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ANALYSIS OF PERFLUOROALKYL SUBSTANCES AND GLYCINE BETAINE: CONTRIBUTION TO THE ASSESSMENT OF HEALTH RISKS AND BENEFITS OF SEAFOOD CONSUMPTION

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Appendix

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During her training in CABA-Lab, Dr. Farabegoli took part in several other research projects, which led to these scientific publications:

- Lugoboni B, Barbarossa A, Gazzotti T, Zironi E, Farabegoli F, Pagliuca G (2013)
  "A quick LC-MS/MS method for determination of flunixin in bovine muscle"
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  "Determination of vitamin B12 in dairy products by ultra-performance liquid chromatography-tandem mass spectrometry"
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List of abbreviations

AHA	American Heart Association
ALA	alpha-linoleic acid
ANPC	aqueous-normal phase chromatography
As	arsenic
BAD	betaine aldehyde dehydrogenase
BfR	Bundensinstitut für Risikobewertung (German Federal Institute for Risk Assessment)
BFRs	brominated flame retardants
BHMT	betaine homocysteine methyl transferase
CA	choline acetyltransferase
CABA-Lab	Laboratorio di Chimica Analitica Bio-Agroalimentare – Dipartimento di Scienze Mediche Veterinarie, Università di Bologna
Cd	cadmium
CHD	cardiovascular heart diseases
CK	choline kinase
CO	choline oxidase
CRC	colorectal cancer
CVD	cardiovascular disease
CV%	coefficient of variation (%)
DGAC	Dietary Guidelines Advisory Committee
DHA	docosahexaenoic acid
DMG	dimethylglycine
DNBC	Danish National Birth Cohort
EDCs	endocrine disruptor compounds
EFSA	European Food Safety Authority
EPA	eicosapentaenoic acid
ESI	electrospray ionization
FAB	fast atom bombardment
FAO	Food and Agriculture Organization of the United Nations
GB	glycine betaine
GC	gas chromatography
GC-MS	gas chromatography coupled to mass spectrometry
HCB	hexachlorobenzene

HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HPLC-MS	high performance liquid chromatography coupled with mass spectrometry
IARC	International Agency for Research on Cancer
IEC	ion-exchange chromatography
IOM	Institute of Medicine
IPE	ion pairing extraction
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LA	linoleic acid
LC	liquid chromatography
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LCPUFAs	long chain polyunsaturated fatty acids
LDL	low density lipoprotein
LOD	limit of detection
LOQ	limit of quantification
MAC	Maximum Allowable Concentration
MeHg	methylmercury
MFD	Marine Framework Directive
MRM	multiple reaction monitoring
MS	mass spectrometry
MSFD	Marine Strategy Framework Directive
MS/MS	tandem quadrupole mass spectrometry
MSPD	matrix solid-phase dispersion
MTBE	methyl tert-butyl ether
NDA	Panel of Dietetic Products, Nutrition and Allergies
NDL PCBs	non-dioxin like polychlorinated biphenyls
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
NP	normal-phase
OECD	Organisation for Economic Co-operation and Development
PAHs	polycyclic aromatic hydrocarbons
Pb	lead
PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
PCDDs	polychlorinated dibenzo-p-dioxins
PCDEs	polychlorinated diphenyl ethers
PCDFs	polychlorinated dibenzofurans
PCNs	polychlorinated naphthalenes

PD	plasma desorption
PEEK	polyether ether ketone
PFAS	perfluoroalkylated substances
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonate
PFTE	polytetrafluoroethylene
PLE	pressurized liquid extraction
POPs	persistent organic pollutants
PPARα	peroxisome proliferator-activated receptors α
PTMI	provisional tolerable monthly intake
PTWI	provisional tolerable weekly intake
PUFA	polyunsaturated fatty acids
QuEChERS	quick, easy, cheap, rugged and safe
RDI	reference daily intake
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RP	reversed-phase
SACN	Scientific Advisory Committee on Nutrition
SCF	Scientific Committee on Food
SCHER	Scientific Committee on Health and Environmental Risks
SDA	stearidonic acid
SLE	solid-liquid extraction
SNURs	Significant New Use Rules
SPE	solid phase extraction
SPME	solid phase microextraction
SWOT	strengths, weaknesses, opportunities, and threats
TBA	tetrabutylammonium hydrogen sulphate
TCDCA	taurochenodeoxycholic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDI	tolerable daily intake
TEF	toxic equivalency factor
TEQ	toxic equivalent
THF	tetrahydrofolate
TTP	time to pregnancy
UHPLC	ultra high performance liquid chromatography
US EPA	Environmental Protection Agency of United States
US FDA	Food and Drug Administration of United states
UVB	ultraviolet B waves
VLDL	very low density lipoprotein
WHO	World Health Organization

