:6n-3) and confirm that the
30 molar percentage of each FA is 50/50."
31

32 We therefore feel that no modification is needed here.
33

34
35 Line 889 As I already mentioned best results is achieved with GC-MS when using specific ions (major
36 ions: 74, 69, 79)

37 Agreed_ this is addressed in a previous comment above
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Reviewer: 3

Comments to the Author

This study reviews the current state of knowledge surrounding lipid and fatty acid analysis of aquatic organism samples. This includes how to properly take and store samples, shipping/transporting, lipid extraction, separations, transesterification, analysis, and data handling. A very thorough and informative review which was enjoyable to read. The review will especially be useful for those that are new to conducting lipid analyses. I have only minor suggestions.

Title should reflect aquatic nutrition... otherwise it opens up to human nutrition... aquatic nutrition or aquatic animal nutrition?

Agreed_ the title was changed to "State of art and best practices for fatty acid analysis in aquatic sciences"

Line 125: What is 'deep-freeze'? Why not just 'freeze' or 'flash freeze'? Please clarify

Agreed_ we changed the term for flash-freeze

Line 151: Regarding sample handling, what about flash freezing in liquid N₂ before transfer to freezer; and what about ensuring lipid-cleaned tools and vials?

Agreed_ we added in section 1.1, line 154, the sentence: "All material should be lipid-cleaned prior any sampling to avoid cross contamination (see section 3.1)."

and the sentence in section 1.2, line 162, "Ideally, samples should be immediately flash-frozen in liquid N₂ in the field and transported to a freezer using a dry shipper."

Line 175: I would add "and sample/tissue types" after species... different tissues are more susceptible to oxidation due to lipid storage in that organ/tissue and pattern of LC-PUFA storage

Agreed _ added

Line 213: Change 'rapidely' to rapidly

Agreed _ done

Line 213: Again, why deep freeze

Agreed_ changed lines 224-225 to "Conversely, lowering water activity below 0.2 through freeze-drying process may be a factor promoting lipid degradation through oxidation (Labuza et al., 1972)"

Line 224: Lipid degradation is minimal. Yes, but still with an expiry date, what might that be?

Agreed_ we added the sentence line 236 'To our knowledge, maximum storage duration of extracted lipids has not been formally investigated. However, samples are likely to remain useable for months to years.'

Line 306: Reference for this

Agreed_ added line 321 "United Nations Convention on Biological Diversity. 2011. Nagoya Protocol on access to genetic resources and the fair and equitable sharing of benefits arising from their utilization. 25pp."

Lines 302-304: Most institutions have a customs broker that can assist with ensuring proper paperwork so that samples are shipped and received safely. This should be mentioned.

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3 Agreed_ added the sentence line 319 "Some research institutions may have specialised services
4 available to ensure proper paperwork so that samples are shipped and received safely."
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7 Lines 309-310: Reference for this

8 Agreed but CITES is composed of many appendices that are all listed on the official website, there are
9 no dedicated reference for this.
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12 Line 351: Isn't that the purpose of this review, to consolidate information so that the reader doesn't
13 have to scour the literature to determine their methods? I would remove this

14 Disagree_ We strongly feel that it is important to remind readers that reading original methods is
15 essential when applying these protocols. We do not enter in the details of the protocol in this review
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18 Line 368-369: Also requires more solvent... \$\$ consideration

19 Disagree_ It depends and it isn't always true that more solvent is required. We voluntary avoided
20 notion of expensive/cheap within the review as it is a subjective notion.
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23 Line 448: The 2 in 'N2' should be subscript

24 Agreed_changed
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26
27 Line 688-695: The description about heating and loss of sample – not just BF3... something to this effect
28 also needs to be said for other catalysts, maybe the end of line 720. Someone that doesn't know much
29 about transesterification may assume that the heating step is only when using BF3 as a catalyst.

30 Agreed_added sentence line 742 "As with BF3, caution is needed during heating to prevent
31 evaporation."
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34 Line 906-907: I understand the focus on multivariate. Nonetheless, researchers will still analyze
35 individual FA. What about mentioning adjustments for multiple testing, i.e., Bonferroni corrections

36 We agree that univariate statistics can, in some instances, be useful for FA data as well. However, we
37 felt like discussing specific points such as recommending adjustments procedures was outside the
38 scope of this review. Instead, we cited a general statistics and data analysis handbook that readers
39 could use if they need guidance on the topic (lines 949-950).
40

41
42 Lines 908-916: I can appreciate what the authors are saying. One FA cannot represent an entire process
43 or organism. But certain marker FA can be very informative when taken in context of the study. I don't
44 think that is well captured in this paragraph, that FA biomarkers can be useful and have been the focus
45 of many studies.

46 Agreed _ biomarkers can be useful and, provided some conditions are met, deserve to be widely used.
47 We have rephrased this section to ensure that this message is properly conveyed.
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50 Lines 908-916: Also surprising that QFASA is not mentioned somewhere, or maybe in section 8.4

51 Agreed _ QFASA model (Iverson et al. 2004) and more recent developments are now presented in the
52 article's introduction, as also suggested by reviewer 2 (lines 83-88).
53

54
55 Line 1023: Should specify 'aquatic nutrition' or something to that effect... obviously FA are studied in
56 human nutrition but is not a topic of this review

57 Agreed_done
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60 Line 1042: What is fresh-frozen? Freshly frozen? Fresh or frozen?

Agreed_done

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4 Line 1061: Should say "multivariate statistical procedures"
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6 **Agreed_done**
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10 **FATTY ACID ANALYSES IN AQUATIC TROPHIC ECOLOGY, AQUACULTURE AND**
11 **NUTRITION: STATE OF THE ART AND BEST PRACTICES**
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15 4 Lydie I.E. Couturier^{1*}; Loïc N. Michel²; Teresa Amaro³; Suzanne M. Budge⁴; Elisabete da Costa⁵;
16 5 Marleen De Troch⁶; Valeria Di Dato⁷; Patrick Fink⁸; Carolina Giraldo⁹; Fabienne Le Grand¹; Iván
17 6 Loaiza⁶; Margaux Mathieu-Resuge¹⁰; Peter D. Nichols¹¹; Christopher C. Parrish¹²; Fany Sardenne¹;
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60**Abstract**

Determining the lipid content and fatty acid composition of aquatic organisms has been of major interest in trophic ecology, aquaculture and nutrition for over half a century. Although protocols for lipid analysis are well-described, their application to aquatic sciences often requires modifications to adapt to field conditions and to sample type. Here, we present the current state of knowledge of methods dedicated to both marine and freshwater lipid analyses, from sampling to data treatment. We review: (i) sample preservation, storage and transport protocols and their effects on lipids, (ii) lipid extraction, separation of polar and neutral lipids, derivatisation and detection methods, and (iii) available tools for statistical analysis of fatty acid data. We provide recommendations for best-practices in field situations and advocate for protocol standardisation and inter-laboratory calibration.

Keywords: Marine and freshwater lipids, fatty acids, methods, recommended practices, biomarkers, nutrition

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10 47 **Abbreviations:**

11	4-HHE	4-Hydroxy-2-hexenal	HPLC	High performance liquid chromatography
12	4-HNE	4-Hydroxy-2-nonenal		
13	ALC	Fatty alcohols	HPLC-ELSD	High performance liquid chromatography-
14	AV	Acid value		
15	BCl ₃	Boron trichloride		Electrospray light scattering detector
16	BF ₃	Boron trifluoride		
17	BHT	Butylated hydroxytoluene	HPTLC	High performance thin layer chromatography
18	BSTFA	Bis(trimethylsilyl) trifluoroacetamide	LC	Liquid chromatography
19	CH ₂ Cl ₂	Dichloromethane	LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
20	CHCl ₃	Chloroform (Trichloromethane)		
21	CI	Chemical ionisation	LI	Lipolysis index
22	CITES	Convention on International Trade in Endangered Species	MAG	Monoacylglycerols
23			MeOH	Methanol
24	COP	Cholesterol oxidation products	MDA	Malondialdehyde
25	DAG	Diacylglycerol	MUFA	Monounsaturated fatty acid
26	DHA	Docosahexaenoic acid (22:6n-3)	NCI	Negative chemical ionisation
27			O-TMSi	O-Trimethylsilyl
28	DMA	Dimethyl acetal	pAV	Para-Anisidine value
29	DMDS	Dimethyl disulphide	PEG	Polyethyleneglycol
30	DMOX	4,4-Dimethyloxazolazine	PFE	Pressurised fluid extraction
31	FA	Fatty acid	PL	Phospholipids
32	FAME	Fatty acid methyl ester	PUFA	Polyunsaturated fatty acid
33	FATM	Fatty acid trophic markers	PTV	Programmed temperature vaporising injection
34	FFA	Free fatty acids	PV	Peroxide value
35	FID	Flame ionisation detector	SCOT	Support coated open tubular
36	FTIR	Fourier transform infra-red	SE	Steryl ester
37	GC-FID	Gas chromatography-flame ionisation detection	SFA	Saturated fatty acid
38	GC-MS	Gas chromatography-mass spectrometry	SPE	Solid phase extraction
39	GF/C GF/F	Glass microfibre filters	TAG	Triacylglycerol
40	H ₂ SO ₄	Sulfuric acid	TBARS	Thiobarbituric acid reactive substances
41	HCl	Hydrochloric acid	TLC	Thin layer chromatography
42	HI	Hydrolysis index	WCOT	Wall coated open tubular
43			WE	Wax ester

Introduction

Lipids and their ~~derived~~ fatty acids (FA) are ubiquitous components of all living organisms. ~~Fatty acids~~ generally consist of a linear hydrocarbon chain with a carboxyl group (-COOH) at its end. They are characterised by the length of their carbon chain, generally from 14 to 24 (up to 36) atoms, as well as by the position and the number of double bonds (usually from 0 to 6) that determine their degree of unsaturation. ~~Fatty acids~~ are constituents of lipids such as wax and sterol esters (WE, SE), triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG), glycolipids and phospholipids (PL), which show different levels of regulation from ingestion to incorporation. One or several FA are linked either with an ester (or ether / vinyl-ether) bond to an alcohol, mainly glycerol, or with an amide linkage to a long-chain nitrogen base, mainly sphingosine. All of these molecules are categorised as either neutral lipids or polar lipids, with reference to chromatographic methods of lipid class separation but also to their metabolic roles (Christie, 1984). Among neutral lipids, FA-containing compounds include TAG and WE, which are the main storage lipids, whereas DAG, free FA (FFA), and MAG are generally minor constituents. Sterols (both in their free and esterified form) and fatty alcohols (ALC) are also found in neutral lipids. ~~Free~~ FA are rarely present in living organisms, as they may be toxic in high concentrations (Jüttner, 2001). Polar lipids include PL, glycolipids, and sphingolipids. Polar lipids are characterised by a hydrophilic polar head group which, in contrast to their hydrophobic aliphatic chains, allows for the formation of lipid bilayers found in cell membranes. Polar lipid properties are determined by the nature of the polar head groups, the nature of the linkage between the polar head group and aliphatic chain, the length of the carbon chain, as well as the position and the number of double bonds within the FA. These different properties influence the functioning of membrane receptors, the fluidity of membranes, and the stability of the bilayer structure (Hazel and Williams, 1990).

Fatty acids have been analysed in aquatic sciences for over 50 years (Ackman *et al.*, 1968) with the first evidence that FA composition of consumers resembles that of its food (Lee *et al.*, 1971). Over the next 30 years, the concept of FA trophic markers (FATM) gained in popularity in aquatic

Commented [MO1]: Concentration is the abundance of a constituent related to the total volume of a mixture, not per unit biomass. Fatty acids per unit biomass may be called FA 'mass fractions' or FA contents.

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10 76 trophic ecology (Dalsgaard *et al.*, 2003). The concept of FATM is based on the fact that the FA
11 77 composition of some primary producers is characterised by specific FA that can be transferred to
12 78 upper trophic levels and that aquatic consumers generally lack the ability to synthesise long-chain
13 79 polyunsaturated FA (LC-PUFA, $\geq C_{20}$) and have limited capacity for bioconversion (Castell *et al.*,
14 80 1972; Langdon and Waldock, 1981; Sargent *et al.*, 1999), although this view has recently been
15 81 challenged (Kabeya *et al.*, 2018). As such, the FA composition of the food is expected to be reflected
16 82 in the FA profile of the consumer, according to the 'you are what you eat' principle. Today, FA are
17 83 increasingly used to study energy flow in food webs in freshwater, estuarine, coastal and deep-sea,
18 84 environments in all latitudes, and to examine functional responses to stressors (Kelly and Scheibling,
19 85 2012). However, diet is not the only determinant of the FA composition of aquatic consumers. FA
20 86 composition varies with intrinsic factors such as phylogeny and developmental/reproductive stages (
21 87 e.g. Galloway and Winder, 2015), and extrinsic factors such as temperature, salinity and hydrostatic
22 88 pressure (e.g. Wodtke, 1981; Cossins and Macdonald, 1989; Hazel and Williams, 1990), thus
23 89 providing information about physiological condition and habitat type of aquatic organisms (e.g.
24 90 Meyer *et al.*, 2019).

