Fatty acid analysis in European flounder muscle: a promising tool to assess the impact of eutrophication on estuarine health

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Abstract

In the present paper, we developed an approach combining lipid class and fatty acid analyses on European flounder muscle, watershed geographic metrics and pollutant analyses in sediments, to assess the ecological status of seven small French estuaries. The watersheds were differentiated by contrasted fatty acids compositions in flounder muscle. The analysis of fatty acids, and more specifically polar lipids, provided a good understanding of the physiological responses of fish to their environment. Within polar lipids, differences in polyunsaturated fatty acid (PUFA) proportions, particularly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), reflected not only the state of cell membranes but also variations in the quality of food sources. High levels of PUFA were associated with better ecological conditions, while reduced PUFA availability was clearly linked to eutrophication. In addition, higher lipid reserve content was clearly identified in systems impacted by fish farming, reflecting a potentially lipid-rich diet. Neutral lipid fatty acids were particularly useful for studying trophic relationships in aquatic ecosystems. Thus, the integration of polar and neutral fatty acids analyses, offered a more comprehensive assessment of biochemical and physiological interactions in the ecosystem. The originality of this research lies in the use of fish lipid markers, which have highlighted the importance of fatty acid profiles as bioindicators to evaluate the health status of estuarine systems and in particular their eutrophication levels, considering an estuarine sentinel species widely distributed over Europe.

Keywords: Estuary, Fatty acids, Environmental stressors, *Platichthys flesus*

1. Introduction

Lipids, including fatty acids (FAs) are key molecules transferred across aquatic food webs to the fish (Galloway & Budge, 2020), to which they are essential for life cycle and physiology (Parrish, 2013). Fatty acids, the main components of lipids, can be part of reserve (e.g. neutral) lipids or membrane (e.g. polar) lipids. Reserve lipids are used as fuel in all metabolic systems and play therefore an important role in fish growth, reproduction and migration. Membrane lipids are the main components of cell membranes and play a major role in biochemical and physiological responses (Filimonova et al. 2016).

Living organisms are mainly capable of synthesizing saturated fatty acids (SFA), such as palmitic acid (16:0) and stearic acid (18:0). SFA are the basis for the biosynthesis of monounsaturated fatty acids (MUFA) such as 16:1n-7, 18:1n-7, 20:1n-7, 22:1n-7 or 24:1n-9 and some polyunsaturated fatty acids (PUFA). In aquatic food webs, PUFA are mainly produced by phytoplankton, transferred to higher trophic levels and accumulated in consumers such as fish (Saito & Aono, 2014; Gonçalves et al. 2012). In fish, some HUFA (highly unsaturated fatty acids, having more than 3 double bonds, such as eicosapentaenoic acid (20:5n-3, EPA), docosahexaenoic acid (22:6n-3, DHA) and arachidonic acid (20:4n-6, AA), are considered as essential (Saito & Aono, 2014). They have a key role in the health and function of organisms. For example, EPA is an excellent source of energy and precursor of eicosanoids (Calder, 2020), DHA is involved in the support of membrane structures and functions (Sherratt et al. 2021), and AA is implicated in the growth and survival of larval stages (Bessonart et al. 1999). In addition, organisms that feed on HUFA exhibit higher growth rates (Neves et al. 2015). However, these cannot be synthesized *de novo*, or not in sufficient quantities by fish.

PUFA fish composition is therefore largely determined by the quality of their diet (Arts and Kohler, 2009). Algae and diatoms, rich in DHA, EPA and AA, are considered a high-quality food source for consumers, improving the efficiency of energy transfer to higher trophic consumers (Lau et al. 2012; Müller- Navarre et al. 2004). Conversely, terrestrial organic matter and cyanobacteria generally lack PUFA and are considered as poor quality food source (Müller-Navarra et al. 2004). Fish FA composition is thus considered as good bioindicators of fish physiology and nutrition (De Carvalho & Caramujo, 2018).

Other studies have shown that certain pollutants can operate as lipid disruptors, impacting normal lipid metabolic processes in organisms (Bennett et al. 2021; Bernier-Graveline et al. 2021). FA profiles can be altered by exposure to organohalogen contaminants in marine mammals (Xie et al. 2023; Zhang et al. 2022), polychlorinated biphenyls (PCBs) in freshwater species (Huang et al. 2022) and pesticides in fish (Gonçalves et al. 2021; Zang et al. 2019; Filimonova et al. 2016). Thus, a positive correlation has been observed between PCBs and DDT concentrations and increased EPA and DHA levels, as well as between DDT and HCH concentrations and SFAs and MUFAs, in freshwater fishes muscles (Zhang et al. 2019). Similarly, exposure to metals (Cd, Hg, Ni) induces a change in FA profiles in sea snail, and in particular in AA, EPA and DHA contents (Silva et al. 2017). FAs therefore appear to be relevant bioindicators not only in fish physiology and nutrition but also in fish ecotoxicology (Liu et al. 2023; Bernier-Graveline et al. 2021; Gonçalves et al. 2021). Thus, lipids and FA are widely considered as good bioindicators of aquatic ecosystem health (Maazouzi et al. 2008, Ramírez et al. 2013) and stress (Sánchez-Muros et al. 2013, Gonçalves et al. 2016).