1. General Introduction

It is widely acknowledged that dietary habits, as well as a good lifestyle, is related to health. A balanced diet, avoiding the excess or the deficiency of nutrients, is essential to prevent lifestyle-related diseases. Nutrition influences lipid composition, oxidative stress and cellular antioxidant status; modulation of the nutritional intake can provoke strong effects on biological processes; for this reason, nutrition could be considered an important instrument able to improve preventive strategies against pathologies associated with environmental toxic insults (Hennig *et al.*, 2007).

Seafood is known for its beneficial properties and is considered a healthier alternative to almost any kind of meat (FAO, 2014).

The term "seafood" is defined as "vertebrate and invertebrate aquatic animals whether of marine or freshwater origin, whether farmed or wild-caught", as used by the European Food safety Agency (EFSA) in its recent opinion (EFSA, 2014). In this work, the term "fish" is considered as a synonym of "seafood" and it includes both finfish and shellfish (crustaceans and molluscs).

Seafood, from a nutritional point of view, provides the intake of protein with high biological value together with other essential nutrients, such as vitamins A and D, selenium, calcium, and iodine (EFSA, 2014). In addition, unlike meat and dairy products, seafood is not high in saturated fat; fatty fish are especially rich in n-3 long chain polyunsaturated fatty acids (LCPUFA). Among LCPUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the most representative and are commonly related to the health benefits on the neurodevelopment and on the prevention of cardiovascular diseases (FAO/WHO, 2011; Domingo, 2014).

In poor Countries fish could be considered an irreplaceable animal source of essential nutrients of high bioavailability, important for people who suffer from malnutrition

and micronutrient deficiencies (FAO, 2014). These statements are strongly supported by several scientific researches carried out in the last 60 years, which are even more oriented to the identification of single beneficial nutrients of seafood and to the quantification of their health action on human body. Some recent investigations have ascribed some cardioprotective and neuroprotective properties also to other non n-3 LCPUFA, and there is an emerging evidence stating that the nutritional impact of fish in is higher than the sum of the benefits derived from the individual intake of nutrients. In fact, the concept of multiple nutritional benefit derived from fish consumption has become increasingly globally accepted (FAO/WHO, 2011).

At the same time, scientific studies started to investigate the presence of environmental pollutants, demonstrating the unavoidable presence of environmental contaminants in fish and shellfish. Mercury and persistent organic pollutants (POPs) were the most investigated contaminants, alerting for their toxicity on human body and their persistence in the environment (FAO/WHO, 2011).

The risk of contamination by environmental pollutants is traditionally associated with the direct contact with a specific contaminant throughout a subject's life span; what should be considered during the evaluation of this risk is the role of the diet: an healthy nutrition, as lifestyle choice, can reduce health risks associated with pollutants (Hennig *et al.*, 2012). Diet contributes, together with other lifestyle choices and physio-pathological conditions, to influence the health status of a subject. Nutrition can also modulate the exposure extent to environmental pollutants, exacerbating or attenuating many disease indicators, such as inflammation and oxidative stress; this means that individuals who are, for example, compromised nutritionally due to poor dietary habits, can be more vulnerable to contaminants throughout their life span. On the other hand, diets rich in antioxidants and beneficial nutrients can improve health and decrease vulnerability to chemical stressors (Hennig *et al.*, 2012).

