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Review Article

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

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Determining the lipid content and fatty acid (FA) 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, derivatization, and detection methods, and (iii)

available tools for the statistical analysis of FA data. We provide recommendations for best practices in field situations and advocate for protocol standardization and interlaboratory calibration.

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

Introduction

Lipids and their 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. FA generally consist of a linear hydrocarbon chain with a carboxyl group (–COOH) at its end. They are characterized 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. FA 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 categorized 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 forms) and fatty alcohols (ALC) are also found in neutral lipids. FFA 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 characterized 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, and 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).

FA 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 trophic ecology (Dalsgaard *et al.*, 2003). According to this concept, FA composition of some primary producers is characterized by specific compounds that can be transferred to upper trophic levels (e.g. Kelly and Scheibling, 2012). The FA composition of a consumer is therefore expected to reflect the FA composition of its food sources ("you are what you eat" principle). FA have accordingly been used to study energy flow in food webs in freshwater, estuarine, coastal, and deep-sea environments in all latitudes and to examine functional responses to stressors (Kelly and Scheibling, 2012). More recently, the FATM concept has culminated in quantitative FA-based diet modelling (Iverson *et al.*, 2004; Galloway *et al.*, 2014; Bromaghin *et al.*, 2017), and recent methodological developments allow for accurate estimation of prey contribution to consumer diet (Litmanen *et al.*, 2020). However, diet modelling is based on the fact that aquatic consumers generally lack the ability to synthesize long-chain polyunsaturated FA (LC-PUFA, ≥C20) and have limited capacity for the bioconversion of short-chain PUFA into long-chain PUFA (Castell *et al.*, 1972; Langdon and Waldock, 1981; Sargent *et al.*, 1999; Taipale *et al.*, 2011). This view has recently been challenged (Kabeya *et al.*, 2018).

Diet is not the only determinant of the FA composition of aquatic consumers. FA composition varies with intrinsic factors such as phylogeny and developmental/reproductive stages (e.g. Galloway and Winder, 2015) and extrinsic factors such as temperature, salinity, and hydrostatic pressure (e.g. Wodtke, 1981; Cossins and Macdonald, 1989; Hazel and Williams, 1990), thus providing information about physiological condition and habitat type of aquatic organisms (e.g. Meyer *et al.*, 2019).

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

There are likely to be as many variations in procedures as there are laboratories (Parrish, 1999). Yet, not all may be applicable to aquatic samples. Procedures involved in sample collection, handling, and storage, as well as extraction and transesterification steps, can cause biochemical modifications in 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 article aims to guide both novice and advanced scientists in identifying the most