25 91 The analysis of the FA composition of aquatic organisms is based on procedures originally
26 92 developed for applications in food science and medicine. Briefly, once a sample is collected, it is
27 93 immediately stored in the dark at the lowest possible temperature for the shortest period of time with
28 94 added antioxidants, and under a nitrogen atmosphere or vacuum. This is to avoid alteration caused by
29 95 enzymes, heat, oxygen and light. Lipids are then extracted, and lipid classes may be separated using
30 96 chromatography. Next, derivatisation of FA is performed (methylation in most cases) and FA
31 97 composition is determined by gas chromatography. Finally, data are treated to obtain the quantitative
32 98 and qualitative FA profiles of the sample (Figure 1).
33

34 99 There are likely to be as many variations of procedures as there are laboratories (Parrish, 1999).
35 100 Yet, not all may be applicable to aquatic samples. Procedures involved in sample collection, handling
36 101 and storage, as well as extraction and transesterification steps, can cause biochemical modifications
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of FA and differential loss, leading to false or misleading interpretations. Identifying the most appropriate protocols and the best-practices in FA analysis of aquatic organisms may prove to be a difficult task for novices, as available literature is widespread and highly diverse, and many studies do not fully describe their methodology (e.g., “modified Folch extraction”).

Our objective is to provide an up-to-date review of the procedures used for a large number and variety of aquatic samples and laboratories around the world. This review emerged from technical workshops of the conference “*Lipids in the oceans*” held in Brest (France) in November 2018. We answer practical questions that are not necessarily formulated in papers and textbooks, but essential to obtain a reliable FA profile. For example, which organ/tissue of particular specimens should be analysed to be representative in the context of trophic ecology? How are samples properly collected, transported or shipped? What are the most appropriate extraction/transesterification methods or solvent systems? How can one ensure reliable FA identification and quantification? How are FA data analysed? This paper aims to guide both novice and advanced scientists in identifying the most appropriate protocols and best-practices for FA analysis of aquatic samples. We hope that this comparative synthesis will stimulate further international collaborations and facilitate standardisation of lipid analyses.

Only

1 Sample handling and storage before lipid extraction

The choice of biological material, its collection and handling techniques, as well as storage methods, are among the most important steps in lipid analysis (Fig. 1). Lipid degradation can occur by hydrolysis (release of FFA from acyl lipids through the enzymatic activity of lipases) or oxidation of carbon-carbon double bonds. In view of the labile nature of lipids, these processes often occur very rapidly, leading to analytical errors and increased sample variability (Christie, 1984). Handling and storage conditions and methods can help minimise sample degradation and analytical bias. Ideally, lipids should be extracted immediately after collection. The second best option is to deep-freeze samples in liquid nitrogen immediately after collection. As sampling of aquatic organisms is often performed in the field and in remote areas, optimal conditions can rarely be achieved, but samples need to be stored adequately. A broad set of procedures exists, including: (i) sample maintenance on ice, (ii) frozen sample storage from -18°C (standard freezer), to -80°C (laboratory freezers) and/or to -196°C (liquid nitrogen), (iii) freeze-drying of samples, or (iv) sample storage in organic solvents for varying storage periods, from a few hours to years prior to analysis. Adequate storage conditions will prevent (or at least limit) the degradation of lipids through hydrolysis and oxidation processes induced by oxygen, temperature, light and/or enzymatic processes. The rate of oxidation and hydrolysis processes (hereafter referred to as “lipid degradation”) is likely to vary greatly across species and tissue types. These processes can be revealed by an increase in FFA and a decrease in glycerolipids and polyunsaturated FA (PUFA) in samples (Christie, 1984, see **section 4**). Here, we provide an overview of different methods used in the literature and discuss their strengths and limitations.

1.1 Sample type/ sampling strategy

Aquatic samples are highly diverse, ranging from bacteria to whales, and sampling procedures need to be adapted to the nature of the studied organisms/functional groups. Samples of seston and

Commented [MO2]: Adding this paper would be appropriate here:
Rudy MD, Kainz MJ, Graeve M, Colombo SM, Arts MT (2016) Handling and Storage Procedures Have Variable Effects on Fatty Acid Content in Fishes with Different Lipid Quantities. *PLoS ONE* 11(8): e0160497.

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phytoplankton are generally collected through water filtration on pre-combusted glass microfiber filters (GF/C or GF/F), while zooplankton can be sorted manually. Such small organisms or functional groups are often directly extracted as whole organisms and stored in solvent. For other organisms such as larger invertebrates, fish, and aquatic mammals, tissue dissection or biopsy is usually required. In the case of multi-purpose samples, it is possible to grind them under liquid nitrogen and subsample the powder (Soudant *et al.*, 1996). Because it is not possible to describe all sampling strategies here, we highly recommend that any study involving lipid and FA analysis must start with a literature review of available information on targeted organisms or on species that are phylogenetically comparable.

1.2 Sample handling

Maintaining samples on ice is often the first and only practical option available to limit lipid degradation between collection and further processing. Whenever possible, long-lasting dry ice is preferable to water ice. Crushed ice is useful for transportation between locations such as sample transport from the ocean (e.g., small and large vessels) to land, or transport between laboratories. Crushed ice should preferably be used for a few hours, as this method only slows, but does not stop, lipid and FA degradation (Wood and Hintz, 1971; Chaijan *et al.*, 2006), and samples should therefore be frozen as soon as possible. As an alternative, deep-frozen, long-lasting ice packs are a convenient option for short-term storage in the field (e.g., TechniIce™). These ice packs maintain temperatures around 0 °C for a few hours in a well-insulated container (L. Couturier pers. obs.). In the particular case of studies on thermal acclimation or adaptation, it may be preferable to maintain samples at environment temperature rather than to cool them before freezing as it may induce a short-term cold-response. Freezing / thawing samples more than once should be avoided.

1.3 Storage

1.3.1 Frozen storage (−20 °C to −196 °C)

~~Frozen~~ Storing samples at cryogenic temperatures is likely the most common method of preservation before performing FA analyses. Yet, depending on the temperature, storage duration and type of biological material, this may not be sufficient to limit lipid degradation. It can lead to increased FFA and products of oxidation (e.g., Christie, 1984; Gullian-Klanian *et al.*, 2017; Tenyang *et al.*, 2019). Lower freezing temperatures and short-term storage are generally associated with lower lipid degradation (e.g., Baron *et al.*, 2007; Rudy *et al.*, 2016; Gullian-Klanian *et al.*, 2017). The level of lipid degradation in samples is highly dependent on initial freezing temperature and temperature variation during storage, as well as storage duration prior to analyses (Han and Liston, 1987; Ramalhosa *et al.*, 2012; Romotowska *et al.*, 2017). When long-term storage is required, the safest approach is to freeze samples at −80 °C immediately after collection. Although not ideal, long-term storage at −20 °C can be suitable for some species, in particular when samples are kept frozen within the organic extraction solvent. Levels of *n*-3 and *n*-6 PUFA in fish muscle, stored at −30 °C and −80 °C, did not change for up to 12 months in both TAG and phospholipid fractions (Passi *et al.*, 2005). Shark muscle samples archived for up to 16 years at −20 °C retained their FA profiles, while lipid class composition was not analysed (Meyer *et al.* 2017). Impacts of frozen storage on FA content and composition are species-specific and can depend on the total lipid content of the organism and tissue (Rudy *et al.*, 2016). Species with high total lipid content (>10% wet weight) must be treated with increased care (low temperature, and short storage period) to avoid changes in FA content during freezing (Rudy *et al.*, 2016; Sardenne *et al.*, 2019).

Based on the literature cited, samples should be stored for less than 6 months prior to analysis at temperature lower than −20 °C when no information is available on the collected biological material. Note that lipid analysis is also possible for samples stored using brine immersion freezing technique (e.g., fisheries-issued material) (Bodin *et al.*, 2014). The sensitivity of lipids to storage varies

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10¹⁸⁹ according to species and tissues. We therefore recommend evaluating the sensitivity of the samples
11¹⁹⁰ for which there is no information available before the start of the study. For example, a temporal
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13¹⁹¹ monitoring of the lipid composition of the biological material of interest at the available storage
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15¹⁹² temperatures will make it possible to define the optimal conservation strategy.

17¹⁹³ 1.3.2 Freeze-drying

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20¹⁹⁴ Freeze-drying (or lyophilisation) is a low temperature dehydration process of frozen material that is
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22¹⁹⁵ used to reduce enzymatic activity and thus lipid degradation in samples. Freeze-drying samples
23¹⁹⁶ immediately prior to extraction does not affect lipid class or FA compositions of aquatic animal
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25¹⁹⁷ tissues (e.g., Dunstan *et al.*, 1993; Murphy *et al.*, 2003; Sardenne *et al.*, 2019). This can be a good
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27¹⁹⁸ alternative to frozen storage, as freeze-dried tissues can be easier to grind and to transport than frozen
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29¹⁹⁹ tissues. Rehydration may be required to improve the lipid extraction of TAG (e.g., Dunstan *et al.*,
30²⁰⁰ 1993) and to comply with extraction procedures that rely on a particular proportion of water in the
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32²⁰¹ sample. Nonetheless, freeze-dried tissues still need to be carefully stored to prevent lipid degradation.
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34²⁰² Storage duration can also have detrimental effects on lipid composition of samples. In fish, crustacean
35²⁰³ and molluscs, the total duration between freeze-drying and lipid extraction should not exceed one
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37²⁰⁴ month at $-20\text{ }^{\circ}\text{C}$ for quantitative analysis (Sardenne *et al.*, 2019). Similarly, freeze-dried microalgae
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39²⁰⁵ samples should not be stored for extended period of time. The PUFA proportion of filtered *Isochrysis*
40²⁰⁶ *galbana* biomass decreased from 36% to 26% after 3 months storage at $-76\text{ }^{\circ}\text{C}$ (Babarro *et al.*, 2001).
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42²⁰⁷ Proportions of FFA in freeze-dried *Phaeodactylum tricornerutum* increased from 4 mg/g to 6 mg/g DW
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44²⁰⁸ after 35 days at $-20\text{ }^{\circ}\text{C}$ while the degree of oxidation was unaffected (Ryckebosch *et al.*, 2011).
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46²⁰⁹ Variability in freeze-dried sample stability/viability for lipid analysis is likely to reflect differences
47²¹⁰ of biological material and length/duration of the freeze-drying process.

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49²¹¹ Overall, the quality of the freeze-dried storage depends on: (i) the storage temperature (lower
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51²¹² temperatures are the best), (ii) the storage duration (shorter durations are better) and (iii) the lipid
52²¹³ content of samples (lean samples are conserved better than lipid-rich samples). Lipid oxidation in

Commented [MO3]: It may be noted here that sampled loaded onto filters can be both freeze-dried, but for subsequent quantification of lipids/FA it is critical that the empty filters are weighed BEFORE loading them with samples. Then, after lyophilisation, the sample weight can be obtained by subtracting the filter+sample weight from the empty filter weight. This is important to mention because if scientists want to quantify lipids/FA of their phytoplankton or other particles retained on filters, they need to weigh filters before too. This can't be repeated often enough especially for collaborative work. I have learned it the hard way ;-)

Commented [MO4]: This is interesting and suggest that the higher the total lipids the more prone FA are to degrade even in dry samples at $-20\text{ }^{\circ}\text{C}$. Rudy *et al.* (2015) found very little effect of freeze-dried samples on FA (%).

Commented [MO5]: But see Rudy (2015).

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10¹⁴ food products occurs more ~~rapidely~~rapidly when water activity is high (Labuza *et al.*, 1972). Deep
11²¹⁵ freeze-drying may be a factor promoting lipid degradation through oxidation and specific testing
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13¹⁶ should be considered for aquatic samples. Freeze-drying may offer some benefits when used as a
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15¹⁷ short-term preservation method (e.g., during transport), but it can be expensive (necessary equipment)
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17¹⁸ and time-consuming, while potentially shortening the viability of samples for lipid analyses.

Commented [MO6]: What is 'deep freeze-drying'?