Estuaries, highly valued for their productivity and role as critical nurseries for marine species (Beck et al. 2001), are facing severe degradations due to various human activities. These coastal ecosystems are under significant threat from factors such as pollution, overexploitation, habitat fragmentation, and the expansion of industries and urban areas (Elliott, 2011). Moreover, estuaries worldwide are vulnerable to multiple anthropogenic pressures, including chemical pollution, dredging, eutrophication, and urban expansion, which compromise water quality and increase the risk of hypoxia (Bárcena et al. 2017; Elliott et al. 2014). Additionally, global climate change has exposed these shallow estuarine ecosystems to heat stress, subjecting them to a growing number of environmental stressors (Alfonso et al. 2021; Cabral et al. 2019).

Consequently, it is now necessary to precisely determine the health status of estuaries defined in terms of organization, resilience, vigour, and the absence of signs of distress and the presence of essential functions that support life systems (Haskell et al. 1992). Thus, a healthy ecosystem is defined as "stable and sustainable", retaining its organization and autonomy over time and its resilience to stress (Costanza, 1992).

Assessing the health status of estuaries is a challenge due to their inherent diversity and the various pressures these ecosystems face, often influenced by the size of the estuary. Small *vs* large estuaries are generally subjected to fewer human-induced changes, which can adversely affect estuarine habitats. Consequently, in small estuaries, the main driver of change is usually degradation of water quality linked to human activities over the watersheds. The degree of human impact on small estuaries is thus mainly assessed by studying the effects of eutrophication and pollution on water, sediments and biota (Laurent et al. 2023). The fish health mirrors the ecosystem health, and lipids are biomarkers that provide information about physiological state and level of contamination of organisms (Parrish et al. 2013). Thus, we hypothesize that fatty acids could be relevant indicators of estuarine health status.

The objectives of the present study were to explore the relevance of lipids and fatty acids as proxies for the ecological status of estuaries by determining fatty acid profiles in flounder muscle in a range of different estuaries. Furthermore, the originality of this study lies in the coupling of fatty acid analyses with geographical metrics on the watershed and chemical analyses in sediments and fish, in order to identify the typology of human activities in the different hydrosystems. This survey focused on seven hydrosystems located in Brittany, chosen for their small size (between 70 and 450 km²) and their contrasting levels of eutrophication induced by human activities and land use. The biological model used in this survey is the European flounder (*Platichthys flesus*), a key sentinel species (Laurent et al. 2022; Borcier et al. 2020). The choice of this species is attributed to its estuarine life cycle (Dando, 2011) and its predominantly benthic lifestyle, exposing flounders to pollutants in sediment (Chiffoleau, 2017; Williams et al. 2014), as well as substances related to runoff over river basins and urban discharges (Defo et al. 2021; Tetreault et al. 2021).

2. Materials and methods

2.1. Study sites, *in situ* fish sampling and tissues collection

Seven watersheds located along the French Atlantic coast were considered in this study: Gouessant, Guillec, Flèche, Quillimadec, Aber Wrac'h, Douffine and Aven (Fig. 1). These hydrosystems showed contrasted environments and stressors. The Gouessant, Guillec, Quillimadec and Flèche catchments show a high agricultural pressure and are considered as eutrophicated systems. The Aber Wrac'h watershed displays a moderate agricultural pressure and a reduced risk of eutrophication. The Douffine system is mainly impacted by a high fish farming activity in the lower part of its watershed. Finally, the Aven catchment is weakly affected by eutrophication and could be considered as a "reference system" (Laurent et al. 2023).

At the end of September 2020, juvenile flounders were sampled by electric fishing in the upstream part of the estuaries. A set of 20 fish (total length 9.16 ± 1.15 cm) per estuary was collected, excepted for the Gouessant estuary where only seven individuals were caught, fish being very rare. Immediately after fishing, fish were sacrificed in the field and dissected to recover the white muscle. Tissues were promptly flash-frozen in liquid nitrogen.

2.2. Geographical metrics and chemical analysis

Several geographical indicators have been developed from a set of geographical reference data as in Laurent et al. (2023), to characterize the studied systems and to identify potential contamination sources (Table S1). Briefly, three metrics were linked to agricultural pressures. Firstly, livestock per watershed was assessed using the total feed index of the livestock unit (Agreste database - Ministry of Agriculture), which facilitates comparison of species based on feed consumption. Secondly, agricultural surfaces were determined on the basis of agricultural land cover in watersheds (Theia Land database). Thirdly, phytosanitary treatments provided by Agreste (Crisan, 2020; Pujol, 2015) allowed calculating the number and quantity of phytosanitary products applied per catchment (Treatment Frequency Index, IFT - European Union). The IFT represents the number of doses applied per hectare during a crop year, which corresponds to the recommended treatment quantities. Urban pressure was gauged using two indicators: population density from INSEE Census data and the proportion of artificial surfaces per watershed (Theia Land database). Lastly, three indicators evaluate watershed ecological health: the percentage of natural surfaces, encompassing forests (Theia Land database), the hedge density (BDTopo database - OFB/IGN), and the percentage of riparian vegetation over a 100 m river band (CBNB map of major vegetation types in Brittany).