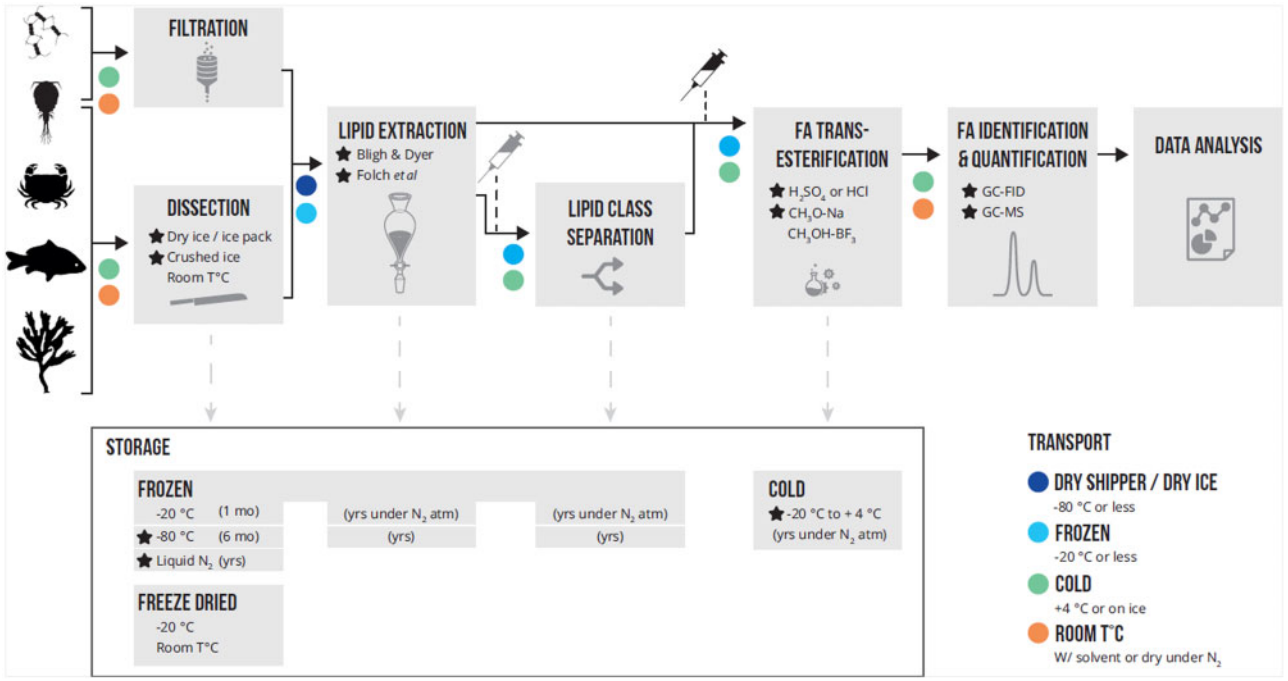

Figure 1. Workflow for the analysis of FA in aquatic samples. The star symbol indicates recommended methods. Dashed arrows point to suggested storage precautions after each analytical step. Transport requirements are colour-coded, and the coldest transport temperature is always the best option regardless 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.

appropriate protocols and best practices for FA analysis of aquatic samples. We hope that this comparative synthesis will stimulate further international collaborations and facilitate the standardization of lipid analyses.

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 (Figure 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 minimize 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°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 (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 PUFA in samples (Christie, 1984, see section Indicators of sample degradation). Here, we provide an overview of different methods used in the literature and discuss their strengths and limitations.

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 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). 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. All material should be lipid-cleaned prior any sampling to avoid cross-contamination (see section Handling cautions). 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.

Sample handling

Ideally, samples should be immediately flash-frozen in liquid N_2 in the field and transported to a freezer using a dry shipper. However, 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 only 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 (i.e. −80°C), long-lasting ice packs are a convenient option for short-term storage in the field (e.g. TechniIce^{TM}). These ice packs can 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.

Storage

Frozen storage (−20°C to −196°C)

Storing samples at cryogenic temperature 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 and sample/tissue types, 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 <6 months prior to analysis at temperature <−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 according to species and tissues. We therefore recommend evaluating the sensitivity of the samples for which there is no information available before the start of the study. For example, a temporal monitoring of the lipid composition of the biological material of interest at the available storage temperatures will make it possible to define the optimal conservation strategy.

Freeze drying

Freeze drying (or lyophilization) is a low temperature dehydration process of frozen material that is used to reduce enzymatic activity and thus lipid degradation in samples. Freeze-drying samples immediately prior to extraction do not affect lipid class or FA compositions of aquatic animal tissues (e.g. Dunstan *et al.*, 1993; Murphy *et al.*, 2003; Sardenne *et al.*, 2019). This can be a good alternative to frozen storage, as freeze-dried tissues can be easier to grind and to transport than frozen tissues. Rehydration may be required to improve the lipid extraction of TAG (e.g. Dunstan *et al.*, 1993) and to comply with extraction procedures that rely on a particular proportion of water in the sample. Nonetheless, freeze-dried tissues still need to be carefully stored to prevent lipid degradation. Storage duration can have detrimental effects on lipid composition of samples. In fish, crustacean and molluscs, the total duration between freeze drying and lipid extraction should not exceed 1 month at −20°C for quantitative analysis (Sardenne *et al.*, 2019). Similarly, freeze-dried microalgae samples should not be stored for extended period of time. The PUFA proportion of filtered *Isochrysis galbana* biomass decreased from 36 to 26% after 3 months storage at −76°C (Babarro *et al.*, 2001). Proportions of FFA in freeze-dried *Phaeodactylum tricornutum* increased from 4 to 6 mg/g DW after 35 days at −20°C while the degree of oxidation was unaffected (Ryckebosch *et al.*, 2011). Variability in freeze-dried sample stability/viability for lipid 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 rapidly when water activity is high (Labuza *et al.*, 1972). Conversely, lowering water activity below 0.2 through freeze-drying process 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.

Solvent and fixative storage

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 $CHCl_3$ 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 of synthetic antioxidant is unnecessary in samples containing large amount of natural antioxidants such as microalgae (Ryckebosch *et al.*, 2012). Note that BHT elute with short-chain FA and when present in large quantities, it can overlap with FA of interest.