18 19¹⁹ 1.3.3 Solvent and fixative storage

20 21 22²⁰ 1.3.3.1 Organic solvent

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24²¹ One common storage strategy is to directly immerse small organisms such as phytoplankton collected
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26²² on filters or small-bodied zooplankton into the lipid extraction solvent (e.g., Marty *et al.*, 1992;
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28²²³ Soudant *et al.*, 1998; Windisch and Fink, 2018). If samples in the extraction solvent (generally
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30²²⁴ chloroform CHCl₃ and methanol MeOH) are kept in the dark and at low (preferably freezing)
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32²²⁵ temperatures, lipid degradation is minimal. For microalgae samples, it is advised to deactivate lipases
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34²²⁶ by a rapid addition of boiling water or isopropanol prior to extraction (Berge *et al.*, 1995; Budge and
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36²²⁷ Parrish, 1999; Ryckebosch *et al.*, 2012).

Commented [MO7]: But see my comment above re 'pre-weighing of filters'!

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38²²⁸ Synthetic antioxidants (0.01% butylated hydroxytoluene, BHT or 0.1% tertiary
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40²²⁹ butylhydroquinone, TBHQ) can be added to solvent-stored samples to reduce radical formation and
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42²³⁰ oxidative losses of unsaturated lipids (Christie, 2003; Ryckebosch *et al.*, 2012). However, addition
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44²³¹ of synthetic antioxidant is unnecessary in samples containing large amount of natural antioxidants
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46²³² such as microalgae (Ryckebosch *et al.*, 2012). Note that BHT elute with short-chain FA and when
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48²³³ present in large quantities, it can overlap with FA of interest.

49²³⁴ 1.3.3.2 Fixatives

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51²³⁵ Fixatives such as ethanol or formalin are commonly used for biodiversity, taxonomy, morphological,
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53²³⁶ or DNA analyses. The use of fixed samples opens new research opportunities, by making it possible
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55²³⁷ to analyse historical samples collected for purposes other than lipid analysis. However, fixatives

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influence the chemical composition of the samples and should therefore not be used as a routine procedure for lipid analyses. For example, FA composition of crustacean tissues is altered in formalin-preserved samples, but not in ethanol preserved samples (Phleger *et al.*, 2001). Direct evaluation of fixative effects on lipids are scarce and preliminary tests on different sample types are highly recommended.

For Review Only

2 Transport and shipping

The packaging, transport and shipping of samples for lipid analysis is a complex process, driven by safety laws, financial costs, stringent storage conditions, and limited duration of storage. Specific legal regulations, including quarantine, must be followed and they can vary, depending on whether the samples are shipped domestically or internationally, and whether there are stopovers during long distance travels. Shipping recommendations to ensure that samples arrive at their destination in good condition include triple packaging with a rigid outer layer and absorbent material, appropriate cooling, accurate labelling (i.e., name, address, phone number, e-mail address, size and weight of the package), and complete legal documentation (e.g., itemised list of content, permits) (IATA, 2010). This section reviews different options of packaging for shipping and traveling, and gives an overview of the main flying regulations and key legal information.

2.1 Shipping samples

Long-distance shipping from the sampling site to the laboratory is challenging. Multiple issues need consideration, such as the conservation state of the samples prior to transport (cooled, frozen, deep-frozen, freeze-dried), the distance, the duration and the type of transport. As samples are generally prone to degradation, temperatures must be kept low and oxidative conditions reduced as much as possible. Regardless of the method used, we recommend only to ship a part of the original sample and keep a backup sample when possible, and to take great care in the packaging (minimum empty space, adapted container to accommodate samples and cooling methods).

When possible, frozen samples should be shipped using dry shippers, which are aluminium containers flooded with liquid nitrogen. They contain adsorbent material that prevents spills of liquid nitrogen during shipment and have attached temperature loggers. Samples can be stored in these conditions for several weeks. However, dry shippers may not be readily available at sampling locations or in laboratories. Restrictions for the transport of liquid nitrogen also need to be considered

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(e.g. in airplanes or closed vehicles). With transporter authorisation, frozen samples can also be shipped on dry ice depending on transport duration (about 24 h for 2.5 kg of dry ice). Alternatively, deep frozen (-80°C) aqueous ice packs or reusable technical ice packs (e.g. TechniIce™, 2019) can be used. A cost-effective option is to freeze-dry samples prior to shipping, as it reduces both the volume to be shipped and the risk of lipid hydrolysis by removing water (see section 1). When dry ice packaging or deep-frozen ice packs are not available at the sampling location, freeze-dried samples should be kept cool and in an oxygen-free atmosphere by flushing containers with nitrogen gas and then sealing prior to packaging and shipping. It is also possible to ship samples with small amounts of solvents with respect to international regulations. Note however that depressurisation in aircrafts promotes solvent leakage (F. Pernet pers. obs). To limit this phenomenon, tubes and caps can be coated with Teflon. Alternatively, preserved and/or extracted samples in solvent may be evaporated and flushed with nitrogen so that dried extracts can be air-transported as frozen biological material. Dried lipid extracts will have to be re-immersed in solvent as soon as possible upon arrival. We however recommend to perform preliminary testing before proceeding with this method.

The gold standard for shipping samples are specialised transport companies that maintain samples on dry ice and guarantee express delivery to the final destination. However, these options may not be practical for some laboratories and/or available for all sampling sites and destinations. Care should be taken when selecting the shipping company, as several unfortunate experiences have shown that some may not be reliable in maintaining the cold transportation chain.

2.2 Flying with samples

Although courier is preferable, one might consider putting samples in checked luggage. One of the safest options to transport samples as checked luggage is to use a dry shipper. However, airlines may have different regulations in transporting liquid nitrogen. Non-harmful materials, such as dry ice or technical ice packs are the best option to meet standard luggage regulations (e.g., < 2.5 kg of dry ice

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10²⁹¹ per person) and safety (e.g. the package permitting the release of carbon dioxide gas when
11²⁹² transporting dry ice).

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13²⁹³ Transporting freeze-dried samples kept in a hermetically closed bag (or a thermos filled with ice)
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15²⁹⁴ is likely the safest and cheapest option. Upon arrival, samples need to be stored in a freezer
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17²⁹⁵ (preferentially at -80°C) or immediately put in a solvent solution for lipid extraction (see **section 1**
18²⁹⁶ for more details on storage duration). Dried lipid extract should be transported as frozen samples and
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20²⁹⁷ be returned to solvent as soon as possible (see section 2.1). Note that preservation methods requiring
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22²⁹⁸ flammable solvents (e.g. ethanol, methanol) are not permitted on aircraft within passenger luggage.
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24²⁹⁹ Recently, the emerging technology of drones opens new opportunities for a rapid and customisable
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26³⁰⁰ aerial transport of sensitive samples from remote areas to laboratory facilities (e.g., Amukele *et al.*,
27³⁰¹ 2017).

28 29 30³⁰² 2.3 Regulations and permits

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33³⁰³ Prior to initiating sample collection and considering transporting them, it is necessary to be well
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35³⁰⁴ informed on import and export regulations that differ widely across countries and studied organisms'
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37³⁰⁵ status. It can be difficult to obtain the necessary information within custom departments and the
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39³⁰⁶ paperwork involved. To transport samples out of a country, it is often necessary to follow The Nagoya
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41³⁰⁷ Protocol on Access and Benefit Sharing. Although it was initially stated that this only applies to
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43³⁰⁸ samples for molecular research, all transports of tissue for biochemical screening are now included.
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45³⁰⁹ This applies only for countries that have signed and ratified the Nagoya Protocol.

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47³¹⁰ When samples contain species listed in the Convention on International Trade in Endangered
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49³¹¹ Species of wild fauna and flora (CITES), it is necessary to request an import/export CITES permit
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51³¹² from each of the countries involved. This needs to be organised well ahead of sample collection, as
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53³¹³ it can take up to 3 years to obtain these permits. Additional research permits may be required for the
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55³¹⁴ import and export of samples depending on national regulations and target species.

3 Lipid extraction

Because of their diverse structures, lipids range from non-polar to highly polar, which makes the selection of a solvent with matching polarity challenging. Methods based on chloroform and methanol (Folch *et al.*, 1957; Bligh and Dyer, 1959) have long been used across a wide range of matrix types with great success. Current alterations in environmental regulations have brought restrictions on the type and amount of solvent usage in laboratories worldwide, encouraging the exploitation of alternative organic solvents and novel eco-friendly methodologies for extraction (e.g., accelerated solvent extraction). Here, we ~~will~~ review both the well-established techniques and the newly developed methods; we caution readers that full validation must be carried out with a given biological material before adopting any modified or adapted method.

3.1 Handling cautions

Contamination is an issue that needs to be carefully addressed at every stage of sample handling. It is recommended to: i) avoid plastic containers, tools or tips, ii) combust at 450 °C for 6 h or solvent-rinse all glassware and glass fibre filters, iii) use glass tubes fitted with a Teflon-lined screw caps, iv) prefer amber glassware (or aluminium foil protected) to limit solvent photo-degradation, v) use the highest quality solvents for material/tool cleaning, extraction, or elution, vi) perform blank and reference samples for each batch of samples, and vii) wear laboratory gloves to limit exposure to toxic solvents and cross-contamination.

3.2 Sample preparation

Regardless of the technique used, extraction of lipids from aquatic samples requires that the solvent penetrates the sample particles so that it can make contact and solubilise the lipids present. The most efficient extractions ensure that the particle size is as small as possible. The gold standard is to

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10³³⁸ homogenise samples with a ball mill homogenizer (at $-196\text{ }^{\circ}\text{C}$ using liquid nitrogen with wet
11³³⁹ samples). With wet plant or animal tissue samples at macro-scales, this may be as simple as chopping
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13³⁴⁰ or blending. For wet microalgae, grinding of samples after immersion in solvent works well to break
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15³⁴¹ open cells. Small organisms (roughly between $1\text{ }\mu\text{m}$ and 1 mm ; on a filter or ~~not~~without) can also be
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17³⁴² directly extracted by direct immersion in the solvent mixture. The use of ultrasound-assisted
18³⁴³ extraction with a probe or bath may aid in breaking cell walls (Pernet and Tremblay, 2003). When
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20³⁴⁴ samples have been freeze-dried (**section 1**) for preservation, they may be most conveniently ground
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22³⁴⁵ with mortar and pestle and it may be necessary to rehydrate prior to carrying out the extraction. Any
23³⁴⁶ of these techniques, when applied prior to subsampling for analysis, should also serve to homogenise
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25³⁴⁷ samples effectively, to ensure representative sampling. These diverse techniques also illustrate the
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27³⁴⁸ need to adapt the preparatory steps and approach to the sample matrix.

28 29 30³⁴⁹ 3.3 Traditional extraction methods

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33³⁵⁰ While many researchers consider Folch et al. (1957) and Bligh and Dyer (1959) extraction methods
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35³⁵¹ to be similar, based on their use of chloroform and methanol, the two are quite different and were
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37³⁵² developed for different objectives. Prior to using those methods, one would do well to read the
38³⁵³ original publications. Both methods rely on the formation of a biphasic mixture, with a lower layer
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40³⁵⁴ principally consisting of chloroform, and containing the isolated lipids, and an upper phase consisting
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42³⁵⁵ of methanol and water and containing the non-lipids. The Folch et al. procedure, using $\text{CHCl}_3\text{:MeOH}$
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44³⁵⁶ (2:1, v:v) and a solvent to sample ratio of at least 20:1, was designed as a general and robust method
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46³⁵⁷ for recovery of lipids from animal tissues (i.e., brain, liver, muscle), now applicable to several tissues
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48³⁵⁸ and species with a range of lipid ~~concentration~~ content (Iverson et al. 2001). The Bligh and Dyer
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50³⁶⁰ method uses a more polar solvent mixture of $\text{CHCl}_3\text{:MeOH: H}_2\text{O}$ (1:2:0.8, v:v:v) with a 4:1 solvent
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52³⁶¹ to sample ratio, and was originally developed to rapidly extract lipids, principally polar lipids, from
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While the Folch *et al.* method is more complicated to perform, it is more reliable and applicable to almost all sample types. A key aspect of the original Folch *et al.* method that is often overlooked, is the washing of the bottom phase that contains lipids with a mixture of solvents of the same proportions as originally found in the upper phases. As originally described by Folch *et al.* (1957), the upper phase consists of CHCl₃:MeOH:H₂O at 3:48:47 and while some groups do use a 50:50 MeOH: H₂O wash as recommended by Christie (1982), it is exceedingly rare that the correct mixture is used. Thus, almost all Folch *et al.* procedures are best described as ‘modified’. The Bligh and Dyer method is easier to carry out, but requires greater consideration of sample type and potential modification of the procedure. For instance, Christie (1982) notes that with high fat samples, an initial pre-extraction with chloroform or diethyl ether is necessary to extract TAG that would otherwise remain unrecovered. The use of the correct proportions of solvents and water are critical for quantitative lipid recovery with both methods. One must take care to adjust the volume of water added when samples with varying moisture contents are analysed (i.e., freeze-dried material vs fresh tissue) and it is often necessary to determine moisture content before beginning extraction.

