Chapter 17
Polyunsaturated Fatty Acids in Decomposing Leaf Litter

Eric von Elert

Keywords Aquatic hyphomycetes · Eicosapentaenoic acid · EPA · Food quality · GC · Invertebrates · Lipid extraction · Litter quality · PUFA · Shredders

1 Introduction

Polyunsaturated fatty acids (PUFAs) are a subclass of fatty acids characterized by more than one double bond arranged in *cis*-configuration and separated by a single $–CH_2–$ group. PUFAs are divided into two major classes distinguished by the position of the first double bond when counted from the terminal methyl group. In n-3 PUFAs, this double bond is at the third position from the terminal carbon atom, and in n-6 PUFAs it is at the sixth position. As this difference reflects distinct biosynthetic pathways, PUFAs can be converted within but not between these two classes. One function of PUFAs is that they serve as precursors for hormones. For example, long-chain PUFAs such as arachidonic acid (ARA; C20:4n-6) and eicosapentaenoic acid (EPA; C20:5n-3) serve as precursors of prostaglandins, which have several hormonal functions in arthropod reproduction (Stanley 2000; Schlotz et al. 2012). Furthermore, PUFAs are an integral constituent of cell membranes, where they mainly occur in phospholipids.

Arthropods are unable to synthesize long-chain PUFAs *de novo* and hence require a dietary source to satisfy their physiological needs (Harrison 1990). This makes PUFAs an essential class of lipids and suggests constraints on consumers when diets have low PUFA contents. Evidence for such a limitation in freshwater arthropods includes strong correlations between PUFA contents of natural phytoplankton and the growth rate of *Daphnia* (Müller-Navarra 1995; Müller-Navarra et al. 2000; Wacker and von Elert 2001; Hartwich et al. 2012), with EPA and

E. von Elert (✉)
Institute of Zoology, University of Cologne, Cologne, Germany
e-mail: evelert@uni-koeln.de

© Springer Nature Switzerland AG 2020

F. Bärlocher et al. (eds.), *Methods to Study Litter Decomposition*,
https://doi.org/10.1007/978-3-030-30515-4_17

α-linolenic acid (α-LA, C18:3n-3) producing the strongest relationship. Supplementation experiments have confirmed that low concentrations of EPA or α-LA in food algae can indeed limit not only the somatic growth of *Daphnia* (von Elert 2002; Becker and Boersma 2005) but also parthenogenetic egg production (Wacker and Martin-Creuzburg 2007; Martin-Creuzburg and von Elert 2009), and similar effects have also been demonstrated for the zebra mussel, *Dreissena polymorpha* (Wacker and von Elert 2003a, b).

PUFA deficiency in one food item can be mitigated by another that is rich in PUFAs (Marzetz et al. 2017). Similarly, temporal fluctuations in dietary PUFA content can be buffered (Koussoroplis et al. 2017), because consumers can store PUFAs and thus profit from ingesting a PUFA-rich diet slightly earlier or later than a PUFA-deficient diet (Koussoroplis et al. 2017). Such mitigating effects can mask the potentially limiting nature of low dietary PUFA contents. At low temperatures, PUFA limitation becomes more severe (Sperfeld and Wacker 2012). This effect has been attributed to reduced membrane fluidity (Hazel 1995), which poikilotherms try to counter by increasing the content of unsaturated fatty acids in their membranes (Farkas 1979; Hazel 1995; Hazel and Williams 1990). Thus, PUFA-deficient diets can constrain consumer fitness and limit the use of cold habitats (Brzezinski and von Elert 2015).