Fixatives. Fixatives such as ethanol or formalin are commonly used for biodiversity, taxonomy, morphological, or DNA analyses. The use of fixed samples opens new research opportunities, by making it possible to analyse historical samples collected for purposes other than lipid analysis. However, fixatives 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 is scarce, and preliminary tests on different sample types are highly recommended.

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. itemized list of content, permits) (see International Air Transport Association IATA recommendations). This section reviews different options of packaging for shipping and travelling and gives an overview of the main flying regulations and key legal information.

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 (e.g. in aeroplanes or closed vehicles). With transporter authorization, 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^{TM}, 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 Sample handling and storage before lipid extraction). 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 depressurization 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 specialized 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.

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

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

Regulations and permits

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

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

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.

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.

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 solubilize the lipids present. The most efficient extractions ensure that the particle size is as small as possible. The gold standard is to homogenize samples with a ball mill homogenizer (at −196°C using liquid nitrogen with wet samples). With wet plant or animal tissue samples at macroscales, 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 µm 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 Sample handling and storage before lipid extraction) 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 homogenize samples effectively, to ensure representative sampling. These diverse techniques also illustrate the need to adapt the preparatory steps and approach to the sample matrix.

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 the recovery of lipids from animal tissues (i.e. brain, liver, muscle), now applicable to several tissues and species with a range of lipid content (Iverson *et al.*, 2001). The Bligh and Dyer method uses a more polar solvent mixture of $CHCl_3$:MeOH:H_2O (1:2:0.8, v:v:v) with a 4:1 solvent to sample ratio and was originally developed to rapidly extract lipids, principally polar lipids, from lean fish tissues.

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 $CHCl_3$:MeOH:H_2O at 3:48:47 and while some groups do use a 50:50 MeOH:H_2O wash as recommended by Christie (1982b), 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 (1982b) 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 is 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 in solvent proportions, or cell disruption (e.g. Kumari *et al.*, 2011; Ryckebosch *et al.*, 2012). In a general manner, authors must 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.

Alternatives to chloroform: towards less toxic solvents

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 hydrochloric acid (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 stabilized 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 stabilized with either ethanol or amylene, with ethanol being the more effective stabilizer. Because dichloromethane is less susceptible to degradation than chloroform, it is typically stabilized with alkenes only. Safe laboratory 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.

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).

The special case of ethanol-preserved samples

The availability of historically preserved samples can often occur for a range of lipid studies (see section Organic solvent in Sample handling and storage before lipid extraction). 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.

Other methods

Accelerated Solvent Extraction (ASE, Dionex), also known as Pressurized Fluid Extraction, is a 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 optimize 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 is as quantitatively and qualitatively efficient as classical lipid extraction methods (Dodds *et al.*, 2004).

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 handling and storage methods. When working on new matrices and developing new or optimized extraction protocols, we recommend to compare them with Folch *et al.* (1957) or Bligh and Dyer (1959) methods, which generally remain the gold standards for lipid recovery. Ensuring complete lipid extraction is critical when applying new protocols. Performing successive lipid extractions on a same sample and measuring its lipid content at each step are the suitable approaches to establish at which stage the sample is totally lipid-depleted. We recommend monitoring extraction efficiency values and ideally reporting them in publications. Alternatively to successive extractions, the fatty acid methyl ester (FAME) content of the lipid extract can be compared to FAME recovered after direct transesterification (section Transesterification). A number of studies have compared the effect of extraction techniques (ASE, Soxhlet, manual) on lipid recovery and FA content (e.g. Ewald *et al.*, 1998; Mulbry *et al.*, 2009; Balasubramanian *et al.*, 2013). While all report differences, there is little agreement as to which may be an optimal method, likely due to substantial differences in the followed protocols.

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 oxidized 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 N_2 atmosphere with solvent (Folch mixture is generally preferred).