In Figure 1 (in the next page) is explained graphically how nutrition can modulate the paradigm of risk assessment in the field of environmental pollutants

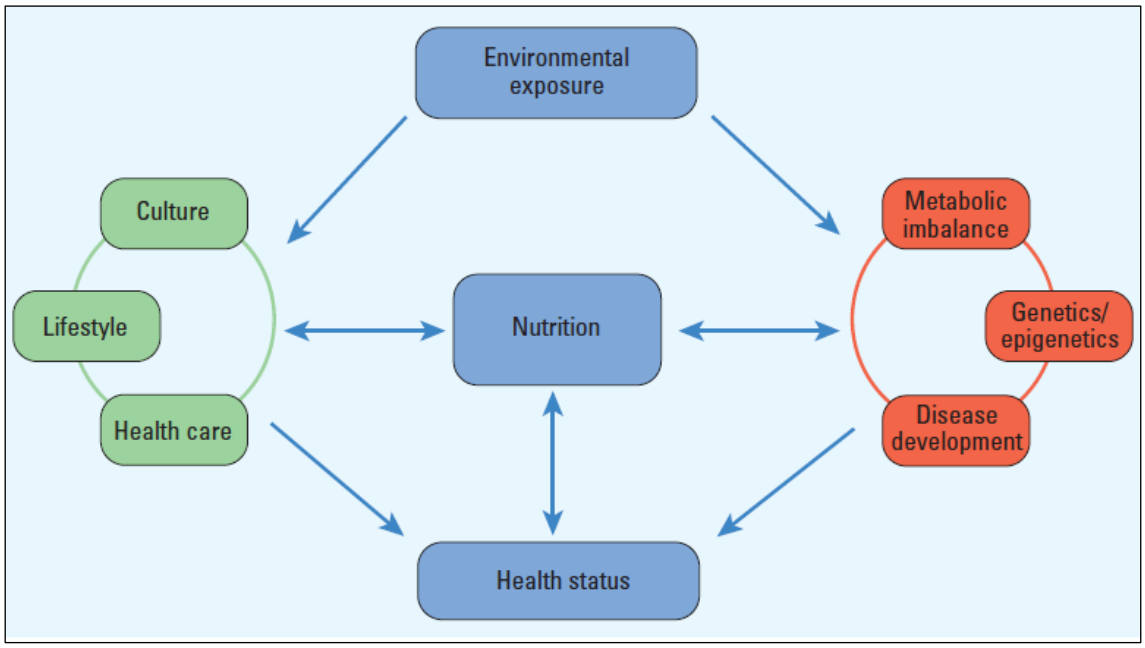


Figure 1: Nutrition role in the modulation of health status related to exposure to environmental contaminants (Hennig et al., 2012)

The debate concerning the benefits and risks of consuming fish resulted in doubts and confusion in how much, or even if, it should be consumed, especially for sensible groups of population (pregnant or nursing women, infants and young children). There is the need to provide useful and clear information to conduce consumers making the healthiest choices for their diet (FAO/WHO, 2011).

Owing to the increased interest and awareness regarding the importance of risk-benefit analysis of foods, the experts of the European Food Safety Authority (EFSA), in 2006, proposed a guideline document describing the risk-benefit analysis procedure, which was split in risk-benefit assessment, risk-benefit management, and risk-benefit communication. For what concern the assessment, they proposed to separate the procedure in risk characterization and benefit characterization, and to conclude with a risk-benefit comparison (EFSA, 2006; Hellberg, 2012). A risk-benefit assessment may be realized both for the general population and for subpopulations with higher sensitivity to the component evaluated, and should include a SWOT analysis (identification of strengths, weaknesses, opportunities, threats of the method). The

need to elaborate several health outcomes, for both environmental contaminants and fish nutrients, makes the risk-benefit assessment of seafood consumption a challenging task (EFSA, 2006; Hellberg, 2012).

The growing public concern induced, in 2006, the Codex Committee on Food Additives and Contaminants to request the Joint Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) Expert Consultation a scientific advice on the health benefits of fish consumption as well as the health risks associated with the presence of methylmercury (MeHg) and dioxins in fish. The aim of this report was to provide a framework to assist the Authorities in preparing proper advices for consumers (FAO/WHO, 2011).

Recently the European Commission asked the Panel of Dietetic Products, Nutrition and Allergies (NDA) of the European Food Safety Agency (EFSA) to deliver a Scientific Opinion comparing the health benefits of seafood consumption with the risk of contamination from MeHg (EFSA, 2014).