Given the importance of PUFAs as a determinant of food quality and huge amounts accumulating in some benthic invertebrates (*Hydropsyche* spp., *Ephemerella* spp., isopods, oligochaetes; Torres-Ruiz et al. 2007; gammaridae; Makhutova et al. 2016), these lipids could also play an important role for stream detritivores. Indeed, one hypothesis to explain the observation that microbial colonization and partial decomposition of litter improve the palatability to, and promote the growth of, litter consumers in streams (Graça 2001) is that microbial lipids enhance litter quality as food (Cargill et al. 1985). This hypothesis is supported by the high relative content of the PUFAs C18:2n-6 and C18:3n-3 in aquatic hyphomycetes (Arce Funck et al. 2015). Poor growth of *Gammarus fossarum* feeding on undecomposed leaf litter from alder (*Alnus glutinosa*) was indeed significantly increased by supplementing the food with a diatom (*Nitzschia palea*) rich in PUFAs, although supplementation with aquatic hyphomycetes affected neither consumption (Aßmann et al. 2011) nor growth (Crenier et al. 2017). The aquatic hyphomycetes were rich in C18:2n-6, whereas the diatom was rich in C20:5n-3, suggesting a limitation of *Gammarus* growth on leaf litter by C20:5n-3 or possibly other n-3 PUFAs. Notably, however, PUFA contents in submerged leaves colonized by microbes were found to be very low (Torres-Ruiz and Wehr 2010), and *G. roeselii* tested in food-choice experiments showed no clear preference for leaf litter of alder (*Alnus glutinosa*) coated with lipid extracts of fungi and an oomycete (Aßmann and von Elert 2009; see also Rong et al. 1995). In view of these mixed results and the fact that the

Table 17.1 Fatty acid content of abscissed maple leaves incubated in tap water for 1 week (*Acer platanoides*), freshly fallen leaf litter of common hornbeam (*Carpinus betulus*) (1) and a leaf-litter mixture of *Acer rubrum*, *Quercus rubra*, and *Platanus occidentalis*

	Fatty acid content (μg g^{-1} dry mass)		
Fatty acid	*A. platanoides* (1)	*C. betulus* (1)	Leaf-litter mixture (2)
C 14:0	439 (26)	50 (5)	
C 14:1 n-9	279 (13)	208 (32)	
C 16:0	4466 (128)	1467 (55)	
C 16:1 n-9	388 (23)	20 (1)	
C 18:0	747 (1)	502 (36)	
C 18:1 n-9 c	126 (8)	0 (0)	
C 18:2 n-6 c	2160 (70)	525 (50)	670 (50)
C 18:3 n-6	17 (1)	29 (1)	
C 18:3 n-3	20,777 (762)	2112 (144)	2050 (220)
C 22:0	62 (1)	105 (2)	
C 24:0	442 (1)	238 (12)	
Total	29,902 (750)	5258 (340)	

Values are means (SE) of three replicates
1 = von Elert (unpublished data), 2 = Torres-Ruiz and Wehr (2010)

role of PUFAs is still poorly investigated in detritus-based food webs, general conclusions about the importance of PUFAs in these systems are currently premature.

Some data are available on the composition of fatty acids in leaf litter (e.g., Guo et al. 2018). Information on the fatty acid content is scarce, however; the total fatty acid content in freshly fallen leaves ranges from 5 to 30 mg g^{-1} dry mass in the few available studies to date (Table 17.1). In maple leaves (*A. platanoides*) and leaf litter of common hornbeam (*C. betulus*), the most abundant PUFAs were α-LA (C18:3n-3) and C18:2n-6, whereas C_{20} PUFAs were undetectable (unpublished data). This result is in accordance with Torres-Ruiz and Wehr (2010) and suggests that C_{20} PUFAs are scarce in microbially conditioned leaf litter. Choice experiments have indicated that α-LA (C18:3n-3) can serve as an indicator of food palatability (Vonk et al. 2016). Although this finding points to a role of PUFAs in determining detritivore food preference, supplementation experiments with specific PUFAs are needed to assess the potential importance for growth and reproduction of litter consumers. The high PUFA contents of some benthic invertebrates mentioned above (Torres-Ruiz et al. 2007) might even point to PUFA sources other than leaf litter to mitigate PUFA deficiency for litter-consuming detritivores (Guo et al. 2018).