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 catalyzed 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 the quantitative analysis of major lipid classes including FFA. FFA 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, high-performance liquid chromatography (HPLC) with electrospray light-scattering detection 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 TLC coupled with densitometry or TLC coupled with flame ionization detection (TLC-FID, Iatroscan, Shantha, 1992) has 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 (HI) of non-polar acyl lipids and the lipolysis index of all acyl lipids (LI) can be used as degradation indicators (Weeks *et al.*, 1993; Parrish *et al.*, 1995) and are calculated as:

$$\mathrm{HI} = \frac{\mathrm{FFA} + \mathrm{ALC}}{\mathrm{TAG} + \mathrm{WE} + \mathrm{FFA} + \mathrm{ALC}} \times 100,$$

$$\mathrm{LI} = \frac{\mathrm{FFA} + \mathrm{ALC}}{\mathrm{Total\ acyl\ lipids} + \mathrm{ALC}} \times 100.$$

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

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 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 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. Malondialdehyde 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–mass spectrometry (MS) [using chemical ionization (CI)] (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 necessarily reflect PV and AVs (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).

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.

Separation of neutral and polar lipids

FA 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.

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

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

Commonly used separation methods

Storage lipids (SE, WE, TAG, DAG, ALC) and FFA are defined as "neutral" because they are hydrophobic molecules lacking charged groups, whereas PL are defined as "polar" because they have a charged hydrophilic head. Neutral and polar lipids can be separated from each other by TLC or by adsorption chromatography using either laboratory packed or commercial prepared silica columns. A simple and widely used protocol for the separation of neutral and polar lipids consists of a short column of silica prepared in a glass disposable Pasteur pipette plugged with cotton wool (Christie, 1982b). The sample is loaded on the top of the column and washed at low pressure with chloroform (or 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 the elution of glycolipids and PL (Da Costa *et al.*, 2017). Eluent and sample volumes must be adjusted to optimize 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 been used to separate groups of lipid classes, including neutral lipids such as cholesterol, and non-acidic PL, and they are especially useful for the isolation of the FFA fraction from lipid extracts (Kaluzny *et al.*, 1985; Kim and Salem, 1990).

Practical recommendations

Before starting to use a separation protocol, we recommend reading handbooks describing the use and applications of SPE for lipid separation including the choice of eluting solvents (Christie, 1982b; Perkins, 1991), and consulting technical resources provided by the SPE manufacturers. Specifically, care must be taken to avoid sample overload and subsequent cross-contamination. This problem can be avoided by knowing the lipid composition

of the sample and the carrying capacity of the column prior to SPE. The amount of sample that can be loaded onto a column is governed by the proportion of the retained lipids. For example, 2–5 mg of PL is considered as the maximum load for SPE of neutral and polar lipids on a 100-mg silica column (Christie, 1982b). Although sample overload leads to cross-contamination, an excess of sorbent can also reduce recovery of the retained analytes. It is therefore suggested to experimentally determine the sample load (mass ratio of lipid to sorbent) to optimize purity and recovery (Pernet *et al.*, 2006). To use the SPE system, we suggest replacing the silica originally contained in the pre-conditioned column with rehydrated silica (generally 6% m/m).

Purity and recovery analyses after SPE are crucial, particularly when the sample matrix is new to the laboratory and when the stationary phase changes. Different brands or batches of silica gel may vary in their properties, and cross-contamination of fractions may occur (Christie, 1982b). Purity can be evaluated by TLC of lipid classes of each eluted fraction. The simplest way to evaluate recovery is to quantify the lipid class content of samples before and after SPE by TLC and proceed with recovery calculation (i.e. the amount of collected lipid after SPE/the amount of lipids before SPE, as a percent). Alternatively, the recovery purity and efficiency can be estimated by adding an internal standard of known mass before SPE. As such, lyso-phosphatidylcholine 12:0 (22.8 µg of FA) was added to the lipid extract before SPE to assess PL recovery in oyster samples (Pernet *et al.*, 2006). Occurrence of 12:0 was exclusively associated to LPC as this FA is absent in oysters. A full recovery 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 that corresponds to the least polar lipid, which must be retained in the polar lipid fraction. For example, commercially available 12:0 DAG and 10:0 phosphatidic acid (PA) can be added to the lipid extracts of oysters (F. Pernet, unpublished data). Because these two shorter chain FA are naturally absent in oysters, their occurrence was exclusively associated with DAG 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 the mass quantification of FA (see section Analysis of FA). 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.

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 GC is to hydrolyze the ester linkage and to convert the released non-volatile FA into their volatile derivatives, usually 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 (see Schlechtriem *et al.*, 2008). 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 *iso*5:0) by increasing aliphatic chain length (Koopman *et al.*, 1996, 2003). Although the use of the boron trifluoride (BF_3) has been recommended by the AOCS (1989), many catalysts can be used in both acid- and base-catalyzed transesterifications, with each method having their own advantages and limits.