1.1 Seafood consumption

Fish is the most important source of protein and essential nutrients for more than one billion people, especially in those Countries where viable substitute foods are not available; it represents the 16.7% of the total animal protein in the global population's diet. For many people, eating seafood is not only a dietary habit but it is also part of cultural traditions (FAO, 2014).

In 2014 the Panel on Dietetic Products, Nutrition and Allergies (NDA) of EFSA collected and analysed information about dietary intake of European Countries. Although the complexity in comparing data from different surveys, the panel confirmed that there is a great variability in fish consumption between age groups and seafood species, and the mean intakes of n-3 LCPUFA, vitamin D, iodine and selenium are generally higher in greater fish consumer Countries, such as Spain or Italy (EFSA, 2014). Data published by FAO show that, in Europe, the annual per capita supply of fish is almost 22 kg (FAO, 2014). The EFSA's Opinion reports that Italian and Spanish populations are those with the highest mean consumption of fish, referring to all seafood categories, except for manufactured fish products, for which the highest mean intakes were found in Sweden, France and Germany (EFSA, 2014).

FAO reports that seafood consumption in European Countries is constantly increasing. The choose of a healthy diet, triggered by various food crises in the past 15 years (e.g. BSE, avian influenza, etc.), is promoting this trend. It was predicted that in 2015 "the Italian fish consumption per capita rises to 26 kg/year and is going to increase till 29 kg/year in 2030" (FAO, 2007). This trend goes hand in hand with the rise in selection of convenience products which need less time for their preparation, as consumers are more and more oriented to a fast meal. A healthy life style, together with good socioeconomic status and educational attainment, may also influence the type of fish consumed, if white or fatty fish (Weichselbaum *et al.*, 2013). The selection of seafood species is mainly orienting towards marine ones, shifting away from traditional freshwater fishes, as the firsts are often easier to cook and offer a wider variety of

taste. Within marine products, high value species (such as diadromous, demersal fish, crustaceans, molluscs and cephalopods) are gradually replacing small pelagic fishes (FAO, 2007).

From the previsions made by FAO, in 2030 the trend of consumption will be more or less the same as today; diadromous species and molluscs will mainly support the increasing total sea production until 2030, but not by capture. From what declared by the European Commission, it's likely that high exploitation of marine stocks make it difficult their reinforcement in the short or midterm. FAO strongly sustains that Countries that more invest in aquaculture will make the biggest profit from the incremental production. Norway and United Kingdom are contributing significantly to the growth of fish farming; in the same way, southern European Countries like Greece, Italy and Spain are requested to contribute, with the production of sea bream and sea bass (FAO, 2007).

1.2 Seafood and health

Dietary habits largely influence the health condition of a population. Seafood is generally associated with good nutrition as considered a “complete and unique source of macro and micronutrients required in an healthy diet” (FAO, 2014). Two portions a week of fish are strongly recommended by Authorities, but only few populations (Japanese and Arctic people) achieve this amount in their dietary habits (Lund, 2013).
Nutritionists traditionally focus on energy content, macronutrients and on biological quality of proteins, but nowadays the role of micronutrients is of increasing interest due to the considerable beneficial effects on development and health (FAO, 2014).

1.2.1 Nutritional content of seafood

The proximate composition of seafood can vary widely among fish species and even within the same species, with the exception of protein content; the moisture generally varies inversely to the lipid content. (EFSA, 2005a).
Fish fillet is considered qualitatively better than cuts from terrestrial species, due to the high biological value of the amino acids, but also for the scarce connective tissue in the muscle, which confers high digestibility to fish meat (EFSA, 2005a).
The energy content of seafood mainly varies due to the fat amount between different species. According to the Scientific Advisory Committee on Nutrition (SACN), fish can be classified as oil-rich (fatty), which contains 5–20% of total lipids, or white (lean) which contains only 1–2% (Weichselbaum *et al.*, 2014). Fatty fishes accumulate lipids in muscle tissue, while lean fishes stock the fat in the liver, which could represent a good source of fish oil (EFA, 2005a).
The biological activity of seafood nutrients is generally attributable to its content in n-3 long chain polyunsaturated fatty acid (n-3 LCPUFA), especially for fatty species (salmon, herring and mackerel); it is also well-known that oil-rich species are source of

fat soluble vitamins, A and D. Seafood also provides large amounts of all amino acids, in balanced rates, especially lysine, an essential amino acid necessary for the production of hormones, enzyme and antibodies. Its deficiency is often associated with anaemia, fatigue and hair loss (Usydus and Szlinder-Richert, 2012).