Chemical analyses included measurements of nitrates and nitrites in water, organic pollutants in sediment, and trace elements in sediment and fish (Table S1). Briefly, nitrate and nitrite concentrations were assessed in filtered water samples (0.2 µm) of approximately 15 mL using a Bran + Luebbe AAIII autoanalyser, according to the method of Aminot & Kérouel (2007). Organic pollutants (24 PAHs and 26 PCBs) were quantified in samples 100 mg (w.w.) of sediment and flounder tissue, by sorptive stir bar extraction-thermal desorption-gas chromatography-tandem mass spectrometry (SBSE-GC-MS/MS) according to the method adapted from Lacroix et al. (2014). Trace elements were analysed using an ICP-quadrupole mass spectrometer (X-series II, Thermo Scientific) operated by the Pôle Spectrométrie Océan Brest (PSO, Brest, France), using sediments that had been dried (at 60°C), ground, mineralised (65 % nitric acid and 30 % hydrogen peroxide) and hydrolysed (105°C, EasyDigest® ANALAB).

2.3. Analysis of flounder lipid composition

2.3.1. Lipid extraction

Lipid extractions were performed on white muscle following a method described by Folch et al. (1957) and modified by Mathieu-Resuge et al. (2019). A representative portion of fresh fish white muscle (few g of wet weight) were ground into a fine and homogeneous powder by ball milling (MM400, RETSCH, Germany) during 30 sec at 30 Hz under liquid nitrogen. Then, approximatively 150 mg of fresh muscle tissue powder (162.36 ± 8.79 mg) were put in glass tubes with 6 mL of chloroform/methanol (2:1; v/v; HPLC grade). To prevent lipid oxidation, vials were immediately flushed under N_2. To improve lipid extraction, samples were then sonicated 10 min and maintained under agitation during 20 min at 6.7 Hz. Lipid extracts were stored at −20°C in the darkness until further analysis.

2.3.2. Lipid class analysis

Lipid class analyses were performed on flounder white muscle lipid extracts by HPTLC (High-performance Thin Layer Chromatography). This method, adapted from Pédron et al. (2017), used a HPLTC system (CAMAG) composed of an Automatic TLC Sampler 4, a TLC Densitometer Scanner 3 and the VisionCats software (v 2.5)(. Neutral and polar lipid classes were analyzed independently on HPTLC glass plates (10 x 20 cm) pre-coated with silica gel (Merck). After a pre-run and an activation step, lipid extracts were spotted on the plates. Neutral lipids were separated with hexane:diethyl ether:acetic acid (20:5:0.5, v/v) followed by hexane:diethyl ether (97:3, v/v). Polar lipids were separated with methyl-acetate:iso-propanol:chloroform:methanol:KCl 0.25% (10:10:10:4:3.6, v/v). After revelation in cupric sulfate phosphoric-acid and heating for 20 min at 180°C, lipid classes appeared as black spots and plates were scanned at 370 nm. Commercial standards, spotted at different concentrations on the same plates, allowed to identify and quantify (calibration curves) six neutral lipid classes (sterol esters, glyceride ethers, triacylglycerols, free fatty acids, fatty alcohols, free sterols) and seven polar lipid classes (sphingomyelin, lysophosphatidylcholine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, cardiolipin, phosphatidylethanolamine). Storage lipids are composed of neutral lipids, except free sterols, whereas membrane lipids include polar lipids and free sterols. The lipid storage index based on the ratio of the quantity of triacylglycerols (proxy of reserve lipids) over the quantity of free sterols (proxy of structural lipids) was also calculated as this ratio is commonly consider as a relevant proxy of fish fitness (Kerambrun et al. 2013).