The efficiency of these transesterification methods differs as a function of the bound between the aliphatic chains and the glycerol or sphingosine backbones (Christie and Han, 2010). 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-catalyzed transesterification of plasmalogens leads to the formation of FAME (from the acyl lipids) and dimethyl acetals (DMA) (from vinyl ether lipids). Dimethyl acetals 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-catalyzed 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-catalyzed transesterification based on ISO standard methodology incorporates an acid wash to neutralize the catalyst. This acidic wash may lead to DMA formation (Gómez-Cortés *et al.*, 2019). Moreover, neither acid transesterification nor base transesterification has 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-catalyzed and is achieved by increasing the reaction time (Eder, 1995; Le Grand *et al.*, 2011). Finally, esterification of FFA is only possible by acid-catalyzed transesterification (Christie, 2003).

Depending on the scientific question (e.g. nutritional value, trophic relationships, physiological responses), FA transesterification can be realized 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.

Boron trifluoride

The Lewis acid BF_3 in methanol (12–14%, w/v) is a powerful acidic catalyst for the transesterification of FA. In general, 800 µl

to 1 ml of BF_3-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 1.5–5 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-catalyzed esterification by BF_3 (Sattler *et al.*, 1991). It is thus necessary to dry the sample either by nitrogen evaporation or by the 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 BF_3 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 BF_3-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 BF_3 as a transesterification catalyst, the difficulty in purchasing guaranteed anhydrous BF_3-MeOH, its high cost, and its high toxicity have led to a decreasing use of this reagent (Liu, 1994; Carrapiso and García, 2000; Cavonius *et al.*, 2014). In samples containing very high levels of storage lipids, such as salmon flesh, copepod oil, and cod liver, the derivatization efficiency can be quite low, leading to small but significant differences in some FA proportions (Schlechtriem *et al.*, 2008). For these reasons, BF_3 is no longer recommended as an acidic catalyst in the preparation of FAME.

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) and also from marine plankton (e.g. Troedsson *et al.*, 2005). It can easily be produced by adding 24 ml of acetyl chloride to 100 ml of methanol, which leads to the formation of 10% (approximately equivalent to 3 N) methanolic HCl. Sulphuric acid (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-catalyzed reagents, such as HCl or H_2SO_4, are not associated with the issues described for BF_3. Caution is, however, needed during heating to prevent evaporation. 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.

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-catalyzed method described above with the substitution of the catalyst and the introduction of an additional step after the reaction is quenched to neutralize the remaining base. All base-catalyzed 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 the use of basic catalysts. As noted above, basic catalysts are unable to esterify FFA so these approaches should only be used when one can be confident that FFA are present in very low amounts, or else when the analyst is targeting esterified FA exclusively (e.g. studies on membrane lipids or storage lipids).

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

Direct transesterification

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

Different methods have been tested and provided reproducible and similar results to the classical extraction–transesterification method (Castro-Gómez *et al.*, 2014; Parrish *et al.*, 2015a). Careful consideration is needed when using this approach as it may not be appropriate to the study goal and/or the matrices analysed. It may also be preferentially applied to small and homogenous (representative of the whole) samples. For example, while it is possible to carry out direct analyses on a small amount of tissue, accurate determination of total blubber FA composition in marine mammals was not possible with this approach due to significant variation in FA composition within the layers of the blubber (Thiemann *et al.*, 2008). Although this rapid direct transesterification approach is less time-consuming and cheaper, once the

entire sample is transesterified, it is not possible to redo the analysis, or to analyse the FA composition of different lipid fractions.

Alternatives to hexane

Hexane, iso-hexane (2-methyl-pentane), or iso-octane are most frequently used to recover FAME after transesterification and before injection. However, hexane is carcinogenic, mutagenic, and reprotoxic and is a hazardous airborne pollutant. Less toxic solvents such as pentane and heptane can be used as alternatives to hexane (Alfonsi *et al.*, 2008). However, recovery of FA from macroalgae is lower with heptane than with hexane, with PUFA tending to be more affected than saturated fatty acid and monounsaturated fatty acid (MUFA) (Le Grand, unpublished data). This preliminary result needs to be verified on other matrices. Pentane rather than hexane may also be used as the solvent in the BF_3/MeOH procedure to reduce the reaction temperature from 85–100°C to 80°C to decrease artefact risk (Parrish, 1999).