Seafood is also a good source of B vitamins, minerals (as iodine, selenium, zinc, calcium, chopper, iron and phosphorus) and others essential compounds, like choline and taurine (Lund, 2013; EFSA, 2014). It is well known that such nutrients could be obtained also from other dietary sources: for instance, n-3 fatty acids are constituents of many vegetables oil in the form of essential fatty acids, such as alpha-linoleic acid (ALA) and stearidonic acid (SDA) (EFSA, 2010; Lund, 2013). In human body ALA and SDA are converted by a specific metabolic pathway into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), by chain length extension and desaturation; this conversion is poorly efficient (<5%, Wang *et al.*, 2006) because humans have limited amounts of the enzymes able to introduce double bonds in n-6 and n-3 positions, called Δ12- and Δ15-desaturase respectively. ALA is abundant in human diet but a diet integration is necessary during critical periods of life, such as pregnancy and the first two years of life (EFSA, 2010; EFSA, 2014).

It is important to highlight that ALA and linoleic acid (LA, 18:2n-6) compete for the same enzymes in the conversion into their respective LCPUFA; indeed, the synthesis of EPA and DHA was found to be inversely proportioned to the LA dietary intake. Moreover, LA also inhibits the incorporation of n-3 LCPUFA into tissues; this make high LA levels in the diet generally associated with a low EPA and DHA status (EFSA, 2010).

EPA is the precursor of prostaglandins, prostacyclins and leukotrienes, biologically active molecules which help the modulation of many physiological functions, including inflammatory and immunological reactions, blood coagulation, blood pressure and renal function. Moreover, n-3 LCPUFA are essential components of cellular membranes, regulating its structure (fluidity and permeability) and function (activity of membrane-bound enzymes, membrane receptors and signal transduction) (EFSA, 2010). In contrast to what happens for other nutrients, n-3 LCPUFA are obtained mainly from fish consumption (EFSA, 2014). The higher amounts of n-3 LCPUFA

derives from marine species (Schneedorferová *et al.*, 2015), especially pelagic (Sioen *et al.*, 2009).

The highest levels of n-3 LCPUFA were found in mackerel and salmon (Mahaffey, 2004; Smith and Sahyuond, 2005) shark, herring and sardine (Ismail, 2005). Sidhu (2003) found high levels of n-3 LCPUFA also in anchovy, while Smith and Sahyoun (2005) reported that clams and lobsters contain the lowest concentrations. Salmonoids species (Atlantic and Pacific salmon, trout and char) can synthesise a little of their own n-3 LCPUFA from 18 carbon n-3 precursors present in plant oil, but this ability is strongly reduced during the migration to seawater (EFSA, 2005a).

The NDA Panel of EFSA observed that eating seafood provides the recommended amounts of n-3 LCPUFA in most of the European Countries as well as significantly contributes to the intake of the other essential nutrients (Hellberg, 2012; FAO, 2014). Moreover, Weichselbaum *et al.* (2013) sustain that, when n-3 LCPUFA dietary intakes are calculated, the potential contribution from shellfish may also be considered.

The n-3 LCPUFA content of seafood species reported by EFSA in its latest Scientific Opinion are indicated in the table below (Table 1):

n-3 LCPUFA (mg/100g)					
Fish		***Crustaceans***		***Molluscs***	
herring	2500	general mean	370-520	general mean	160-350
tuna	2500	crayfish	60		
Atlantic salmon	1800				
trout	600				
carp	300				
cod	200				
whiting	200				

Table 1: Lipid content in seafood species most commonly consumed in Europe (EFSA, 2014)