2.4. Neutral and polar lipid fatty acids analysis

Neutral and polar lipids fractions were separated using the procedure described by Le Grand et al. (2014). Briefly, 1 mL of muscle lipid extract was evaporated to dryness. Lipids were recovered with three washings of 0.5 mL of chloroform/methanol (98:2; v:v) and deposited at the top of a silica gel micro-column (40 mm × 4 mm, silica gel 60A 63–200 µm rehydrated with 6 % H_2O (70–230 mesh)). Neutral lipids were eluted with 10 mL of chloroform/methanol (98:2; v:v) while the polar lipids were recovered with 20 mL of methanol. In each fraction, internal standard was added (2.3 µg of tricosanoic acid (C23:0)). After evaporation to dryness, neutral and polar lipids fractions were transesterified for 10 min at 100°C after the addition of 0.8 mL of methanol/H_2SO_4 (3.4 %; v:v) according to Le Grand et al. (2014). The transesterification allowed the formation of fatty acids methyl esters (FAME) and dimethylacetals (DMA) that were recovered with hexane and washed with hexane-saturated waterFinally, FAME and DMA were analysed with a Varian CP8400 gas chromatograph (GC) equipped with two splitless injectors programmed at 220°C, and two flame-ionization detectors programmed at 280°C, with hydrogen as vector gas. Temperature programme was as followed, from 0°C to 150°C at 50°C min^{-1}, then to 170°C at 3.5°C min^{-1}, to 185°C at 1.5°C min^{-1}, to 225°C at 2.4°C min^{-1} and finally to 250°C at 5.5°C min^{-1} and maintained for 15 min. FAME and DMA were separated on two columns with different polarities, one polar (ZBWAX: 30 m × 0.25 mm ID × 0.2 µm, Phenomenex) and another one apolar (ZB5HT: 30 m × 0.25 mm ID × 0.2 µm, Phenomenex), to avoid co-elution issues. FAME and DMA were identified by comparing their retention time with commercial (37-Component FAME mix, Supelco ; PUFA N◦1 and N◦3, and Bacterial Acid Methyl Ester Mix, Sigma) and lab-made references and quantified relatively to the internal standard. The use of an unique internal standard to quantify FA by GC-FID is a widespread method (Couturier et al 2020). The accuracy of the method was checked with the 37-Component FAME mix (Supelco). The relative proportions of FA and DMA were expressed as mass percentages over the total FA+DMA content. The sum of polar and neutral lipid FA quantities allowed obtaining total lipid FA quantities.

2.5. Statistical analysis

Only FAs accounting for > 0.5 % in at least one station and/or period were considered for the statistical analysis. Statistical analyses were performed with R software (v.3.5.0) implemented in Rstudio (v. 1.1.453). Normality and homoscedasticity of variances were investigated with a Shapiro-Wilk test and a Bartlett test, respectively. Because the data were not normally distributed, a nonparametric Kruskal-Wallis test followed by a Dunn's post hoc test (for multiple comparisons) were applied to compare the means. Data integration was conducted by principal component analyses (PCA) using the FactorMineR package with default settings. Graphical analyses were performed with "ggplot" package of R. A p-value lower than 0.05 was considered as a significant difference.

3. Results

3.1. Geographical, hydrobiological and chemical data: a typology of the watersheds

We performed a PCA combining geographical catchment metrics with hydrobiology and chemical data (Table S1 & Fig. 2).

Axis 1 (horizontal) of the figure revealed two distinct groups of watersheds. On the left, we observed a first group of hydrosystems characterized by high agricultural pressure, while on the right, the Douffine and Aven catchments showed a moderate agricultural pressure. Furthermore, within the first group, the Gouessant, Flèche and Guillec basins were mainly characterized by high livestock production and phytosanitary treatments, water nitrogen enrichment and metallic contamination of fish muscle; the Quillimadec and Aber Wrac'h basins in the upper left part of the diagram showing higher human population density and urbanization.

In the second group on the right, the Douffine system was characterized by high fish farming activity, metallic contamination of the sediment and large proportion of well-preserved natural environments. The Aven watershed, isolated in the upper right part of the graph, stood out due to high density of hedges and contamination of the sediment by PAHs and fish muscle by Arsenic. Overall, the PCA (Fig. 2) highlighted a moderate eutrophication in the Aven basin compared to other six watersheds.

3.2. Fish lipid classes

The highest levels of total lipids were observed in flounders from Guillec and Douffine (≈ 14 $\mu g.mg^{-1}$), while the lowest amounts being measured in Flèche (11 $\mu g.mg^{-1}$) (Table 1). Overall, the storage lipids were also higher in Douffine fish (3.85 $\mu g.mg^{-1}$) compared to other hydrosystems; fish from Aven and Aber Wrac'h showing the lowest values (≈ 1 $\mu g.mg^{-1}$). The range of variation was lower in membrane *vs* storage lipids, from 9.66 $\mu g.mg^{-1}$ in Flèche to 12.08 $\mu g.mg^{-1}$ in Guillec. The ratio of reserve lipids to membrane lipids (TG:FS) showed a moderate fish lipid reserve for Aven and Aber Wrac'h (1.18 - 1.23), unlike flounders sampled in Douffine showing the highest value (5.6). Finally, the fish condition factors were rather similar (≈ 1) over the seven watersheds (Table 1).

3.3. Fish fatty acid profiles

3.3.1. Total lipid fatty acids

PCA on total lipid fatty acids (TL FA) in flounder muscle (Table S2) revealed significant differences in FAs composition between estuaries (Fig. 3), the first and second principal components accounting for 45.1% of the total variance of the data set.