Analysis of FA

The full process of FAME analysis consists of injection, separation, identification, and quantification of FAME and each of these steps has to be optimized to achieve high accuracy and precision (see Eder, 1995; Christie, 2003 for review). GC with 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, artefacts, 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.

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 vaporizing chamber (classical vaporizing injection), (ii) injected into a cool chamber, which is subsequently heated to vaporize the sample (programmed temperature vaporizing injection), or (iii) directly injected into the column inlet (direct injection or on-column).

Once vaporized, 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 vaporized 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. FA 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 vaporizing injection method for suitability and flexibility reasons. High-speed injection, high injector temperature (>220°C) and proper injector inserts all contribute to decreasing discrimination problems. However, we recommend regular checks of FAME discrimination factors using certified reference mixtures (e.g. Supelco 37).

Separation

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

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

Many researchers use polar columns, which allow for a better separation of aquatic FA than non-polar phases, with only some rare and minor co-elutions. Ackman (1986) was a strong advocate of the polyethylene glycol (PEG) phase. Columns coated with PEG are easily recognizable because of their commercial name with the suffix "wax". With this polar phase, FAME continue to elute in order of increasing boiling point, and also roughly in order of increasing numbers of double bonds. However, polar columns are highly sensitive to temperature (max 250°C), water, and oxygen and are thus less stable over time than non-polar columns. Retention times may also vary (decrease) over time, especially for highly unsaturated compounds. Consequently, peak identification (based on elution time) needs to be checked regularly and rigorously.

An alternative phase consists of substituting 50% dimethylpolysiloxane (non-polar) with the polar cyanopropylmethyl (e.g. Iverson *et al.*, 2004; Pedro *et al.*, 2019). This phase provides similar resolution as PEG phases, although it is much less sensitive to oxygen, resulting in longer column lifetimes. The chemistry of this phase is more influenced by temperature than other polar columns. Separation of co-eluting peaks can sometimes easily be achieved simply by subtle adjustments to oven temperature ramps.

Identification

The most common way to identify FAME by GC-FID analysis is to compare their retention times with those of commercially

available individual purified standards. Among the most used standards for the FA identification in aquatic samples, there are PUFA No. 3, a complex qualitative standard mixture extracted from Menhaden oil, and Supelco® 37 Component FAME Mix, a comprehensive mixture of 37 FAME ranging from 4:0 to 24:1*n*-9. Several long-chain and (highly) unsaturated FAME occurring in some samples are not commercially available but can be identified using fully characterized natural products (e.g. the non-methylene interrupted FA typical of marine molluscs in Zhukova and Svetashev, 1986; Ackman, 1989; Kraffe *et al.*, 2004).

In addition to these protocols, confirmation of FA identification is best achieved using GC–MS. Peak identification is achieved using commercial spectral libraries (e.g. The LipidWeb http://lipidhome.co.uk), or via personal/laboratory FA mass spectral libraries to assist in identification of often complex environmental FAME samples. Further details on various GC–MS protocols together with specific 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 CI 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 an *O*-trimethyl silyl (*O*-TMSi) ether or other derivative is generally used. Bistrimethylsilyltrifluoroacetamide 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 *O*-TMSi derivative occurs alpha to the carbon containing the hydroxyl group, with the main cleavage on the side adjacent to the carboxyl group.

Derivatization 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. Polyunsaturated FA 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 characterization 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 22:4*n*-9t in scallop (Marty *et al.*, 1999). MonounsaturatedFA 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 characterization of a range of novel C_{16} and C_{18} MUFA, including both *cis*- and *trans*-isomers, from microbial monocultures and complex soil consortia (Nichols *et al.*, 1986). Other derivatization procedures are also available, although they are not covered here, and are as detailed in Christie (1989).

Quantification

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

The internal standard must not coelute with the FAME, its chemical structure must be close to the majority of the FAME, and it must be absent from the sample. A known mass of internal standard should be added either after SPE or before transesterification to account for the derivatization yield and recovery of FAME (see section Separation of neutral and polar lipids). The most widely used standards are the carboxylic form of nonadecanoic acid (19:0), heneicosanoic acid (21:0), and tricosanoic acid (23:0). Data are generally expressed as relative (area %, mass % or mole %) or absolute contents (e.g. mg FA/g sample dry mass).