The method presented here details procedures to quantify individual PUFAs in leaf litter, invertebrates, and other types of samples, complementing a method to determine the total lipid content in leaf litter as described in Chap. 16.

2 Equipment, Chemicals, and Solutions

2.1 Equipment and Materials

- Freezer (−20 or −80 °C)
- Refrigerator (4 °C)
- Stream of nitrogen gas under a fume hood
- Heating block or water bath (40 and 70 °C)
- Vortex
- Ultrasonic bath
- Centrifuge suited for glass reagent tubes (e.g., 16 mm × 100 mm; 3000 *g*)
- Glass reagent tubes (e.g., 16 mm × 100 mm) with screw caps
- Sample vials with micro-inserts (200 µl) and septum-lined caps
- Pasteur pipettes
- Gas chromatograph (GC) with a splitless injector and a flame ionization detector (FID), equipped with a capillary GC column suited for the analysis of fatty acid methyl esters (FAMEs), e.g., an Agilent J&W DB-225 column (length 30 m, ID 0.25 mm, film thickness 0.25 µm; von Elert 2002).

2.2 Chemicals

- Liquid nitrogen
- Dichloromethane, HPLC grade
- Methanol, HPLC gradient grade
- Isohexane, gas chromatography FID grade
- HCl in methanol (3 M), CAS number 7647-01-0
- Reference compounds
  - Heptadecanoid acid methyl ester (C17:0 ME), CAS number 1731-92-6
  - Nonadecanoid acid methyl ester (C19:0 ME), CAS number 1731-94-8
  - Tricosanoic acid methyl ester (C23:0 ME), CAS number 2433-97-8
  - Commercially available mixture of fatty acid methyl esters (FAME), such as the Sigma™ 37 Component FAME Mix, menhaden oil, or the bacterial acid methyl ester (BAME) mix

2.3 Solutions

- Extraction solvent: dichloromethane/methanol (2:1, v:v)
- C17:0 ME, C19:0 ME and/or C23:0 ME (each 200 µg ml^{-1} isohexane)

3 Experimental Procedures

3.1 Sample Preparation

1. Collect leaf litter or animal sample in the field or from a laboratory experiment and protect from light and elevated temperatures throughout the analysis to avoid oxidation and auto-degradation of polyunsaturated lipids.
2. Blot-dry samples when water adheres to the surface (e.g., on Kim-Wipes™) and extract lipids from these fresh samples (the solvent efficiently extracts lipids from living tissue, so that freeze-drying of samples is not critical).
3. If results are to be related to litter dry mass, best split samples and use one portion for dry mass determination, the other for fatty acid analysis.
4. If a sample is used for both dry mass and fatty acid analyses, freeze the sample in liquid N_2, and store at −20 or −80 °C if immediate processing is not possible.
5. Freeze-dry and homogenize samples using pestle and mortar. Minimize storage at this stage (even at − 20 or −80 °C) to avoid a possible slow oxidation of unsaturated fatty acids; however, freeze-dried and homogenized samples immersed immediately in solvent for lipid extraction can be stored at −20 °C for several weeks.
6. Determine the dry mass of samples prior to adding solvent for lipid extraction.

3.2 Lipid Extraction

1. Transfer leaf litter or animal sample in screw-cap reagent tube and add 5 ml of extraction solvent with a Pasteur pipette.
2. Add internal standards (IS), with the type and amounts depending on the sample (e.g., 100 µl C17:0 ME, C19:0 ME, and/or C23:0 ME per 2–4 mg of litter dry mass or per 200–600 µg of animal dry mass).
3. Incubate overnight at 4–8 °C.
4. Perform all subsequent steps at low light and especially avoid exposure to direct sunlight.
5. Vortex the sample tubes and place them in an ultrasonic bath for 1 min.
6. Centrifuge for 5 min at 3000 *g* (without brake!)
7. Use a Pasteur pipette to transfer the extract into a clean screw-cap reagent tube, add another 3 ml of extraction solvent to the original tube, close the tube, and repeat the sample extraction by ultrasonication for 1 min and subsequent centrifugation.
8. Use a Pasteur pipette to combine the second extract with the first.
9. Spin down any debris in the combined extract by centrifuging the reagent tubes at 3000 *g* for 5 min (without brake!).