PCA showed that Douffine flounders were mainly discriminated by the presence of 18:1n-9 (OA oleic acid), 18:2n-6 (LA linoleic acid) and 18:3n-3 (ALA alpha-linoleic acid), in opposition to Aven individuals characterized by 20:1n-7, 22:4n-6 (DTA) and 22:5n-3. PCA also revealed that fish from Aber Wrac'h were discriminated by

16:1n-9, 20:2n-6 and 22:6n-3 (DHA), while fish from the Gouessant were characterized by 20:2n-6, branched FAs, 20:4n-6 (AA). PCA also revealed that individuals caught in Quillimadec were rather close to those of Aber Wrac'h. Finally, PCA showed that flounders from Flèche showed high levels of 20:5n-3 (EPA), 18:1n-7 and 16:1n-7; a convergent but less marked trend being observed for the Guillec fish.

3.3.2. Neutral lipid fatty acids profiles

We examined neutral lipid fatty acid profiles (NL FA) in flounder muscle (Table S3). According to the PCA results (Fig. 4), the first and second principal components explain 42.4 % of the total variance of the data set. A group of hydrosystems was located in the left part of the NL FA PCA diagram, including Douffine, Flèche and Guillec. It was discriminated by high levels of OA (18:1n-9), LA (18:2n-6) and ALA (18:3n-3); LA and ALA being positively correlated with level of nitrites, fish farm production, proportion of natural habitats and metallic contamination in the sediment (Fig. S1b). Furthermore, Flèche and Guillec fish showed high levels of MUFA (16:1n-7 and 18:1n-7) that were positively correlated with water nitrate and livestock (Fig. S1b). Moreover, NL PCA showed that fish caught in Aber Wrac'h were enriched with AA (20:4n-6), osbond acid (22:5n-6), DHA (22:6n-3), nervonic acid (24:1n-9) and DMA. Statistical analysis revealed that all these FAs were correlated with As in sediment (Fig. S1b). NL PCA highlighted that individuals from Gouessant located in the lower part of the diagram (Fig. 4) were mainly characterized by Branched FAs negatively correlated with the hedge density (Fig. S1b). Fish from Quillimadec were relatively close to the gravity centre of the NL PCA and showed mainly high levels of 20:1n-7 and 20:1n-11 positively correlated with levels of PAHs in the sediment (Fig. S1b). Finally, fish from Aven were clearly located in the right part of the NL PCA diagram (Fig. 4), and mainly characterized by high levels of DTA (22:4n-6) and DPA (22:5n-3); these two fatty acids being negatively correlated with livestock and positively correlated with PAHs in sediment (Fig. S1b).

3.3.3. Polar lipid fatty acids profiles

The PCA carried out on polar lipid fatty acid profiles (PL FA) of flounder muscle (Table S4) showed contrasted differences of FAs composition between hydrosystems (Fig. 5), the two first principal components explaining 47.4 % of the total variance of the data set.

The PL PCA showed that flounder from Douffine were still discriminated by 18:1n-9, 18:2n-6 and 18:3n-3, additionally accompanied by 18:1n-7 and 20:4n-6 (AA). Statistical analyses and Spearman correlations showed positive correlations between 18:1n-9, 18:2n-6 and 18:3n-3 and fish farm, natural surfaces, riparian vegetation and metals (Fig. S1a). Flèche fish were characterized by palmitoleic acid (16:1n-7), a fatty acid positively correlated with fish farm, natural surfaces, riparian vegetation and metal concentrations (Fig. S1a). High values of vaccenic acid (18:1n-7) and DMA were also detected in Flèche fish and were positively correlated with nitrates and livestock (Fig. S1a). Some Guillec fish showed high values of myristic acid (14:0), stearic acid (18:0) and EPA (20:5n-3). Myristic acid was positively correlated with fish farm, natural surfaces, riparian vegetation and metal concentrations (Fig. S1a). EPA was positively correlated with nitrate concentrations, livestock, agricultural surfaces and hedges density (Fig. S1a). The PL PCA showed that Aber Wrac'h flounders showed high levels of DPA (22:5n-6) and DHA (22:6n-3), positively correlated with As in sediment and urbanized surfaces (Fig. S1a). In addition, the Aven fish were characterized by high proportions of margaric acid (17:0), 18:1n-11, 20:1n-11, and DTA (22:4n-6), positively correlated with PAHs in sediments. FAs 18:1n-11 and 22:4n-6 were also correlated with hedges density (Fig. S1a). No particular trends were detected for the FAs composition of fish from Gouessant and Quillimadec, the individuals being positioned close to the gravity centre of the PL PCA (Fig. 5).

4. Discussion

4.1 Typology of the human activities in the watersheds

In the present study, the environmental metrics (geography, hydrobiology and contaminants) (Fig. 2) highlighted that the seven studied hydrosystems were submitted mainly to agricultural pressure, the classical signatures of industrial activities like PCBs levels in sediment and fish being very weak. Significant PAHs concentrations detected in particular systems were related to their high levels of car traffic and in the Aven to the presence of a busy car park close to the estuary.