With GC–MS analyses, one approach is to use the total ion chromatogram in a similar fashion as peak area is used with GC-FID, by comparing the peak area of an internal standard with peak areas of compounds of interest, and relative response factors determined individually using authentic standards. Note here that response factors vary with structure (carbon and unsaturation numbers, mostly notably), ionization methods (electronic vs. chemical) and mass detector (e.g. quadrupole vs. ion trap) (Dodds *et al.*, 2005; Quehenberger *et al.*, 2011). Consequently, the range of response factors is greater in MS than with FID. Response factors for FA, where standards are not available, are assumed the same as those of structurally similar and available compounds but this is arguable.

For greater sensitivity, calibration can be based on prominent ion(s) using selected ion monitoring (SIM) as a single ion can be scanned many more times in a cycle than the full mass spectrum, giving a higher signal to noise ratio (Dodds *et al.*, 2005; Thurnhofer and Vetter, 2005; Quehenberger *et al.*, 2011). It involves the exclusive acquisition of a group of selected ions during a given period if the identity and retention time of a given analyte have been established through full scan mode. Since fragmentation can vary depending on chain length and degree of unsaturation, careful assignments of selected ions group to analytes and appropriate adjustments of SIM parameters are necessary to achieve accurate quantification and complete discrimination when some FA are co-eluting.

Quantification of FA can be further improved by using mixtures of isotopically labelled FA internal standards (e.g. deuterated FA). 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 components

is the same (Quehenberger *et al.*, 2011). The main limitation to this approach is the commercial availability of isotope-labelled FA that could "match" all FA present in aquatic samples.

Data treatment

As emphasized 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 valorize 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 standardization and/or transformation, FA data can be analysed in different, non-mutually exclusive ways. Unconstrained ordination methods can help visualizing 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.

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 be chosen carefully with respect to the ecological context and scientific question of interest. When used sensibly, biomarker compounds can be very useful, as proven by their routine use in ecological literature (see Dalsgaard *et al.*, 2003; Kelly and Scheibling, 2012 for review).

Standardization and transformation

Choosing standardization 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). Fatty acids can be expressed either as an absolute content or mass fraction, such as mg/g 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 are the commonly used standardization 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 varies across studies and FA analysis methodologies, limiting inter-study comparison.

Choice of data standardization 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 allow one to circumvent the violation of the multivariate normality assumption in constrained ordination methods such as redundancy analysis (RDA, Legendre and Legendre, 2012; see section Multivariate hypothesis tests and modelling).

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 emphasizing the presence and the role of rare FA, or if focusing on the dominant FA will give a more global pattern within and across organisms.

Exploring FA data through ordination

Ordination methods are essential to describe and visualize sample dispersion in a complex multidimensional dataset and to identify groups of co-varying variables. Those are computed based on a matrix of resemblances (distance, dissimilarities, similarities), or derived ranks, rather than raw (or transformed) data. In FA literature, the most commonly used resemblance measure between samples are either the Euclidean distance or percentage difference (also called Bray–Curtis dissimilarity; Legendre and Legendre, 2012). Euclidean distance is generally preferred over Bray–Curtis for FA analysis (Happel *et al.*, 2017). However, just like data transformations, the choice of the resemblance measure can have important impacts on the final results. The choice of matrix will depend on whether a dataset includes null/absent values and if they need to be included in the analysis according to the study question. The percentage difference coefficient is double-zero asymmetric, i.e. the absence of one FA from two individuals is not considered as contributing to their similarity. The Euclidean distance, on the contrary, is double-zero symmetric, i.e. will be affected by double null values (Legendre and Legendre, 2012). Other properties of resemblance measures should also be considered. Choice of the resemblance coefficient has received extensive

attention in multivariate community ecology (e.g. Legendre and Legendre, 2012).

Commonly used methods to plot ordinations include principal components analysis (PCA), principal coordinates analysis (PCoA), and non-metric multidimensional scaling (nMDS). PCA can only use Euclidean distance, while PCoA and nMDS can use any resemblance measure. PCoA and nMDS use the ranks of similarities or dissimilarities matrix to find a spatial arrangement of individuals and represent relationships among them (Kelly and Scheibling, 2012). NMDS is conceptually simple, generally applicable, and well adapted to ecological data (Clarke and Warwick, 2001). However, it is crucial to take into account the stress coefficient, which is measured from the relationship between ranks of dissimilarities and ranks of sample distances on the plot. A high stress score (>0.05) indicates that data have been highly distorted to fit in the visualization, which may not be representative and should be considered with caution (Clarke and Warwick, 2001; Buttigieg and Ramette, 2014). Principal components analysis also describes relationships among samples, reducing large numbers of variables to a few components that represent most of the variance and that are built by combining correlated variables (Budge *et al.*, 2006; Kelly and Scheibling, 2012). It also helps to visualize variables that contribute the most to the dissimilarity among observed groups and FA that are highly correlated (Budge *et al.*, 2006). After ordination, several procedures (similarity percentage, also called SIMPER, analysis; comparison of PCA loadings) can be applied to identify FA-driving resemblance or dissimilarity among groups (Clarke and Warwick, 2001).