10. Carefully (sensitive pellet!) transfer the supernatant with a Pasteur pipette to a clean screw-cap reagent tube.
11. Resuspend the pellet with another 3 ml of extraction solvent, vortex, and then centrifuge for 5 min at 3000 *g* (without brake!) and combine the supernatants.
12. Place the reagent tube containing the combined supernatants into a heating block or water bath (max. temperature 40 °C) and evaporate the solvent under a stream of nitrogen gas, immediately removing the tube from the heating block when dry.

3.3 Transesterification

1. Proceed immediately with transesterification by adding 5 ml of 3 M methanolic HCl to the reagent tube, closing the screw cap, and incubating the sample for 20 min at 70 °C in a heating block or water bath.
2. Let the sample cool down to room temperature or lower (e.g., by placing the tube at 4–8 °C in a refrigerator).
3. Add 2 ml of isohexane with a Pasteur pipette, and vortex three times for several seconds, allowing the phases to separate each time between mixings.
4. Transfer the upper hexane phase with a Pasteur pipette into a new screw-cap reagent tube.
5. Repeat this isohexane extraction two more times, ensuring that no solvent from the lower phase is transferred by leaving a few microliters of the upper layer in the sample.
6. Evaporate the combined isohexane extracts under a stream of nitrogen gas at max. 40 °C in a heating block or water bath.
7. Dissolve the dry deposit in the reagent tube in 100 µl isohexane and transfer the solution to a microliter-insert in a sample vial.
8. Repeat this step to obtain a total volume of 200 µl in the sample vial.
9. Gently evaporate the solvent again under a stream of nitrogen and dissolve the dry deposit in the microliter-insert of a sample in a final volume of 100 µl isohexane.
10. Close the vial with a septum cap and store at −20 °C until measurement by gas chromatography.

3.4 Gas Chromatography

1. Set the flow of the carrier gas (e.g., 35 cm s^{-1}, helium).
2. Set FID detector and injector to 220 °C.
3. Set the temperature program for the GC oven, e.g., 60 °C (hold for 1 min) and then to 180 °C at 120 °C min^{-1}, next to 200 °C at 50 °C min^{-1} (hold for 10.5 min), and then to 220 °C at 120 °C min^{-1} (hold for 7.5 min), according to von Elert (2002), resulting in a total run time per sample of 20.6 min.

4. Inject a 1 µl aliquot of each sample in splitless mode.
5. Inject reference compounds to identify peaks in a given sample as FAMEs with retention times identical to those of the reference compounds.
6. For quantification by means of internal standards (IS), establish dose-response curves for each of the FAMEs of interest, based on different ratios of IS with the FAME of interest; this is most conveniently achieved by preparing a mixture of all FAMEs of interest with known concentrations and mixing increasing aliquots with known amounts of IS.
7. Subsequently, derive calibration curves for each FAME of interest from splitless injections of 1 µl aliquots of these solutions.

4 Final Remarks

Several types of reference compounds can be used to identify FAMEs by comparing retention times. For routine analyses of fatty acids with an even number of carbon atoms, these include a commercially available mixture containing 37 components (Sigma™ 37 Component FAME mix), menhaden oil, and specifically prepared PUFA mixes. Bacterial acid methyl ester (BAME) mixtures serve well for fatty acids with an odd number of carbon atoms (e.g., bacterial fatty acids). Petroselinic acid (C18:1n-12) and oleic acid (C18:1n-9) cannot be distinguished.