The high agricultural pressure in Gouessant, Flèche and Guillec was mainly linked to high production of corn for the first and vegetables (potatoes, carrots, shallots…) for the second and third, over more than 70% of the catchment surface; these systems being also impacted by intensive breeding. Quillimadec and Aber Wrac'h showed a lower agricultural pressure but a higher urbanisation, whereas Aven and Douffine were characterized by highest proportions of preserved areas and riparian vegetation. However, the specificity of the anthropization in the Aven and Douffine was a production of canned vegetables and a high fish farm activity, respectively. Thus, over the whole data set, the Aven watershed could be considered the system least impacted by human activities.

4.2. Total lipid classes in fish muscle

The highest lipid reserves (total lipids, storage lipids) were detected in fish sampled in Douffine. The highest TG:FS ratio, the lipid storage index commonly used in fish ecology (Kerambrun et al. 2013), was also highlighted in this estuary. Globally, fish from Douffine hydrosystem therefore stored more lipids than individuals in the other basins, which could indicate a more positive energetic balance than in the other systems, potentially due to a more abundant or energetic diet (quality and/or quantity) or due to less energy expenditure (response to stress and/or water temperature). The storage of lipids could also be related to an excess of food, thus conducting to an imbalance between energy acquired and energy expended. This trend could be related in the Douffine, to its very high fish farming activity leading to a distribution of lipid-rich feeds and to a heavy eutrophication of the hydrosystem (Pickova & Mørkøre, 2007).

The remaining study sites showed only slight lipid differences between them, which can be explained by variations in the abundance and diversity of prey in the environment. Water pollution can also influence fish body composition, including lipid content, as certain pollutants can disrupt lipid metabolism, leading to lipid accumulation or depletion in fish tissues (Filimonova et al. 2016). The Aber Wrac'h and Aven showed the lowest values for storage lipids and ratio TG:FS that could be a signal of a moderate eutrophication. The fish condition factor did not differentiate the seven flounder populations, thus underlining that no major fitness loss was detected over the watersheds.

4.3. Fatty acids profiles in fish muscle

4.3.1. Total lipid fatty acids (TL)

The PCA (Fig. 3) clearly highlighted on Dim 1 a gradient of the estuarine environment quality (along horizontal axis), from Douffine to Aven. Fish in Douffine *vs* Aven showed higher MUFA and lower PUFA proportions, reflecting poorer muscle lipid quality. On the contrary, the PUFA higher values in Aven could indicate a better quality of food web components (Keva et al. 2020). In addition, fish from Douffine showed a combination of oleic (OA), linoleic (LA) and alpha-linoleic (ALA) acids, which are the dominant C18 FAs in terrestrial seeds and animal oils. These FAs are the most relevant biomarkers for linking aquaculture waste in the environment and its potential effects (White et al. 2019). Douffine is characterized by three fish farms producing 900 T.yr^{-1} of trout. Furthermore, 20:3n-3 slightly more abundant in Douffine was also highly accumulated in fish fed intensively on artificial feeds (Jankowska et al. 2004). On the other hand, LC-PUFA, such as 22:5n-3, abundant in Aven reflect the marine origin of flounder food sources (Závorka et al. 2022). Fish from Aven were also enriched in n-3 and n-6 LC-PUFA, such as DHA (22:6n-3) and DTA (22:4n-6). The level of LC-HUFAs such as DHA is reduced in highly eutrophicated environments (Taipale et al. 2016) and high in oligotrophic systems, confirming the limited eutrophication in the Aven hydrosystem (Laurent et al. 2023). The distribution of FAs appeared rather close in Aber Wrac'h - Quillimadec *vs* Aven, indicating a moderate eutrophication in these two estuaries.

The PCA (Fig. 3) on Dim 2 also revealed a vertical gradient separating Gouessant and Flèche hydrosystems, the Guillec fish group being located in intermediate position. Fish from Gouessant were characterized by a particular AA abundance, this FA and its metabolites playing a major role in proper functioning of the immune system and inflammatory response (Hanna & Hafez, 2018; Denisenko et al. 2015). Gouessant fish also contained high proportions of branched FAs, commonly used to evaluate the bacterial contribution on the marine environment (Prato et al. 2010; Alfaro et al. 2006). Two successive large dams fragment the Gouessant river downstream, which favours eutrophication, increase of water temperature, and probable proliferation of cyanobacteria in the reservoirs (Chinyama et al. 2016); these particular conditions could explain the specificity of the FA signals in this hydrosystem. Individuals from Flèche and Guillec both showed high levels of DMA and EPA (20:5n-3) but reduced levels of DHA that could impact the healthy state of cell membrane (Costa et al. 2015; Saito & Aono, 2014). Furthermore, the high proportion of dimethyl acetals (DMA) detected in Flèche and Guillec could be related to the effect of eutrophication and hypoxia in these systems. Indeed, the plasmalogens from which DMA are derived play a protective role during oxidative and hypoxic stress, and confer stress resistance (Nagan & Zoeller, 2001). Individuals from Flèche and Guillec also showed high levels of vaccenic acid (18:1n-7), used to assess the bacterial contribution in the marine food web (Meziane & Tsuchiya, 2000; Kharlamenko et al. 2001; Alfaro et al. 2006). Thus, a very active livestock farming in Flèche and Guillec basins could lead to a bacterial proliferation in the estuaries.