Vitamin D is a fat-soluble element with a key role in the control of skeletal metabolism, together with calcium and phosphate, and is essential for a correct body development and bone mass accumulation (Usydus and Szlinder-Richert, 2012). Its endogenous synthesis takes place in the skin, after the absorption of ultraviolet B waves (UVB) radiation by its precursor; therefore its biological activation needs sunlight exposure (Lund, 2013; Slusher *et al.*, 2015). Since the production of endogen vitamin D is directly proportional to UVB radiation, dietary intake is of primary importance in case of scarce sunlight exposure (Slusher *et al.*, 2015). The assumption through diet is particularly important for people living in northern latitudes, where the photoperiod is highly variable along the year, and especially in winter, when UV radiation is insufficient for vitamin D production (Weichselbaum *et al.*, 2013; Lund, 2013). The deficiency has been related to rickets in children (Usydus and Szlinder-Richert, 2012), some chronic diseases in adults, such as obesity and type II diabetes mellitus (Slusher *et al.*, 2015), and increased susceptibility to bone fractures (osteoporosis) in elderly people (Usydus and Szlinder-Richert, 2012). Moreover, vitamin D deficiency has been recently considered a contributing factor to increased risk of cardiovascular disease (Slusher *et al.*, 2015) and some cancers (colon, breast, prostate or esophagus), as well as some auto-immunological diseases (Usydus and Szlinder-Richert, 2012).

The exogenous intake mainly occurs through seafood consumption, especially fatty fish, which could be of great support in case of limited exposure to UVB radiation (Weichselbaum *et al.*, 2013; FAO, 2014; EFSA, 2014). The recommended intake for adults is 10 μg/day, but this value is still under evaluation (Lund, 2013). Considering a diet composed only by seafood, the adult recommended daily intake would be met by consuming between 20 and 40 g of salmon per day (Usydus and Szlinder-Richert, 2012).

The vitamin D levels in seafood species, published in the latest report of EFSA, are reported in the table in the next page (Table 2).

Vitamin D (µg/100g)			
Fish		***Crustaceans***	***Molluscs***
trout	10-18	general mean 0.5	clams and mussels 5
anchovies	10-18		
herring	10-18		
carp	0.5-2		
hake	0.5-2		
mackerel	0.5-2		
plaice	0.5-2		

Table 2: Vitamin D content in fish species most commonly consumed in Europe (EFSA, 2014)

Fish also contains a good deal of B-complex vitamins, such as vitamin B_3 (niacin). All of them play an important role in contributing to energy-yielding metabolism and in preserving physiologic functions, such as normal performances of the nervous system. Vitamin B_{12} and B_6 act as cofactors for enzymes in the production of red blood cell and are involved in the regulation of a normal homocysteinemia. Their deficiency is rare because are provided by several foods of animal origin, such as meat, dairy products and fish. Niacin can be also endogenously synthetized (Weichselbaum *et al.*, 2013).

Taurine is another important nutrient provided by seafood. Generally included among amino acids, it does not present any carboxylic group, but it is a sulfonic acid derived from cysteine. Taurine, like n-3 LCPUFA and vitamin D, is a conditionally essential nutrient for humans: its synthesis does not compensate the physiological needs and it must be integrated with diet, especially during lactation, to ensure a correct child's neurodevelopment. The fish species with higher content of taurine are mussels, scallops and crabs (Lund, 2013).

Glycine betaine is a small zwitterionic compound produced by a wide variety of organism (bacteria, plants, invertebrates and mammals) to play the role of osmoprotectant (de Zwart *et al.*, 2003; Lenky *et al.*, 2012). In mammals it preserves the

cell volume regulation, especially in the inner medulla of the kidney. It also enhance protein stability, maintaining the tertiary structure of macromolecules, a key role in renal medulla to contrast the denaturing effect of the urea on tubular cells. Glycine betaine is also a methyl donor, supplying methyl groups for homocysteine catabolism to methionine in the liver. It thus stabilises the normal blood concentrations of homocysteine which is a positive marker of heart and vascular health (de Zwart *et al.*, 2003; Lever and Slow, 2010; EFSA, 2011a). Our supply of glycine betaine is almost entirely from the diet, either from food or from the biosynthesis through the catabolism of choline in the liver (de Zwart *et al.*, 2003; Lever and Slow, 2010). The richest animal source of glycine betaine are shellfish (de Zwart *et al.*, 2003).

The nutritional value of fish may also vary depending on whether bones of small fishes are eaten or not. This could influence the macro- and microelements content of seafood, particularly for small sized fish usually consumed whole, which provide highly bioavailable calcium, iron and zinc in significant amounts (FAO, 2014).