Multivariate hypothesis tests and modelling

Multivariate analyses are commonly applied in ecological studies to discriminate the influence of different factors on FA composition, such as physiological, spatial, or even temporal variations (Anderson, 2001). Statistical comparison of groups based on FA composition is generally performed through non-parametric or randomization procedures, as FA datasets rarely match the prerequisites of parametric statistical tests (Anderson and Walsh, 2013). Analysis of similarity (ANOSIM; Clarke, 1993) or permutational analysis of variance (PERMANOVA; Anderson, 2001) is among the most commonly used statistical test (e.g. Hughes *et al.*, 2005; Hixson *et al.*, 2015; Parrish *et al.*, 2015a; Sardenne *et al.*, 2016; Mathieu-Resuge *et al.*, 2019). Analysis of similarity uses ranks of pairwise dissimilarities and generates its own statistic by randomization of rank dissimilarities (Clarke and Warwick, 2001). Permutational analysis of variance tests for differences among group centroids (Anderson, 2001). Both methods are affected by dispersion heterogeneity, with ANOSIM being more sensitive than PERMANOVA (Anderson and Walsh, 2013). Permutational analysis of variance is unaffected by differences in correlation structure and seems more powerful to detect changes in FA composition. As such, applying ANOSIM is appropriate for a one-way test (e.g. to compare one species submitted to different diets), but not for more complex study designs (Kelly and Scheibling, 2012).

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

Coupling FA with other ecological markers

When tackling elaborate ecological questions, combining FA with other tracers has proven to be very powerful. Tracers commonly combined with FA include stable isotope ratios of light biogenic elements (Kharlamenko *et al.*, 1995, 2001; Nyssen *et al.*, 2005), as well as contaminants (Kainz and Fisk, 2009; Le Croizier *et al.*, 2016). In many cases, those combinations are qualitative. However, modern data analysis frameworks allow quantitative coupling of stable isotope and FA in mixing models (Stock *et al.*, 2018) or ecological niche models (Jackson *et al.*, 2011; Sardenne *et al.*, 2016). Such developments hold great promise for ecological research, due to the complementarity of these two methods. More generally, multiple factor analysis (Pagès, 2014) or co-inertia analysis (Dray *et al.*, 2003) could provide flexible, widely applicable and quantitative ways to couple data from different methods by allowing exploration of the links between multiple 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 the 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 $-20°C$ for <1 month, $-80°C$ for <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.

The lipid extract is either separated into classes or directly transesterified. Separation of polar and neutral lipids prior to transesterification allows for a better interpretation of FA profiles when seeking information on nutritional status and/or physiology of organisms. Special attention should be paid to control for the purity and recovery of each fraction. Non-volatile FA are then transesterified into their volatile derivatives, usually FAME, before being analysed by GC. Acid-catalyzed reagents, such as HCl or H_2SO_4, are the most commonly used in aquatic science as they present fewer analytical issues than BF_3. Hexane is the most widely used solvent to recover FAME after transesterification. Less toxic alternatives are emerging but are not applicable to all sample types. The analysis and quantification of FAME is usually conducted by GC-FID. FAME are identified by comparing their retention times with those of individual purified standards. They are quantified as relative proportions using peak areas or by absolute concentration when an internal standard was added before transesterification. When necessary, peak identification can be confirmed using GC–MS, but this process may require additional derivatization steps. Data treatment and statistical analysis of FA should be carefully applied according to the investigated scientific question. We presented common multivariate statistical procedures that we consider widely relevant for FA-related problems. This list is by no means exhaustive. We encourage analysts to explore their data in multiple ways. A good starting point is usually a simple preliminary visualization of data. This will not only help to check for data quality but also to make informed choices about subsequent procedures. When interpreting data and statistical results, it is crucial to take into account the studied organisms and/or ecosystems and their specificities.

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

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