The presented GC method quantifies absolute amounts of fatty acids, which can be related to sample dry mass or organic carbon. The detection limit is 10 ng of FAME mg^{-1} dry mass. Quantification requires the systematic addition of an internal standard (IS) to the samples. If the fatty acid profiles of samples are unknown, it is good practice first to perform a qualitative analysis without IS to check which FAMEs (C17:0 ME, C19:0 ME, C23:0 ME) are best suited as IS. Unless precluded by other constraints, C17:0 ME and C23:0 ME are best used simultaneously to relate FAMEs with a low retention time to C17:0 ME and those with a high retention time to C23:0 ME. Separate calibration curves with different ratios of IS need to be established for all FAMEs of interest. The amount of IS may have to be adjusted to the fatty acid content of the sample, to ensure that the ratios of IS to each fatty acid are covered by the calibration curve. A dry mass of 3–4 mg litter and 200–600 µg animal tissue are good starting points.

References

Arce Funck, J., Bec, A., Perrière, F., Felten, V., & Danger, M. (2015). Aquatic hyphomycetes: A potential source of polyunsaturated fatty acids in detritus-based stream food webs. *Fungal Ecology, 13*, 205–210.

Aßmann, C., & von Elert, E. (2009). The impact of fungal extracts on leaf litter on the food preference of *Gammarus roeselii*. *International Review of Hydrobiology, 94*, 484–496.

Aßmann, C., Rinke, K., Nechwatal, J. A. N., & von Elert, E. (2011). Consequences of the colonisation of leaves by fungi and oomycetes for leaf consumption by a gammarid shredder. *Freshwater Biology, 56*, 839–852.

Becker, C., & Boersma, M. (2005). Differential effects of phosphorus and fatty acids on *Daphnia magna* growth and reproduction. *Limnology and Oceanography, 50*, 388–397.

Brzezinski, T., & von Elert, E. (2015). Predator evasion in zooplankton is suppressed by polyunsaturated fatty acid limitation. *Oecologia, 179*, 687–697.

Cargill, A. S., II, Cummins, K. W., Hanson, B. J., & Lowry, R. R. (1985). The role of lipids, fungi and temperature in the nutrition of a shredder caddisfly, *Clistoronia magnifica*. *Freshwater Invertebrate Biology, 4*, 64–78.

Crenier, C., Arce-Funck, J., Bec, A., Billoir, E., Perrière, F., Leflaive, J., Guérold, F., Felten, V., & Danger, M. (2017). Minor food sources can play a major role in secondary production in detritus-based ecosystems. *Freshwater Biology, 62*, 1155–1167.

Farkas, T. (1979). Adaptation of fatty-acid compositions to temperature - study on planktonic crustaceans. *Comparative Biochemistry and Physiology B – Biochemistry and Molecular Biology, 64*, 71–76.

Graça, M. A. S. (2001). The role of invertebrates on leaf litter decomposition in streams – A review. *International Review of Hydrobiology, 86*, 383–393.

Guo, F., Bunn, S. E., Brett, M. T., Fry, B., Hager, H., Ouyang, X. G., & Kainz, M. J. (2018). Feeding strategies for the acquisition of high-quality food sources in stream macroinvertebrates: Collecting, integrating, and mixed feeding. *Limnology and Oceanography, 63*, 1964–1978.

Harrison, K. E. (1990). The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: A review. *Journal of Shellfish Research, 9*, 1–28.

Hartwich, M., Martin-Creuzburg, D., Rothhaupt, K. O., & Wacker, A. (2012). Oligotrophication of a large, deep lake alters food quantity and quality constraints at the primary producer-consumer interface. *Oikos, 121*, 1702–1712.

Hazel, J. R. (1995). Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation? *Annual Review of Physiology, 57*, 19–42.

Hazel, J. R., & Williams, E. E. (1990). The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in Lipid Research, 29*, 167–227.

Koussoroplis, A. M., Pincebourde, S., & Wacker, A. (2017). Understanding and predicting physiological performance of organisms in fluctuating and multifactorial environments. *Ecological Monographs, 87*, 178–197.