These basic procedures may require some adjustments depending on lipid matrix before and/or during extraction to increase the yield or specificity of lipids to be analysed. For example, it may include inactivation of lipase, changes of solvent proportions, or cell disruption (e.g. Kumari *et al.*, 2011; Ryckeboosch *et al.*, 2012). In a general manner, authors must thus refer to the available literature on their model organisms or matrices. Given the numerous modified extraction procedures that reflect the diversity of aquatic biological matrices, it is also essential that authors detail their protocol in publications when it differs from original methods.

Commented [M08]: This subsection is extremely well written and very informative.

3.4 Alternatives to chloroform: toward less toxic solvents

3.4.1 Dichloromethane

Many laboratories have become increasingly conscious of a range of occupational health and safety areas in recent decades. Lipid studies historically used chloroform as a major solvent in many steps including extraction. The less toxic dichloromethane (CH_2Cl_2) has regularly been used instead of chloroform for lipid extraction in many aquatic labs. This replacement originated from food chemistry research (e.g. Chen *et al.*, 1981) and was later formally reported for aquatic samples (e.g., Cequier-Sánchez *et al.*, 2008). Regardless of solvent choice, both dichloromethane and chloroform may degrade on storage to produce HCl and phosgene (Noweir *et al.*, 1972). Phosgene is a much more acutely toxic chemical than either chloroform or dichloromethane, and inhalation can lead to pulmonary oedema (e.g., Snyder *et al.*, 1992). Chloroform degradation or insufficiently stabilised may lead to lipid degradation (i.e., empty chromatograms, including disappearance of the internal standard) (Le Grand and Pernet, pers. obs). To limit phosgene production, chloroform is stabilised with either ethanol or amylene, with ethanol being the more effective stabiliser. Because dichloromethane is less susceptible to degradation than chloroform, it is typically stabilised with alkenes only. Safe lab practices, including restricting solvent use to fume hoods, can effectively avoid risks associated with chlorinated solvents and phosgene formation. In the field, when samples are preserved by direct immersion in solvent, caution is advised.

3.4.2 Methyl-tert-butyl ether

Methyl-tert-butyl ether (MTBE) was recently used as a less toxic solvent for the extraction of lipids, but had lower recovery rates than chlorinated solvents (Sheng *et al.*, 2011; Ryckebosch *et al.*, 2012). However, the MTBE based extraction protocol appeared especially well adapted to high-throughput lipidomics, as the lipid extract recovered in the upper phase was cleaner with non-extractible material remaining in the lower phase (Matyash *et al.*, 2008).

3.5 The special case of ethanol preserved samples

The availability of historically preserved samples can often occur for a range of lipid studies (see section 1.3.3.1). However, because ethanol extracts some lipids, the sample and all added ethanol must be used in the lipid extraction protocol (Phleger *et al.*, 2001). When using the Bligh and Dyer (1959) method, the extraction should be performed with chloroform (or dichloromethane), ethanol and water, where ethanol replaces methanol. Care is needed when selecting historic ethanol-preserved samples for lipid analysis and in which the ethanol preservative may have been replaced during storage.

3.6 Other methods

Accelerated Solvent Extraction (ASE, Dionex), also known as Pressurised Fluid Extraction (PFE), is widely used method based on a controlled increase in solvent temperature and pressure during extraction. This process maintains the liquid state of solvents near their supercritical region where they have high extraction properties. Several ASE parameters can be adjusted to improve the extraction, such as temperature, pressure, extraction duration (including pre-heating) and number of extraction cycles. All of these parameters affect the final solvent volume and can be adjusted individually to each sample type to optimise the extraction yields and quality (Schäfer, 1998). This sample type-specific adjustment enables high extraction efficiency with a low solvent volume and a short extraction time which is beneficial from a cost and environmental standpoint. Microscale lipid extraction of fish tissues (100 mg) using ASE was shown to be as quantitatively and qualitatively efficient as classical lipid extraction methods (Dodds *et al.*, 2004).

3.7 Concluding remarks

This section did not aim to be exhaustive in describing existing extraction methods. When comparing data from different studies, it is important to consider the lipid extraction techniques used as well as

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10⁴³⁰ handling and storage methods. When working on new matrices and developing new or optimised
11⁴³¹ extraction protocols, we recommend to compare them with Folch et al. (1957) or to Bligh & Dyer
12⁴³² (1959) methods, which generally remain the gold standards for lipid recovery. Ensuring complete
13⁴³³ lipid extraction is critical when applying new protocols. Performing successive lipid extractions on a
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15⁴³⁴ same sample and measuring its lipid content at each step is a suitable approach to establish at which
16⁴³⁵ stage the sample is totally lipid-depleted. Alternatively to successive extractions, the fatty acid methyl
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18⁴³⁶ ester (FAME) content of the lipid extract can be compared to FAME recovered after direct
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20⁴³⁷ transesterification (**section 6**). A number of studies have compared the effect of extraction techniques
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22⁴³⁸ (ASE, Soxhlet, manual) on lipid recovery and FA concentration (e.g. Ewald *et al.*, 1998; Mulbry *et*
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24⁴³⁹ *al.*, 2009; Balasubramanian *et al.*, 2013). While all report differences, there is little agreement as to
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26⁴⁴⁰ which may be an optimal method, likely due to substantial differences in the followed protocols.
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Commented [MO9]: It may also be important to better
explain why complete lipid extraction is important. For
example, some authors may be interested in expressing
'only' relative lipid values (%) and thus may not fully extract
lipids, thinking that lipids and their FA are equally
distributed in samples. Perhaps include extraction efficiency
values (how many percent of total lipids are extracted after
the first, second, third extraction).

4 Indicators of sample degradation

Sample lipids may degrade through hydrolysis of ester bonds or through oxidation of carbon-carbon double bonds. These are two, largely independent processes leading to different compounds or groups of compounds, which may be used as indicators of sample degradation. Therefore, samples showing indicators of hydrolysis may not have been oxidised and *vice versa*. Unless necessary for a short time period (e.g. shipping), purified lipid extracts are susceptible to oxidation and should be kept under N₂ atmosphere with solvent (Folch mixture is generally preferred).

4.1 Hydrolysis

The most frequently encountered aquatic lipids are acylglycerols (TAG, PL, and glycolipids) in which FA are esterified to a glycerol backbone. There are also ether lipids that replace one ester bond with an ether linkage or a vinyl ether linkage. Esters of carboxylic acids undergo hydrolysis in a reversible reaction which is catalysed by hydrolase enzymes called esterases present in biological samples. Lipases, a subclass of esterases, are found in animals, plants, fungi, bacteria and even in certain viruses (Beisson *et al.*, 2000). Lipid hydrolysis can be assessed from the FFA content, and from the lysophospholipid content when samples are dominated by PL. Standard measurements of oil quality are the acid value (AV) and FFA content, determined in the oil industry by titration and chromatography, respectively. However, both these measurements are rarely used outside of the industrial context.

Many chromatographic systems have been used over the past years for quantitative analysis of major lipid classes including FFA. Free FA can be easily separated from extracted lipids by means of well-known methods based on either liquid (LC) or thin layer chromatography (TLC) (reviewed by Fuchs *et al.*, 2011). For example, HPLC with light-scattering detection (HPLC-ELSD) has been successfully used to detect and quantify FFA in fish with a relatively short processing time and highly reproducible measurements (Christie, 1985; Silversand and Haux, 1997). As for LC, a number of

improvements to classic TLC have led to more sophisticated forms, providing better separation efficiencies and quantifications. High-performance thin-layer chromatography (HPTLC) coupled with densitometry or TLC coupled with flame ionisation detection (TLC-FID, Iatroscan, Shantha, 1992) have also been used to quantify lipid classes and FFA in samples from different origins including marine (Lochmann *et al.*, 1996; Parrish *et al.*, 2009), terrestrial (Zoumpoulakis *et al.*, 2012) and freshwater species (Litz *et al.*, 2017). In food studies, FFA determined by TLC-FID is generally equivalent to AV (Nishiba *et al.*, 2000). TLC is normally less expensive and less time-consuming than HPLC-based methods that can, of course, also be used to provide similar results.

The hydrolysis index of non-polar acyl lipids (HI) and the lipolysis index of all acyl lipids (LI) can be used as degradation indicators (Week *et al.*, 1993; Parrish *et al.* 1995) and are calculated as:

$$HI = \frac{FFA + ALC}{TAG + WE + FFA + ALC} * 100$$

$LI = \frac{FFA + ALC}{\text{total acyl lipids} + ALC} * 100$ LI values are always lower than HI values for any sample because all acyl lipids are considered.

4.2 Oxidation

Lipid oxidation mainly occurs through autoxidation when lipids react spontaneously with atmospheric oxygen. The process is usually autocatalytic, with oxidation products accelerating the reaction so that it increases over time. Other catalysts such as light, heat, enzymes and metals lead to photo-oxidation, thermal oxidation and enzymatic oxidation. Fatty acid oxidation is a process in which free radicals cause unsaturated FA to react with molecular oxygen forming acyl hydroperoxides, which can then breakdown into a variety of secondary products. A decrease in PUFA concentration may indicate oxidation as they are particularly susceptible to degradation (Sasaki and Capuzzo, 1984).

Lipid hydrolysis may accelerate lipid oxidation due to the formation of FFA that can be substrates for oxidation reactions, as found *in vivo* (Saponaro *et al.*, 2015). High levels of PUFA in lipids make

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them susceptible to oxidation (Koshio *et al.*, 1994). Oxidation of *n*-3 and *n*-6 PUFA leads to the formation of highly chemically reactive compounds such as malondialdehyde (MDA), 4-hydroxy-2-hexenal (4-HHE) and 4-hydroxy-2-nonenal (4-HNE). HHE is an aldehyde derived from *n*-3 PUFA, and HNE from *n*-6 PUFA.

A number of physical and chemical tests, including instrumental analyses, are available to determine primary and secondary products of oxidation (Shahidi and Zhong, 2005). Oil supplement, food and other industries have a range of standard measurements for determining oil oxidation. These include the internationally recommended peroxide value (PV) and *p*-anisidine value (pAV) (Nichols *et al.*, 2016). For example, PV can be measured by titration and 2-thiobarbituric acid reactive substances (TBARS) by spectrophotometry, and both can be determined using highly precise Fourier transform infrared spectroscopy (FTIR). MDA is commonly monitored using the direct TBARS test (Fernández *et al.*, 1997; Shahidi and Zhong, 2005) and with HPLC (Mendes *et al.*, 2009), while HHE and HNE can be quantified by GC-MS (using chemical ionisation) (Kenmogne-Domguia *et al.*, 2014). Recent analytical developments allowed simultaneous quantification of MDA, HHE and HNE by LC/APCI-MS and LC-MS/MS (Douny *et al.*, 2016; Tullberg *et al.*, 2016). Together with pAV, MDA, HHE and HNE are secondary markers of oxidation while PV, hydroperoxides and conjugated dienes are primary markers. However, it has to be noted that MDA, HHE and HNE quantifications do not necessary reflect PV and anisidine values (Viau *et al.*, 2016).

Sterols are also a source of oxygenated lipids named oxysterols which are sterols bearing a second oxygen functionality. Cholesterol oxidation likely proceeds in conjunction with oxidative decomposition of coexisting PUFA (Ohshima *et al.*, 1993). Cholesterol oxidation products (COP) can be determined by cold saponification of lipids (Ohshima *et al.*, 1993), followed by enrichment of COP by solid-phase extraction (SPE), and then quantification and identification by GC and GC-MS (Pickova and Dutta, 2003).

4.3 Concluding remarks

To conclude, we encourage as a minimum the assessing and reporting of hydrolysis indicators as a quality control criterion of sample handling and extraction procedures. The threshold above which sample/data quality should be questioned depends on the sample matrix, the logistical context and the study objective. In any case, the extent of either form of degradation should ideally be evaluated and compared with baseline values from fresh and properly extracted samples.

5 Separation of neutral and polar lipids

Fatty acids are incorporated almost unaltered into the storage lipids such as WE, TAG, and ALC, reflecting the FA profiles of the consumed food (Dalsgaard *et al.*, 2003). In contrast, polar lipids are primarily involved in cell membranes which regulate and adjust their FA composition in response to environmental factors like temperature, salinity and hydrostatic pressure (Hazel and Williams, 1990). As such, inference of an organism's diet based on total lipids can be biased by the ratio of FA originating from storage (mostly neutral lipids) and membrane lipids (mostly polar lipids). This ratio varies widely across species, ontogenetic stages, reproduction, nutritional status and tissue types. For example, in small pelagic fish from the Mediterranean, the contribution of TAG stored in the muscle in winter was 1.5%, 5.2% and 47.6% of total lipids for sardine, anchovy and sprat, respectively, the remainder being mostly polar lipids (Pethybridge *et al.*, 2014). This demonstrates that bulk FA composition variations among species can be confounded with variations in lipid class composition. However, many studies in trophic ecology have based their trophic interpretation on the FA composition of bulk lipids without considering the FA distribution between storage and structural lipids.