Interestingly, unlike lipid classes, the total lipid fatty acids (TL) appear to provide substantial insights into the effects of environmental stressors on the lipid profiles of flounder. These results emphasize the influential role of the major environmental variables as key determinants shaping the composition of fatty acid profiles in fish tissues. Nevertheless, we sought to deepen this information by separating fatty acid: neutral lipid fatty acids (NL) *vs* polar lipid fatty acids (PL).

4.3.2. Neutral lipid fatty acids profiles (NL)

Neutral lipid fatty acids (NL) stored in muscle tissue partly reflect the fish diet. NL composition of prey, such as plankton (Dalsgaard et al. 2003; Jezyk & Penicnak, 1966), can influence NL composition in fish. Indeed, when invertebrate primary consumers feed on a high-quality source, PUFA contents thus offer a high-quality source to predators, including fish (Brett et al. 2017; Guo et al. 2018). Conversely, when they consume lower-quality foods, such as leaves or cyanobacteria, PUFA content of primary consumers decreases (Kühmayer et al. 2020; Müller-Navarra et al. 2004), negatively impacting food quality for their predators.

The NL PCA (Fig. 4) on Dim 2 highlighted that fish from Aber Wrac'h were enriched in LC-PUFA and especially in DHA, an essential HUFA characterizing the presence of marine diatoms and dinoflagellates. In terrestrial environments, EPA and DHA levels are very low or non-existent (Hixson et al. 2015), but marine diatoms and dinoflagellates can synthesize de novo EPA and DHA (Strandberg et al. 2015; Guedes et al. 2011). Furthermore, fish from Aber Wrac'h were also characterized by high level of AA. In Laurent et al. (2023), we hypothesized arsenic contamination through the spreading of algal sludge in fields (Greger et al. 2007), resulting from the exploitation of algae in the food industry (Piwowar & Harasym, 2020). Algae naturally concentrate arsenic and a range of metals that are incorporated into enzymes, proteins and vitamins (Wahbeh et al. 1985). In addition, AA and DHA are commonly present in red (Gracilaria sp.) and brown (Sargassum sp.) algae, respectively (Kelly and Scheibling, 2012; Shanab et al. 2018; Kumar et al. 2011, 2010; Van Ginneken et al. 2011). However, industrial extraction of molecules of interest generally involves water extraction (Zakaria et al. 2016; Castro-Puyana et al. 2013); the fraction that remains, being often considered as a waste product, is liable to contain many lipids. Thus, algal sludge commonly spread over the Aber Wrac'h watershed could impact the lipid reserves of the entire trophic chain, including flounder.

Fish from Gouessant and Quillimadec showed a low proportion of DHA. These systems displayed water reservoirs that favour the proliferation of cyanobacteria and/or green algae that cannot produce HUFAs (Strandberg et al. 2015; Guedes et al. 2011).

The Dim 1 of the NL PCA mainly refers to environmental quality, as for the TL. Fish from Douffine were constantly typified by oleic, linoleic and alpha-linoleic acids, characteristic of terrestrial food sources (White et al. 2019). NL reflect the fish diet, so it appears that the feeds distributed to fish farms may have contaminated the entire trophic chain. Indeed, these same FAs were observed in greater proportion in fish living in waters surrounding aquaculture facilities, suggesting consumption of feed used in aquaculture (Johnson et al. 2018), confirming the trends observed with TL and PL. These three FAs therefore appear to be reliable indicators of fish farming activity, whatever the type of fatty acid. In addition, fish from Douffine were deficient in DHA, another characteristic of the impact of fish farms (Fernandez-Jover et al. 2011). Individuals from Douffine also showed high levels of particular FAs, myristic acid (14:0) and margaric acid (17:0), both characteristic of bacterial presence (Prato et al. 2010; Virtue et al. 2000). Intensive aquaculture has expanded in recent years, accompanied by waste discharges that can lead to environmental eutrophication (Amirkolaie, 2011; Talbot & Hole, 1994) and bacterial proliferation. Furthermore, in Le Croizier et al. (2016), margaric acid was correlate with Cd, and Douffine system exhibited high Cd concentration in sediments.

In the case of NL, individuals from Flèche and Guillec showed high levels of oleic, linoleic and alpha-linoleic acids in this fraction. As with Douffine, the Guillec system hosts fish farms (450 $T.an^{-1}$), which may explain these FA concentrations. The Flèche river, on the other hand, only supports a very small fish farm (20 $T.an^{-1}$). The proportion of 18:1n-9, 18:2n-6 and 18:3n-3 here could be related to a diet composed mainly of prey derived from terrestrial sources, such as chironomidae (Mendes et al. 2014), gammarus (Vinagre et al. 2008) or harpacticoids (Aarnio, 2000).