Makhutova, O. N., Shulepina, S. P., Sharapova, T. A., Dubovskaya, O. P., Sushchik, N. N., Baturina, M. A., Pryanichnikova, E. G., Kalachova, G. S., & Gladyshev, M. I. (2016). Content of polyunsaturated fatty acids essential for fish nutrition in zoobenthos species. *Freshwater Science, 35*, 1222–1234.

Martin-Creuzburg, D., & von Elert, E. (2009). Good food versus bad food: The role of sterols and polyunsaturated fatty acids in determining growth and reproduction of *Daphnia magna*. *Aquatic Ecology, 43*, 943–950.

Marzetz, V., Koussoroplis, A. M., Martin-Creuzburg, D., Striebel, M., & Wacker, A. (2017). Linking primary producer diversity and food quality effects on herbivores: A biochemical perspective. *Scientific Reports, 7*, 11035.

Müller-Navarra, D. C. (1995). Evidence that a highly unsaturated fatty acid limits *Daphnia* growth in nature. *Archiv für Hydrobiologie, 132*, 297–307.

Müller-Navarra, D. C., Brett, M. T., Liston, A. M., & Goldman, C. R. (2000). A highly unsaturated fatty acid predicts carbon transfer between primary producers and consumers. *Nature, 403*, 74–77.

Rong, Q., Sridhar, K. R., & Bärlocher, F. (1995). Food selection in three leaf-shredding stream invertebrates. *Hydrobiologia, 316*, 173–181.

Schlotz, N., Sørensen, J. G., & Martin-Creuzburg, D. (2012). The potential of dietary polyunsaturated fatty acids to modulate eicosanoid synthesis and reproduction in *Daphnia magna*:

A gene expression approach. *Comparative Biochemistry and Physiology A – Molecular and Integrative Physiology, 162*, 449–454.

Sperfeld, E., & Wacker, A. (2012). Temperature affects the limitation of *Daphnia magna* by eicosapentaenoic acid, and the fatty acid composition of body tissue and eggs. *Freshwater Biology, 57*, 497–508.

Stanley, D. W. (2000). *Eicosanoids in invertebrate signal transduction systems*. Princeton: Princeton University Press.

Torres-Ruiz, M., & Wehr, J. D. (2010). Changes in the nutritional quality of decaying leaf litter in a stream based on fatty acid content. *Hydrobiologia, 651*, 265–278.

Torres-Ruiz, M., Wehr, J. D., & Perrone, A. A. (2007). Trophic relations in a stream food web: Importance of fatty acids for macroinvertebrate consumers. *Journal of the North American Benthological Society, 26*, 509–522.

von Elert, E. (2002). Determination of limiting polyunsaturated fatty acids in *Daphnia galeata* using a new method to enrich food algae with single fatty acids. *Limnology and Oceanography, 47*, 1764–1773.

Vonk, J. A., van Kuijk, B. F., van Beusekom, M., Hunting, E. R., & Kraak, M. H. S. (2016). The significance of linoleic acid in food sources for detritivorous benthic invertebrates. *Scientific Reports, 6*, 35785.

Wacker, A., & Martin-Creuzburg, D. (2007). Allocation of essential lipids in *Daphnia magna* during exposure to poor food quality. *Functional Ecology, 21*, 738–747.

Wacker, A., & von Elert, E. (2001). Polyunsaturated fatty acids: Evidence for non-substitutable biochemical resources in *Daphnia galeata*. *Ecology, 82*, 2507–2520.

Wacker, A., & von Elert, E. (2003a). Effects of larval food conditions on recruitment of *Dreissena polymorpha*. *Ecological Studies, Hazards and Solutions, 6*, 49. Moscow: MAX-Press.

Wacker, A., & von Elert, E. (2003b). Food quality controls reproduction of the zebra mussel (*Dreissena polymorpha*). *Oecologia, 135*, 332–338.