5.1 When should the lipid fraction be separated?

Lipid class composition should be assessed alongside the FA composition to decide whether lipid class separation is necessary for robust dietary inference. Calculating the ratio of storage to structural lipids provides a comprehensive assessment of the cost/benefit of separating neutral and polar lipids fractions. For samples dominated by storage lipids, the FA composition of bulk lipids will mostly mirror the diet, and lipid class fractionation is not mandatory (e.g., fatty fish species, marine mammal blubber, and digestive gland and liver of marine organisms). However, for samples rich in structural lipids, fractionation of lipid classes will provide more reliable dietary indicators. The analysis of FA in polar lipids can inform on nutritional requirements of the study organism and on environmental

Commented [MO11]: Liver FA in freshwater fish do not reflect dietary FA. In freshwater fish, hepatocytes can convert dietary FA to other FA, mostly long-chain PUFA such as DHA, in particular if dietary DHA is scarce as the case in stream fishes (see: [Murray D.S., Hager H.H., Tocher D.R. & Kainz M.J. \(2014\) Effect of partial replacement of dietary fish meal and oil by pumpkin kernel cake and rapeseed oil on fatty acid composition and metabolism in Arctic charr \(*Salvelinus alpinus*\). *Aquaculture*, 431, 85-91.](#))

Murray D.S., Hager H.H., Tocher D.R. & Kainz M.J. (2014) Effect of partial replacement of dietary fish meal and oil by pumpkin kernel cake and rapeseed oil on fatty acid composition and metabolism in Arctic charr (*Salvelinus alpinus*). *Aquaculture*, 431, 85-91.

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10⁵⁴⁶ conditions prevailing at the time of sampling. Comparing the relative FA proportions in polar and
11⁵⁴⁷ neutral lipids allows the identification of the dietary FA that were selectively retained/eliminated to
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13⁵⁴⁸ form cell membranes (e.g. Delaunay *et al.*, 1993; Plante *et al.*, 2007). The unsaturation level of FA
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15⁵⁴⁹ and PUFA incorporated in polar lipids is linked to the temperature in ectothermic organisms (Hazel
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17⁵⁵⁰ and Williams, 1990; Hall *et al.*, 2002; Pernet *et al.*, 2007) and can be associated with the basal
18⁵⁵¹ metabolic rate (Hulbert and Else, 1999; Pernet *et al.*, 2008). Consequently, the separate analysis of
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20⁵⁵² neutral and polar lipids is a practical approach to address questions on trophic ecology as well as on
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22⁵⁵³ nutrition and comparative physiology.

23⁵⁵⁴ To establish trophic relationships of species for which all potential food sources are known, a good
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25⁵⁵⁵ strategy is to analyse the FA composition of total lipids in food sources (e.g., prey) and of neutral
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27⁵⁵⁶ lipids in consumers to characterise their diet (e.g., predators). Such approach was implemented by the
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29⁵⁵⁷ pioneers of the use of FA as trophic indicators (reviewed in Dalsgaard *et al.*, 2003). Alternatively,
30⁵⁵⁸ when study organisms are both prey and predator, or when predator-prey relationships are not well-
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32⁵⁵⁹ known, we suggest at a minimum analysing FA from both neutral and total lipids. Beyond trophic
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34⁵⁶⁰ considerations, quantification of lipid classes provides useful information on the physiological
35⁵⁶¹ condition of organisms (Fraser *et al.*, 1989) and the quality of sample preservation through FFA and
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37⁵⁶² lysophospholipids levels (see **section 4**).

38 39 40⁵⁶³ 5.2 Commonly used separation methods

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43⁵⁶⁴ Storage lipids (SE, WE, TAG, DAG, ALC) and FFA are defined as ‘neutral’ because they are
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45⁵⁶⁵ hydrophobic molecules lacking charged groups, whereas PL are defined as ‘polar’ because they have
46⁵⁶⁶ a charged hydrophilic head. Neutral and polar lipids can be separated from each other by TLC or by
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48⁵⁶⁷ adsorption chromatography using either laboratory packed or commercial prepared silica columns. A
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50⁵⁶⁸ simple and widely used protocol for separation of neutral and polar lipids consists of a short column
51⁵⁶⁹ of silica prepared in a glass disposable Pasteur pipette plugged with cotton wool (Christie, 1982b).
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53⁵⁷⁰ The sample is loaded on the top of the column and washed at low pressure with chloroform (or

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dichloromethane) often mixed with methanol, (e.g., CHCl_3 :MeOH, 98:2 v/v) to elute neutral lipids. It can then be washed with acetone to obtain acetone mobile polar lipids, generally dominated by pigments and glycolipids. Finally, a last wash is performed with methanol to yield polar lipids dominated by PL. Some PL with lower polarity may elute with acetone, while some glycolipids with higher polarity may elute with methanol. Including an elution step with acetone may not be necessary in non-plant samples as they contain negligible amounts of glycolipids (Christie, 1982b). Yet, the latter step (elution step with acetone) can be useful in deciphering trophic links in deep-sea fishes (Şen Özdemir *et al.*, 2019). Diethyl-ether:acetic acid (98:2, v/v) can be used to recover pigments, prior to elution of glycolipids and PL (Da Costa *et al.*, 2017). Eluent and sample volumes must be adjusted to optimise separation. It is also important to note that hydration of silica changes the efficiency of retention through a partition effect between bound water and polar lipids (Christie, 1982b). The use of silica hydrated with 6% water (m/m) can improve the recovery of polar lipids from marine bivalves (e.g., Marty *et al.*, 1992; Pernet *et al.*, 2006).

Commercially pre-packed columns connected to a vacuum manifold are widely used instead of the manually packed Pasteur pipette (Christie, 1992; Ruiz-Gutiérrez and Pérez-Camino, 2000). This procedure, often referred to as SPE, is time effective and provides similar results as classic column chromatography (Juaneda and Rocquelin, 1985). However, it is difficult to achieve quantitative recovery of PL with this approach. Only ~62% of total lipids were recovered from clam tissue using SPE due to the selective retention of phosphatidylcholine as silica is not rehydrated in these pre-packed columns (Pernet *et al.*, 2006). Commercial SPE columns are available with a wide range of chemically-bonded stationary phases. These have great potential for the isolation of specific lipid classes, yet surprisingly few applications have been described to date. For example, an alternative method developed by Kaluzny *et al.* (1985) using aminopropyl-bonded silica is suitable for separating lipid classes in oyster tissues, though particular care is required to recover the acidic lipids quantitatively (Kim and Salem, 1990; Pernet *et al.*, 2006). Aminopropyl bonded phases have also

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10⁵⁹⁶ been used to separate groups of lipid classes, including neutral lipids such as cholesterol, and non-
11⁵⁹⁷ acidic PL, and they are especially useful for the isolation of the FFA fraction from lipid extracts.
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14⁵⁹⁸ 5.3 Practical recommendations

17⁵⁹⁹ Before starting to use a separation protocol, we recommend reading handbooks describing the use
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19⁶⁰⁰ and applications of SPE for lipid separation (Christie, 1982b; Perkins, 1991) and to consult technical
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21⁶⁰¹ resources provided by the SPE manufacturers. Specifically, care must be taken to avoid sample
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23⁶⁰² overload and subsequent cross-contamination. This problem can be avoided by knowing the lipid
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25⁶⁰³ composition of the sample and the carrying capacity of the column prior to SPE. The amount of
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27⁶⁰⁴ sample that can be loaded onto a column is governed by the proportion of the retained lipids. For
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29⁶⁰⁵ example, 2 to 5 mg of PL is considered as the maximum load for SPE of neutral and polar lipids on
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31⁶⁰⁶ a 100 mg silica column (Christie, 1982b). Although sample overload leads to cross-contamination,
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33⁶⁰⁷ an excess of sorbent can also reduce recovery of the retained analytes. It is therefore suggested to
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35⁶⁰⁸ experimentally determine the sample load (mass ratio of lipid to sorbent) to optimise purity and
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37⁶⁰⁹ recovery (Pernet *et al.*, 2006). In order to use the SPE system, we suggest replacing the silica
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39⁶¹⁰ originally contained in the pre-conditioned column with rehydrated silica (generally 6% m/m).

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41⁶¹¹ Purity and recovery analyses after SPE are crucial, particularly when the sample matrix is new to
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43⁶¹² the laboratory and when the stationary phase changes. Different brands or batches of silica gel may
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45⁶¹³ vary in their properties and cross-contamination of fractions may occur (Christie, 1982b). Purity can
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47⁶¹⁴ be evaluated by TLC of lipid classes of each eluted fraction. The simplest way to evaluate recovery
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49⁶¹⁵ is to quantify the lipid class content of samples before and after SPE by TLC and proceed with
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51⁶¹⁶ recovery calculation (i.e., the amount of collected lipid after SPE / the amount of lipids before SPE,
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53⁶¹⁷ as a percent). Alternatively, the recovery purity and efficiency can be estimated by adding an internal
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55⁶¹⁸ standard of known mass before SPE. As such, lyso-phosphatidylcholine 12:0 (22.8 µg of FA) was
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57⁶¹⁹ added to the lipid extract before SPE to assess PL recovery in oyster samples (Pernet *et al.*, 2006).
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59⁶²⁰ Occurrence of 12:0 was exclusively associated to LPC as this FA is absent in oysters. A full recovery
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of LPC following SPE on silica indicated that other PL were fully recovered as LPC is the most strongly retained phospholipid on silica (Pernet *et al.*, 2006). To evaluate the purity of each lipid fraction and the absence of cross-contamination, it is necessary to add one standard based on the least neutral lipid which must remain in the neutral lipid fraction, and another one which corresponds to the least polar lipid which must be retained in the polar lipid fraction. For example, commercially available 12:0 diacylglycerol (DG) and 10:0 phosphatidic acid (PA) can be added to the lipid extracts of oysters (F. Pernet unpub. data). Because these two shorter chain FA are naturally absent in oysters, their occurrence was exclusively associated with DG and PA standards. The FA 12:0 and 10:0 were expected in neutral and polar lipid fractions, respectively. Note that these standards do not replace the traditional 23:0 used for mass quantification of FA (see section 7). In the case of SPE, 23:0 is generally added directly in the elution tubes to avoid passage in the column as this standard would only be distributed in the neutral fraction.

6 Transesterification

Except in highly degraded samples, where FFA proportion can reach high values, FA are generally part of more complex lipid molecules, and are linked to glycerol by an ester linkage (e.g., TAG, PL, glycolipids). Therefore, one approach to analyse FA by gas chromatography (GC) is to hydrolyse the ester linkage and to convert the released non-volatile FA into their volatile derivatives, usually FA methyl esters (FAME). Saponification followed by methylation is the classical method for preparing FAME, and while laborious, it produces FAME without contamination with unsaponifiable lipid compounds (e.g., non-volatiles, sterols, alcohols, hydrocarbons, phthalates and pigments). However, most researchers use transesterification. A number of transesterification methods exist, all based on the presence of excess methanol, a catalyst (acid or base) and heat. To be analysed by GC, FAME are then extracted in a GC compatible solvent (often hexane), and the organic phase is washed several times by water to remove acid and glycerol. Produced FAME are stable for months at 4 °C in the dark under nitrogen. Transesterification using ethanol instead of methanol is also possible, but less used, leading to the formation of FA ethyl esters. Butanol can also be used as a substitute to methanol to quantify very short chain FA (down to *iso5:0*) by increasing aliphatic chain length (Koopman *et al.*, 1996, 2003). Although the use of the boron trifluoride (BF₃) has been recommended by the AOCS (1989), many catalysts can be used in both acid- and base-catalysed transesterification, with each method having their own advantages and limits.

The efficiency of these transesterification methods differs as a function of the nature of the aliphatic chains. This is particularly important in aquatic samples because, depending on the matrix, a considerable part of these aliphatic chains can be linked to the entire lipid molecule not by an ester linkage, but by a vinyl-ether (e.g., plasmalogens), an ether (e.g., alkyl lipids) or an amide (e.g., sphingolipids) linkage. It is thus important to choose the most pertinent transesterification method with respect to the sample nature and the scientific question. For example, the acid-catalysed transesterification of plasmalogens leads to the formation of FAME (from the acyl lipids) and

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dimethyl acetals (DMA) (from vinyl-ether lipids). DMA are mainly detected in polar lipids but are marginally present in neutral lipids, reflecting the contribution of glyceride ether also named “neutral plasmalogens” (TAG containing at least one vinyl ether bound aliphatic chain). Base-catalysed transesterification of these molecules only leads to the formation of FAME, leaving vinyl ether bound aliphatic chains unreacted (Crackel *et al.*, 1988; Cruz-Hernandez *et al.*, 2006). However, attention needs to be paid to the particular procedure followed, as base-catalysed transesterification based on ISO standard methodology incorporates an acid wash to neutralise the catalyst. This acidic wash may lead to DMA formation (Gomez-Cortes *et al.* 2019). Moreover, neither acid- nor base-transesterifications have any effect on ether linkages (Christie 1989). This point is important to consider when analysing samples containing large amounts of alkyl ether lipids, such as alkylglyceryl ether lipids in shark liver oil. Transesterification of FA sphingolipids components is acid-catalysed and is achieved by increasing the reaction time (Eder, 1995; Le Grand *et al.*, 2011). Finally, esterification of FFA is only possible by acid-catalysed transesterification (Christie, 2003).