To conclude, NL analysis did not provide more accurate information than TL, and the systems studied were less well segregated. Moreover, if only NL are taken into account, we obtain information essentially on trophics, which leads to a loss of global information. Some observed results could be related to environmental stressors, such as eutrophication and pollution by PAHs or metals, which could all exert selective pressures on preys on which flounder feed, influencing their lipid and fatty acid composition. Fish that feed on these preys will then be impacted by these variations in their diet composition, which may have consequences for their own lipid reserves and NL fatty acid composition (Keva et al. 2019; Rajasilta et al. 2019; Le Croizier et al. 2016).

4.3.3. Polar lipid fatty acids profiles (PL)

The polar lipid fatty acids (PL) present in fish muscles can reveal information about fish physiology, in particular about the FA composition of their membrane lipids. The FAs composition of fish cell membrane is important for the maintenance of cellular functions and properties, and therefore for the health of the organism. For example, the PL composition can influence membrane fluidity and permeability; the organism's ability to cope with temperature changes being strongly linked to the proportion of PUFA. Thus, analysis of PL in fish muscles can shed light on how fish adapt to their environment.

The PL PCA (Fig. 5) showed that Aven fish muscle presented intermediary levels of EPA and DHA, and the highest level of DPA; these three omega-3 polyunsaturated fatty acids are inter-related through a single pathway, and displayed major neuroprotective properties (Dyall, 2015). This trend could be an indicator of the presence of zooplankton or the good ecological condition of this hydrosystem.

Fish from Douffine were invariably characterised by 18:1n-9, 18:2n-6 and 18:3n-3 correlated with fish farm activity. However, the individuals also showed high proportions of AA (20:4n-6). A previous study showed a predominance of AA in several marine fish species, while they were contaminated with various metals such as Cd, Cu and Zn (Ajeeshkumar et al. 2015). Thus, the heavily metallic contamination of the Douffine sediments by ancient mining activities (Chiffoleau, 2017; Lernière & Clozel, 2002) could explain the high level of AA in the flounder, a bottom dwelling-fish. Furthermore, previous studies have already shown that metal exposure acts in an alternative pathway to maintain the level of certain PUFA such as AA (Fadhlaoui & Couture, 2016).

Analysis of PL fatty acids revealed that Aber Wrac'h showed high levels of PUFA, and particularly DHA, which is essential to cell membranes (Costa et al. 2015; Saito & Aono, 2014). These high proportions of DHA could indicate an efficient cell membrane physiology in Aber Wrac'h individuals.

Individuals from Gouessant and Quillimadec were located in the centre of the PL PCA (Fig. 5), thus did not show clear trends in the distribution of their FAs. Fish from Gouessant were particularly close to the gravity centre of the PCA. The fish being very rare in the Gouessant estuary, we suggest that this equilibrium state between the different types of membrane lipids may be the only way the fish have to ensure their survival in this highly stressed system. Fish from Flèche were still characterised by MUFA and DMA. The high proportion of DMA in PL could be related to the impact of a marked oxidative stress on cell membrane lipids (Nagan & Zoeller, 2001).

To conclude, the present study confirmed that particular changes in the PL fatty acids composition could be related to fish response to environmental stressors, such as pollutants and/or oxidative stress. These changes in the composition of lipid membranes could significantly impact fish physiology and fitness (Liu et al. 2023; Bernier-Graveline et al. 2021; Gonçalves et al. 2021; Zang et al. 2019).

5. Conclusion

The analysis of the main lipid classes of the European flounder did not make it possible to effectively discriminate estuaries showing contrasting states of health; the approaches conducted on fatty acids providing more pertinent informations. The analysis of total fatty acids was relevant, particularly for muscle of young fish such as juvenile flounder which showed limited neutral lipids *vs* polar lipids. However, it is important to point out that lipid composition can vary between stages and species.

Polar lipids, although more related to fish physiology and thus more regulated than neutral lipids, were still partly influenced by diet and therefore provided us with information on both physiological and trophic status. Thus, for fatter fish species, or even adult flounder, it could be imperative to opt for polar fatty acid analysis, offering a deeper understanding of lipid composition, which is particularly valuable for exploring complex aspects of fish physiology and ecology.

The separation of neutral and polar fatty acids is time-consuming and costly. It is therefore essential to strike a balance between the need for in-depth information and the constraints of time and cost.

Neutral fatty acids are useful for analysing trophic relationships in aquatic ecosystems. However, the analysis of polar fatty acids could be particularly relevant to explore fish response to stressors in the environment. Thus, in the context of stress ecology in estuarine systems, it could be highly recommended to integrate the analysis of polar fatty acids in addition to neutral fatty acids, in order to fully grasp the biochemical and physiological interactions at play.

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