Depending on the scientific question (e.g. nutritional value, trophic relationships, physiological responses), FA transesterification can be realised directly on the biomass (direct transesterification), on a total lipid extract or on lipid fractions, such as polar or neutral lipids, lipid classes (e.g., TAG, phosphatidylcholine) or lipid subclasses (e.g., phosphatidylethanolamine plasmalogen) isolated beforehand. It can be useful to purify FAME by HPLC or TLC before GC injection (Marty *et al.*, 1992). This prevents the saturation and the degradation of the column through the injection of non-FAME molecules (e.g. non-volatiles, sterols, hydrocarbons, phthalates, pigments) and thus increases its lifespan. Obtained chromatograms are also “cleaner” (no contaminants) and easier to identify.

6.1 Boron trifluoride (BF₃)

The Lewis acid boron trifluoride (BF₃) in methanol (12-14%, w/v) is a powerful acidic catalyst for the transesterification of FA. In general, 800 µL to 1 mL of BF₃-MeOH (14%, w/v) is added to a dry lipid extract containing a maximum of 1-4 mg of lipids (Liu, 1994; Carrapiso and Garcia, 2000; Le

Commented [MO12]: 12 or 14%? It should be mentioned that the ratio of BF₃ to MeOH needs to be exact to avoid problems with excess or insufficiency of the catalyst.

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10⁶⁸⁴ Grand *et al.*, 2011). This method is fast and efficient (Morrison and Smith, 1964; Carrapiso and
11⁶⁸⁵ García, 2000; Cavonius *et al.*, 2014), and it has been successfully used on marine lipid samples where
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13⁶⁸⁶ almost all the lipid classes, including FFA and plasmalogens, were transesterified in 10 min at 100°C
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15⁶⁸⁷ (Le Grand *et al.*, 2011; Cavonius *et al.*, 2014). For the sphingolipids, such as sphingomyelin and
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17⁶⁸⁸ ceramide aminoethylphosphonate, the transesterification should be prolonged and last for 1.5 h to 5
18⁶⁸⁹ h at 100°C (Eder, 1995; Le Grand *et al.*, 2011).
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20⁶⁹⁰ Heating is generally conducted in a dry bath, water bath or heat block. The higher the temperature
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22⁶⁹¹ used, the shorter the reaction time. An increase in reaction time has no negative effect, provided that
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24⁶⁹² no evaporation occurs (Morrison and Smith, 1964). Presence of residual water in the sample limits
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26⁶⁹³ the acid-catalysed esterification by BF₃ (Sattler *et al.*, 1991). It is thus necessary to dry the sample by
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28⁶⁹⁴ either nitrogen evaporation or by addition of anhydrous sodium sulphate (Molnar-Perl and Pinter-
29⁶⁹⁵ Szakacs, 1986).

30⁶⁹⁶ Because evaporation during the transesterification leads to acid concentration, which degrades FA
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32⁶⁹⁷ and especially PUFA (Lough, 1964), it is crucial to prevent vial caps from unscrewing when heating
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34⁶⁹⁸ samples (Morrison and Smith, 1964). Poor storage conditions and quality of BF₃ cause PUFA
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36⁶⁹⁹ degradation (Morrison and Smith, 1964; Christie, 1993). It is recommended to use fresh reagent and
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38⁷⁰⁰ to store it at 4°C or colder (-20°C). Despite these issues, the use of BF₃-MeOH for transesterification
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40⁷⁰¹ was referenced as an official method (AOCS, 1973; IUPAC, 1979) and Ackman (1998) showed that
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42⁷⁰² issues could be avoided following simple rules. Yet, the potential PUFA degradation induced by using
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44⁷⁰³ BF₃ as a transesterification catalyst, the difficulty in purchasing guaranteed anhydrous BF₃-MeOH,
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46⁷⁰⁴ its high cost and its high toxicity have led to a decreasing use of this reagent (Liu, 1994; Carrapiso
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48⁷⁰⁵ and García, 2000; Cavonius *et al.*, 2014). In samples containing very high levels of storage lipids,
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50⁷⁰⁶ such as salmon flesh, copepod oil and cod liver, the derivatisation efficiency can be quite low, leading
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52⁷⁰⁷ to small but significant differences in some FA proportions (Schlechtriem *et al.*, 2008). For these
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54⁷⁰⁸ reasons, BF₃ is no longer recommended as an acidic catalyst in the preparation of FAME.
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6.2 Acidic alternatives to BF_3

A number of alternatives to Lewis acids have been employed (Christie, 1982a). Methanolic HCl (3 N) appears to be the most commonly used reagent for transesterification of lipid-bound FA to FAME in lipids from freshwater plankton (von Elert, 2002; Martin-Creuzburg *et al.*, 2012; Windisch and Fink, 2018), but also from marine plankton (e.g., Troedsson *et al.*, 2005). It can easily be produced by adding 24 ml acetyl chloride to 100 ml of methanol, which leads to the formation of 10% (approx. equivalent to 3 N) methanolic HCl. H_2SO_4 is often recommended (Christie, 1982a; Budge *et al.*, 2006), as it can easily be prepared by mixing a small amount of concentrated H_2SO_4 (~98%) in methanol to achieve a 1% solution (Schlechtriem *et al.*, 2008).

The most commonly used acid catalysed reagents, such as HCl or H_2SO_4 , are not associated with the issues described for BF_3 . A number of comparisons of acid catalysts for esterification/transesterification have been made (e.g. Medina *et al.*, 1992; Budge and Parrish, 2003; Thiemann *et al.*, 2004; Carvalho and Malcata, 2005), with no clear consensus on optimal methods. The best advice is likely to select a method and consistently apply it.

6.3 Basic alternatives

Several basic catalysts are available (e.g., NaOH, KOH) to transesterify acyl lipids. While a range of procedures have been described (e.g. Christie, 1982a; Medina *et al.*, 1992; Velasco *et al.*, 2002), most are very similar to the acid catalysed method described above with the substitution of the catalyst and the introduction of an additional step after the reaction is quenched to neutralise the remaining base. All base-catalysed transesterifications proceed very rapidly at room temperature (Christie, 1982a), thereby preserving labile structures (i.e., epoxides, conjugated double bonds) that are often destroyed with harsher acid catalysts. This 'gentler' transesterification is the primary motivation for use of basic catalysts. As noted above, basic-catalysts are unable to esterify FFA so these approaches should only

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10⁷³² be used when one can be confident that FFA are present in very low amounts, or else when the analyst
11⁷³³ is targeting esterified FA exclusively (e.g., studies on membrane lipids or storage lipids).
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13⁷³⁴ Finally, there are a number of official and approved methods that employ combined approaches,
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15⁷³⁵ using both basic and acidic catalysts (e.g., AOCS, 1989). While at least one of these methods (i.e.,
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17⁷³⁶ AOAC 969.33-1969, 1997) describes the procedure as first saponification with NaOH, followed by
18⁷³⁷ methylation with BF₃ in methanol, the short reaction time with NaOH in methanol is actually an
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20⁷³⁸ alkali-catalysed transesterification, followed by acid-catalysed methylation of any FFA that remain
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22⁷³⁹ (Ackman, 1998). Although direct acid-catalysed transesterification is sufficient for a typical aquatic
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24⁷⁴⁰ lipid if performed with sufficient time and heat, the alkali-catalysed step may persist because of its
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26⁷⁴¹ adoption by official bodies (Ackman, 1998).
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28⁷⁴² 6.4 Direct transesterification

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31⁷⁴³ The standard procedure for determining FA profiles (e.g. extraction to isolate lipids, followed by
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33⁷⁴⁴ transesterification to yield FAME), can be labour-intensive and increases analytical cost. In some
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35⁷⁴⁵ cases, studies are only interested into the FA composition of total lipids in samples and therefore
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37⁷⁴⁶ perform a direct one-step transesterification that considerably reduces the analytical time (Lewis *et al.*
38⁷⁴⁷ *et al.*, 2000). This method is realised directly on the sample, and differs from the classical
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40⁷⁴⁸ transesterification by the elimination of the lipid extraction step (Lewis *et al.*, 2000; Castro-Gómez
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42⁷⁴⁹ *et al.*, 2014; Parrish *et al.*, 2015).
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43⁷⁵⁰ Different methods have been tested and provided reproducible and similar results to the classical
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45⁷⁵¹ extraction-transesterification method (Castro-Gómez *et al.*, 2014; Parrish *et al.*, 2015). Careful
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47⁷⁵² consideration is needed when using this approach as it may not be appropriate to the study goal and/or
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49⁷⁵³ the matrices analysed. It may also be preferentially applied to small and homogenous (representative
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51⁷⁵⁴ of the whole) samples. For example, while it is possible to carry out direct analyses on a small amount
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53⁷⁵⁵ of tissue, accurate determination of total blubber FA composition in marine mammals was not
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55⁷⁵⁶ possible with this approach due to significant variation in FA composition within the layers of the
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10⁵⁷ blubber (Thiemann *et al.*, 2008). Although this rapid direct transesterification approach is less time-
11⁵⁸ consuming and cheaper, once the entire sample is transesterified, it is not possible to redo the analysis,
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13⁵⁹ or to analyse the FA composition of different lipid fractions.

16⁶⁰ 6.5 Alternatives to hexane

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19⁶¹ Hexane, iso-hexane (2-methyl-pentane), or iso-octane are most frequently used to recover FAME
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21⁶² after transesterification and before injection. However, hexane is carcinogenic, mutagenic and
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23⁶³ reprotoxic, and is a hazardous airborne pollutant. Less toxic solvents such as pentane and heptane can
24⁶⁴ be used as alternatives to hexane (Alfonsi *et al.*, 2008). However, recovery of FA from macroalgae
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26⁶⁵ is lower with heptane than with hexane, with PUFA tending to be more affected than SFA and MUFA
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28⁶⁶ (Le Grand unpub. data). This preliminary result needs to be verified on other matrices. Pentane rather
29⁶⁷ than hexane may also be used as the solvent in the BF₃/MeOH procedure to reduce the reaction
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31⁶⁸ temperature from 85-100 °C to 80 °C to decrease artefact risk (Parrish, 1999).

7 Analysis of fatty acids

The full process of FAME analysis consists of injection, separation, identification and quantification of FAME and each of these steps has to be optimised to achieve high accuracy and precision (see Eder, 1995; Christie, 2003 for review). Gas chromatography (GC) with flame ionisation detection (FID) is generally the preferred method for the quantification of FAME in aquatic samples.

7.1 Injection

Sample injection is a critical step to achieve high accuracy in FAME analysis and we encourage readers to refer to seminal papers of Konrad Grob for a thorough overview of available techniques (e.g., Grob, 2007). There are currently three injection techniques. Before being transferred into the capillary column, the sample can be (i) evaporated in a permanently hot vaporising chamber (classical vaporising injection), (ii) injected into a cool chamber which is subsequently heated to vaporise the sample (programmed temperature vaporising injection, referred to as PTV), or (iii) directly injected into the column inlet (direct injection or on-column).

Once vaporised, the sample can be split (i.e., split injection) with only a small part of the vapour entering the column while the rest is vented. This is the most appropriate technique for concentrated samples (e.g., Shantha and Ackman, 1990). Alternatively, the whole vaporised sample is transferred from the injector to the column without splitting of the sample (i.e., splitless injection). This technique is particularly appropriate for trace analysis. The major drawback of the split or splitless injection techniques is the possible discrimination between high- and low-boiling compounds in the sample.

Fatty acids in aquatic samples have a wide range of boiling points and quantification may require application of relative response factors (Grob, 2007). On-column injection avoids discrimination of FA and therefore provides the best results. Yet, a large number of aquatic scientists use the vaporising injection method for suitability and flexibility reasons. High-speed injection, high injector temperature (> 220 °C) and proper injector inserts all contribute to decreasing discrimination

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10⁷⁹³ problems. However, we recommend regular checks of FAME discrimination factors using certified
11⁷⁹⁴ reference mixtures.

Commented [MO13]: It would be beneficial to provide examples. Or refer to publications that used these.

14⁷⁹⁵ 7.2 Separation

17⁷⁹⁶ Upon injection, FAME are transferred into a wall-coated open tubular (WCOT) capillary column
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19⁷⁹⁷ allowing separation according to their carbon number, the number and location of the double bonds,
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21⁷⁹⁸ and the cis-trans configuration. The column is located in an oven and exposed to temperature ramps
22⁷⁹⁹ ranging from 60 °C to 300 °C (e.g. Tang and Row, 2013 for examples of temperature ramping). The
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24⁸⁰⁰ carrier gas is generally helium or hydrogen. The resolution capability of the column depends on
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26⁸⁰¹ several factors such as polarity of the stationary phase, column length, internal diameter and film
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28⁸⁰² thickness. In view of its importance, this information should be reported in detail in the method
29⁸⁰³ description of any study.

31⁸⁰⁴ Based on the type of FA being investigated, one of the most important steps is the selection of the
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33⁸⁰⁵ stationary phase of the column which influences elution times of FAME, especially PUFA. The
34⁸⁰⁶ polarity of the column ranges from non-polar to extremely polar. On non-polar columns, the elution
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36⁸⁰⁷ time generally increases with increasing boiling points of analytes. Therefore, the elution time of
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38⁸⁰⁸ FAME increases with chain length and with decreasing unsaturation (e.g., 18:2 n -6 elutes first,
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40⁸⁰⁹ followed by 18:1 n -7, and then by 18:0). The major drawback with non-polar columns is that some
41⁸¹⁰ FA isomers (*cis-trans*) or FA pairs may co-elute. Conversely, non-polar columns exhibit low bleed,
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43⁸¹¹ long lifetime, retention time stability and high temperature resistance (to 360 °C), which makes their
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45⁸¹² use suitable for a large range of compounds. These columns are well-suited for separating neutral
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48⁸¹⁴ of 100% dimethylpolysiloxane or 5% phenyl-95% dimethylpolysiloxane.

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50⁸¹⁵ Many researchers use polar columns which allow for a better separation of aquatic FA than non-
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52⁸¹⁶ polar phases, with only some rare and minor co-elutions. Ackman (1986) was a strong advocate of
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54⁸¹⁷ the polyethylene glycol (PEG) phase. Columns coated with PEG are easily recognisable because of

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10¹⁸ their commercial name with the suffix "wax". With this polar phase, FAME continue to elute in order
11¹⁹ of increasing boiling point, but also roughly in order of increasing numbers of double bonds.
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13²⁰ However, polar columns are highly sensitive to temperature (max 250 °C), water and oxygen, and
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15²¹ are thus less stable over time than non-polar columns. Retention times may also vary (decrease) over
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17²² time, especially for highly unsaturated compounds. Consequently, peak identification (based on
18²³ elution time) needs to be checked regularly and rigorously.
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20²⁴ An alternative phase consists of substituting 50% dimethylpolysiloxane (non-polar) with the polar
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22²⁵ cyanopropylmethyl (e.g., Iverson *et al.*, 2004; Pedro *et al.*, 2019). This phase provides similar
23²⁶ resolution as PEG phases, although it is much less sensitive to oxygen, resulting in longer column
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25²⁷ lifetimes. The chemistry of this phase is more influenced by temperature than other polar columns.
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27²⁸ Separation of co-eluting peaks can sometimes easily be achieved simply by subtle adjustments to
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29²⁹ oven temperature ramps.
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31³⁰ 7.3 Identification 32

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34³¹ The most common way to identify FAME by GC-FID analysis is to compare their retention times
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36³² with those of commercially-available individual purified standards. Among the most used standards
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38³³ for the FA identification in aquatic samples, there are PUFA No. 3, a complex qualitative standard
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40³⁴ mixture extracted from Menhaden oil, and Supelco® 37 Component FAME Mix, a comprehensive
41³⁵ mixture of 37 FAME ranging from 4:0 to 24:1*n*-9. Several long-chain and (highly) unsaturated
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43³⁶ FAME occurring in some samples are not commercially available, but can be identified using fully
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45³⁷ characterised natural products (e.g., the non-methylene interrupted fatty acids typical of marine
46³⁸ molluscs in Zhukova and Svetashev, 1986; Ackman, 1989; Kraffe *et al.*, 2004).
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48³⁹ In addition to these protocols, confirmation of FA identification is best achieved using GC-mass
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50⁴⁰ spectrometry (GC-MS). Peak identification is achieved using commercial spectral libraries, or via
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52⁴¹ personal/laboratory FA mass spectral libraries to assist in identification of often complex
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54⁴² environmental FAME samples. Further details on various GC-MS protocols together with specific
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MS information are available in Christie (1989). Briefly, the base peak at m/z 74 is termed the McLafferty rearrangement ion, and is formed by cleavage of the parent FAME molecule beta to the carboxyl group. Where chemical ionisation is used, the mass spectra have a more prominent quasi-molecular ion (MH^+), and minor ions only at $[MH - 32]^+$ and $[MH - 32 - 18]^+$. Because all FA will have the carboxylic acid (or ester) functionality in common, knowledge of the molecular mass is particularly valuable in determining basic structure with respect to numbers of carbon atoms and double bonds. There are unfortunately generally no characteristic ions that indicate the position or geometry (i.e., *cis-trans*) of double bonds in positional isomers of monounsaturated and polyunsaturated FAME (Christie, 1989).

Where hydroxyl groups are present on the FAME, conversion of the hydroxyl group to a trimethyl silyl (O-TMSi) ether or other derivative is generally used. Bistrimethylsilyltrifluoroacetamide (BSTFA) is a useful reagent to accomplish this. The high polarity of the free hydroxyl group is reduced by formation of the O-TMSi ether, thereby enhancing the chromatography of the derivative. Fragmentation of the OTMSi derivative occurs alpha to the carbon containing the hydroxyl group, with the main cleavage on the side adjacent to the carboxyl group.

Derivatisation protocols have been developed to provide more detailed position of double bonds or geometry information. These include, for example, use of pyrrolidine, 3-pyridyl carbinol (picolinyl ester), dimethyl disulphide (DMDS) and 4,4-dimethyloxazolazine (DMOX) derivatives. An advantage of the use of the DMOX protocol is that the FAME and their corresponding DMOX derivatives have the same elution order when run on the same GC column. PUFA DMOX derivatives can be readily formed using the protocols described in Svetashev, 2011 and Lee Chang *et al.*, 2016. Interesting examples of application of the DMOX protocol have been the characterisation of a novel series of C_{18} to C_{22} *trans* n-3 PUFA from a marine haptophyte (Lee Chang *et al.*, 2016) and the 22:4n-9t in scallop (Marty *et al.*, 1999). MUFA double bond position and geometry is readily determined by capillary GC-MS of their DMDS derivatives (Nichols *et al.*, 1986). An example of application of the DMDS protocol has been the characterisation of a range of novel C_{16} and C_{18} MUFA, including

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10⁸⁶⁹ both *cis* and *trans* isomers, from microbial monocultures and complex soil consortia (Nichols *et al.*,
11⁸⁷⁰ 1986). Other derivatisation procedures are also available, although they are not covered here, and are
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13⁸⁷¹ as detailed in Christie (1989).

16⁸⁷² 7.4 Quantification

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19⁸⁷³ In GC-FID analyses, the response intensity is based on the number of oxidisable carbon atoms in the
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21⁸⁷⁴ analyte; equivalent masses of FA having different carbon chain lengths and/or numbers of double
22⁸⁷⁵ bonds will not produce the same response in the FID (Ackman, 1972). FAME are usually quantified
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24⁸⁷⁶ by peak areas, and absolute concentrations are determined by adding an internal standard.
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26⁸⁷⁷ Discrimination of FAME may be associated with injection technique and it is often necessary to
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28⁸⁷⁸ introduce empirical response factors, determined through careful use of commercially available
29⁸⁷⁹ authentic mixed standards (see section 7.1). A way to estimate the importance of bias from different
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31⁸⁸⁰ origins (such as injector, or detector) is for example to analyse a FA mixture of known theoretical
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33⁸⁸¹ mass composition such as the Supelco® 37 Component FAME Mix. It is also possible to analyse a
34⁸⁸² phospholipid with two different aliphatic chains, of different length and saturation (e.g.,
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36⁸⁸³ phosphatidylcholine 16:0/22:6*n*-3) and confirm that the molar percentage of each FA is 50/50.

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38⁸⁸⁴ The internal standard must not coelute with the FAME, its chemical structure must be close to the
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40⁸⁸⁵ majority of the FAME and it must be absent from the sample. A known mass of internal standard
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42⁸⁸⁶ should be added either after SPE or before transesterification to account for derivatisation yield and
43⁸⁸⁷ recovery of FAME (see section 5). The most widely used standards are the carboxylic form of
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45⁸⁸⁸ nonadecanoic acid (19:0), heneicosanoic (21:0) and tricosanoic acid (23:0). Data are generally
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47⁸⁸⁹ expressed as relative (area %, mass % or mole %) or absolute ~~concentrations~~ contents (e.g., mg FA
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49⁸⁹⁰ g⁻¹ sample dry mass).

50⁸⁹¹ With GC-MS analyses, one approach is to use the total ion chromatogram in a similar fashion as
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52⁸⁹² peak area is used with GC-FID, by comparing the peak area of an internal standard with peak areas
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54⁸⁹³ of compounds of interest, and relative response factors determined individually using authentic

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standards. Response factors for analytes where standards are not available are assumed to be the same as those of available compounds. Note here that response also varies with structure, likely due to difference in ionisation so calibration remains important. For greater sensitivity, calibration can be based on a single (or multiple) prominent ion(s) in the spectrum of the FA of interest, typically using an internal standard technique. Another approach with GC-MS is the use of stable isotope dilution methods, where labelled internal standards are used. These offer the advantage of being identical to the analyte of interest in all aspects except mass, so that response, as well as matrix effects, of the labelled and unlabelled component are the same (Quehenberger *et al.*, 2008).

8 Data treatment

As emphasised by the previous sections, obtaining reliable FA data can be a long and complex process. The purpose of this section is therefore to offer guidance in how to valorise these hard-earned data in the best possible way, and to use them to shape robust answers to a wide range of scientific questions. As environmental phenomena are inherently complex, a single variable rarely depicts them in a satisfactory way (Buttigieg and Ramette, 2014). Moreover, FA studies typically deal with datasets containing many variables (i.e., individual FA). This section will therefore mostly focus on multivariate analysis.

Regardless of the data analysis approach, FA studies commonly rely on so-called "biomarker compounds", i.e., FA (or combinations of FA) whose origin can be assigned to a biogeochemical process, an ecosystem compartment and/or an organism group. While this approach has proven very useful and deserves to be routinely applied, it is important to remember that there are no ubiquitous FA biomarkers, and that their applicability will depend on the studied system or organism (e.g., Feiner *et al.*, 2018). To avoid interpretation biases, biomarker compounds should be chosen carefully with respect to the ecological context and scientific question of interest. This topic has been extensively discussed in other reviews (Dalsgaard *et al.*, 2003; Kelly and Scheibling, 2012) and will not be further elaborated upon here.

8.1 Standardisation and transformation

Choosing standardisation or a prior transformation method for FA data needs to be based on the research questions, as it can have important impacts on the final results and interpretations (Mocking *et al.*, 2012). FA can be expressed as either an absolute concentration or mass ratio, such as $\text{mg}\cdot\text{g}^{-1}$ of lipids or of tissue, or as relative proportion (%) of total FA mass or molar concentration. Concentrations are commonly used in nutrition studies, and can be useful in controlled feeding experiments (Elsdon, 2010). Proportions of total FA mass is the commonly used standardisation

Commented [MO14]: This section may benefit from a more structured start. I fully agree that a set of multivariate analysis is typically used for analyzing FA. But each use of a specific analytical method first requires a research question. Perhaps a list/table of possible methods for FA research approaches may be useful. For example, as stated in I. 923, FA used as biomarkers are very common, for which e.g., PCA, nMDS are commonly used. It may be beyond the scope of this paper to go into too much detail, but a list would be helpful for the non-initiated reader to understand which statistical methods is/are appropriate for which kind of FA interpretation/question/field.

Commented [MO15]: Content. Concentrations see above.

Commented [MO16]: This is actually a mass fraction, not a ratio.

Commented [MO17]: But need to be related to volumetric units, otherwise these 'concentrations' are mass fractions.

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method in trophic studies. It avoids occultation of compositional differences in FA profiles by removing the influence of non-FA components from both the lipid fraction (e.g., polar vs neutral lipids) and the total lipid contents of tissues. Yet, proportion data are generally calculated in relation to identified FA in a sample, which vary across studies and FA analysis methodologies, limiting inter-study comparison.

Choice of data standardisation is interdependent with the choice of data transformation method (if needed) and of applied statistical test (univariate vs. multivariate tests). FA data transformation can be recommended in some cases (e.g., Kelly and Scheibling, 2012), but the overall effect of the procedure is highly variable (Happel *et al.*, 2017). Deciding on a transformation method depends on the test used and its assumptions (e.g., normal distribution of data, homoscedasticity). Although these assumptions can be met for univariate analyses (that test the effect of one or several factors on individual FA), this is rarely the case for multivariate analyses using large FA datasets, even after transformation (Budge *et al.*, 2006). However, multivariate methods such as unconstrained ordinations (e.g., principal component analysis, see below) give useful and adequate results when the data are properly transformed (e.g., Legendre and Gallagher, 2001). Moreover, proper testing procedures through permutations allows one to circumvent the violation of the multivariate normality assumption in constrained ordination methods such as redundancy analysis (RDA, Legendre and Legendre, 2012; see subsection 8.3).

Kelly and Scheibling (2012) reviewed some of the most common data transformation methods used in FA studies, including log-ratio (Budge *et al.*, 2006), arcsine and logit transformations. Arcsine transformation has been widely applied to transform proportional FA data, yet its use in biological analysis has been challenged in some studies. Warton and Hui (2011) suggested that applying logistic regression is more appropriate to transform proportion data. It is also important to note that some transformations such as $\log(x)$ or $\log(x+1)$ will give more weight to rare (i.e., found in small amounts) FA, while reducing the influence of the abundant FA (Happel *et al.*, 2017). Depending on the study objectives, it is essential to identify clearly whether the scientific question requires emphasising the

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10 presence and the role of rare FA, or if focusing on the dominant FA will give a more global pattern
11 within and across organisms.
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13 14 8.2 Exploring fatty acid data through ordination 15

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17 Ordination methods are essential to describe and visualise sample dispersion in a complex
18 multidimensional dataset, and to identify groups of co-varying variables. Those are computed based
19 on a matrix of resemblances (distance, dissimilarities, similarities), or derived ranks, rather than raw
20 (or transformed) data. In FA literature, the most commonly used resemblance measure between
21 samples are either the Euclidean distance or Percentage difference (also called Bray-Curtis
22 dissimilarity; Legendre and Legendre, 2012). Euclidean distance is generally preferred over Bray-
23 Curtis for FA analysis (Happel *et al.*, 2017). However, just like data transformations, the choice of
24 the resemblance measure can have important impacts on the final results. The choice of matrix will
25 depend on whether a dataset includes null/absent values and if they need to be included in the analysis
26 according to the study question. The Percentage difference coefficient is double-zero asymmetric, i.e.
27 the absence of one FA from two individuals is not considered as contributing to their similarity. The
28 Euclidean distance, on the contrary, is double-zero symmetric, i.e. will be affected by double null
29 values (Legendre and Legendre, 2012). Other properties of resemblance measures should also be
30 considered. Choice of the resemblance coefficient has received extensive attention in multivariate
31 community ecology (e.g., Legendre and Legendre, 2012).
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34 Commonly used methods to plot ordinations include principal components analysis (PCA),
35 principal coordinates analysis (PCoA) and non-metric multidimensional scaling (nMDS). PCA can
36 only use Euclidean distance, while PCoA and nMDS can use any resemblance measure. PCoA and
37 nMDS use the ranks of similarities or dissimilarities matrix to find a spatial arrangement of
38 individuals and represent relationships among them (Kelly and Scheibling, 2012). They have been
39 used to classify and compare FA compositions of consumers across sampling location (Hughes *et al.*,
40 2005; Parrish *et al.*, 2015), habitats (Hixson *et al.*, 2015) or species (Sardenne *et al.*, 2016). NMDS
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10⁹⁷⁷ is conceptually simple, generally applicable, and well adapted to ecological data (Clarke and
11⁹⁷⁸ Warwick, 2001). However, it is crucial to take into account the stress coefficient, which is measured
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13⁹⁷⁹ from the relationship between ranks of dissimilarities and ranks of sample distances on the plot. A
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15⁹⁸⁰ high stress score (> 0.05) indicates that data have been highly distorted to fit in the visualisation,
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17⁹⁸¹ which may not be representative and should be considered with caution (Clarke and Warwick, 2001;
18⁹⁸² Buttigieg and Ramette, 2014). PCA also describes relationships among samples, reducing large
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20⁹⁸³ numbers of variables to a few components that represent most of the variance and that are built by
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22⁹⁸⁴ combining correlated variables (Budge *et al.*, 2006; Kelly and Scheibling, 2012). It also helps to
23⁹⁸⁵ visualise variables that contribute the most to the dissimilarity among observed groups and FA that
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25⁹⁸⁶ are highly correlated (Budge *et al.*, 2006). After ordination, several procedures (similarity percentage,
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27⁹⁸⁷ also called SIMPER, analysis; comparison of PCA loadings) can be applied to identify FA driving
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29⁹⁸⁸ resemblance or dissimilarity among groups (Clarke and Warwick, 2001).

31⁹⁸⁹ 8.3 Multivariate hypothesis tests and modelling

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34⁹⁹⁰ Multivariate analyses are commonly applied in ecological studies to discriminate the influence of
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36⁹⁹¹ different factors on FA composition, such as physiological, spatial or even temporal variations
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38⁹⁹² (Anderson, 2001). Statistical comparison of groups based on FA composition is generally performed
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40⁹⁹³ through non-parametric or randomisation procedures, as FA datasets rarely match the prerequisites
41⁹⁹⁴ of parametric statistical tests (Anderson and Walsh, 2013). Analysis of similarity (ANOSIM; Clarke,
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43⁹⁹⁵ 1993) or permutational analysis of variance (PERMANOVA; Anderson, 2001) are among the most
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45⁹⁹⁶ commonly used statistical tests (e.g. Hughes *et al.*, 2005; Hixson *et al.*, 2015; Parrish *et al.*, 2015;
46⁹⁹⁷ Sardenne *et al.*, 2016; Mathieu-Resuge *et al.*, 2019). ANOSIM uses ranks of pairwise dissimilarities
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48⁹⁹⁸ and generates its own statistic by randomisation of rank dissimilarities (Clarke and Warwick, 2001).
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50⁹⁹⁹ PERMANOVA tests for differences among group centroids (Anderson, 2001). Both methods are
51¹⁰⁰⁰ affected by dispersion heterogeneity, with ANOSIM being more sensitive than PERMANOVA
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53¹⁰⁰¹ (Anderson and Walsh, 2013). PERMANOVA is unaffected by differences in correlation structure

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10 and seems more powerful to detect changes in FA composition. As such, applying ANOSIM is
11 appropriate for a 1-way test (e.g. to compare one species submitted to different diets), but not for
12 more complex study designs (Kelly and Scheibling, 2012).

13
14 Besides hypothesis testing, constrained ordination (e.g., redundancy analysis, RDA) offers a way
15 to extract and summarise variability from a multivariate dataset (FA data) that can be explained by
16 one or a set of explanatory variables (e.g., locations, species, diets, etc.). In other words, it allows
17 regression of multiple response variables (FA) on multiple explanatory variables (chosen according
18 to the scientific question). More details on this technique can be found elsewhere (Legendre and
19 Legendre, 2012).

20 21 22 23 24 25 26 8.4 Coupling fatty acids with other ecological markers

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29 When tackling elaborate ecological questions, combining FA with other tracers has proven to be very
30 powerful. Tracers commonly combined with FA include stable isotope ratios of light biogenic
31 elements (Kharlamenko *et al.*, 1995, 2001; Nyssen *et al.*, 2005), as well as contaminants
32 concentrations (Kainz and Fisk, 2009; Le Croizier *et al.*, 2016). In many cases, those combinations
33 are qualitative. However, modern data analysis frameworks allow quantitative coupling of stable
34 isotope and fatty acids in mixing models (Stock *et al.*, 2018) or ecological niche models (Jackson *et*
35 *al.*, 2011; Sardenne *et al.*, 2016). Such developments hold great promise for ecological research, due
36 to the complementarity of these two methods. More generally, multiple factor analysis (Pagès, 2014)
37 or co-inertia analysis (Dray *et al.*, 2003) could provide flexible, widely applicable and quantitative
38 ways to couple data from different methods by allowing exploration of the links between multiple
39 multivariate datasets.
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Conclusion

The use of FA analysis in trophic ecology, nutrition and aquaculture research and development holds considerable promise to improve our understanding of the natural world from organism taxonomy and physiology to ecosystem functioning. However, the application of this approach needs to be carefully considered when initiating a new study to ensure that interpretations proposed for FA data are relevant and appropriate. Although protocols for lipid analysis are well-described, their application to aquatic sciences often requires modifications to adapt to field conditions and to sample types. Here, we presented the current state of knowledge on methods dedicated to aquatic lipid analyses, from sampling to data treatment. We reviewed sample preservation, storage and transport protocols and their effects on lipids, lipid extraction methods, separation of polar and neutral lipids, transesterification and quantification techniques, and available tools for treatment and statistical analysis of FA data. A simplified flowchart of all these steps is represented in Figure 1.

Lipids can be degraded by hydrolysis or oxidation at each stage of the lipid analysis process. We thus encourage processing samples at low temperature under nitrogen atmosphere, and reporting hydrolysis indicators. To limit possible deleterious effects, samples should be handled at the lowest suitable temperature before direct extraction, or be immediately frozen for storage, ideally at $-20\text{ }^{\circ}\text{C}$ for less than one month, $-80\text{ }^{\circ}\text{C}$ for less than 6 months or under liquid nitrogen with no time limit. Long-distance transport of samples must be planned well ahead of the start of the study to obtain relevant import/export permits and to choose appropriate transportation to maintain sample viability and respect transport regulations and national laws. When possible, we recommend transporting samples fresh-frozen under a nitrogen atmosphere. Depending on the objectives of the study, samples can be either directly transesterified or undergo extraction to isolate lipids. Lipid extraction is usually performed following well-established methods using either chloroform or dichloromethane (less toxic), mixed with methanol. We strongly encourage scientists to describe in their publication any modifications from original extraction methods.

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10 The lipid extract is either separated into classes or directly transesterified. Separation of polar and
11 neutral lipids prior to transesterification allows for a better interpretation of FA profiles when seeking
12 information on nutritional status and/or physiology of organisms. Special attention should be paid to
13 control for the purity and recovery of each fraction. Non-volatile FA are then transesterified into their
14 volatile derivatives, usually FAME, before being analysed by GC. Acid catalysed reagents, such as
15 HCl or H₂SO₄ are the most commonly used in aquatic science as they present fewer analytical issues
16 than BF₃. Hexane is the most widely used solvent to recover FAME after transesterification. Less
17 toxic alternatives are emerging, but are not applicable to all sample types. The analysis and
18 quantification of FAME is usually conducted by GC-FID. FAME are identified by comparing their
19 retention times with those of individual purified standards. They are quantified as relative proportions
20 using peak areas or by absolute concentration when an internal standard was added before
21 transesterification. When necessary, peak identification can be confirmed using GC-MS, but this
22 process may require additional derivatisation steps. Data treatment and statistical analysis of FA
23 should be carefully applied according to the investigated scientific question. We presented common
24 statistical procedures that we consider widely relevant for FA-related problems. This list is by no
25 means exhaustive. We encourage analysts to explore their data in multiple ways. A good starting
26 point is usually a simple preliminary visualisation of data. This will not only help to check for data
27 quality, but also to make informed choices about subsequent procedures. When interpreting data and
28 statistical results, it is crucial to take into account the studied organisms and/or ecosystems and their
29 specificities.

30 We believe that methodological consistency, controlled experimentation, and interlaboratory
31 comparison and calibration can lead to enhanced progress in FA studies and are particularly important
32 for comparative studies. Standardisation and optimisation of FA methodologies will ultimately
33 improve our understanding on the role of lipids in biological mechanisms, and will enable us to use
34 them as ecological or physiological tracers as efficiently as possible.

Commented [MO18]: Perhaps better to use 'argue' or 'think'.

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1074 **Figure caption**

1075 **Figure 1.** Workflow for the analysis of fatty acids in aquatic samples. The star symbol indicates
1076 recommended methods. Dashed arrows point to suggested storage precautions after each analytical
1077 step. Transport requirements are color-coded, and the coldest transport temperature is always the best
1078 option regardless of the step. The grey syringe symbol represents the step at which the internal
1079 standard for recovery and purity should be added. The black syringe symbol represents the step at
1080 which the internal standard for quantification should be added.

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Authors' contribution:

LC; LM; FP; PS equally contributed to the conception of the paper and its Figure, and led the writing of the paper. All other authors contributed to writing and editing the manuscript and approve the final draft.

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