The most important mineral constituents of seafood are: calcium, phosphorus, iodine, fluorine, selenium and potassium. Calcium is involved in the processes of skeletal and tooth construction and, in combination with phosphorus, it increases bone resistance. In addition it has a crucial role in blood coagulation and in the conduction of nervous system pulses. Phosphorus, together with fluorine, is involved in the improvement of gums and teeth conditions; it takes part in the process of cell regeneration, in body growth and in the regulation of blood pH. Fluorine, together with calcium, promotes the normal function of skeletal system (Usydus and Szlinder-Richert, 2012).

It has been demonstrated that fish is an important source of micronutrients, not commonly available from other foodstuff. For instance, marine species are almost the only natural source of iodine, the whom activity is proved in the prevention of thyroid and cardiovascular diseases and is probable against prostate cancer (EFSA, 2014). Iodine is a primary element for the thyroid hormones synthesis, which regulates the basal metabolic rates and controls the structural and mental development (Weichselbaum *et al.*, 2013). The optimal daily intake for adults ranges from 150 to 250 µg (Zimmermann, 2009) and is influenced by the consumption of demersal fish

species (Sioen *et al.*, 2009). A deficiency in iodine intakes, particularly frequent in pregnant women, has a negative impact on the somatic growth and on the neurodevelopment of the children, and results in increased infant mortality and reduced cognitive and motor functions (Weichselbaum *et al.*, 2013; Zimmermann, 2009). Low levels of iodine in adult people cause lethargy and bulge of thyroid gland (goiter) (Weichselbaum *et al.*, 2013).

Selenium is a micronutrient which owes its importance to the strong anti-oxidant activity, regulating the activity of glutathione peroxidase. It plays an essential role in the production of thyroid hormone and in the physiological function of immune and reproductive system (Weichselbaum *et al.*, 2013); it also fosters the normal function of enzymatic activity (Usydus and Szlinder-Richert, 2012). Together with the protection from oxidative stress, it is also potentially able to contrast the accumulation of Hg both in fish and in humans (Mozaffarian and Rimm, 2006; Hellberg, 2012; Lund, 2013). Seafood represent a good source of selenium, in different chemical forms; the must bioavailable is the organic one (selenomethionine, selenocysteine). A novel organic selenium compound, selenoneine, has been recently identified in tuna (Lund, 2013). Considering seafood as the only source of selenium, the adult daily requirement would be met by 100 g of tuna, anchovy, sardines, Atlantic mackerel, and golden bass daily consumption (Usydus and Szlinder-Richert, 2012).

Additional investigations are requested to verify the interaction between mercury and selenium; this could have relevant public health implications (Mozaffarian and Rimm, 2006).

Potassium is another essential element, ascribed to the micronutrients. It acts as electrolyte balancer, regulating the cellular water content and their physiological operating. Some Authors supposed that increasing potassium intake may partially attenuate the effect of sodium on the cardiovascular system and possibly protect from cardiovascular diseases (Weichselbaum *et al.*, 2013).

Data on mineral composition of fish species from EFSA report are reported in the table in the next page (Table 3).

Calcium (mg/100g)					
Fish		***Crustaceans***		***Molluscs***	
anchovy	100-135	general mean	30-100	general mean	30-100
herring	100-135				
Atlantic salmon	15-20				
cod	15-20				
tuna	15-20				
Iodine (µg/100g)					
Fish		***Crustaceans***		***Molluscs***	
cod	160	lobster	360	clams	120-140
hake	110	crayfish	65	mussels	120-140
mackerel	110			cuttlefish	20
carp	2-12			squid	20
trout	2-12			octopus	20
Selenium (µg/100g)					
Fish		***Crustaceans***		***Molluscs***	
tuna	75	general mean	20-75	general mean	50-65
trout	21				
Zinc (µg/100g)					
Fish		***Crustaceans***		***Molluscs***	
anchovy	2.2	crab	6.5	general mean	1.4-1.5
herring	1.1	general mean	1.4-1.5		
general mean	0.3-0.7				

Table 3: Data regarding mineral compositions of species most commonly consumed in Europe (EFSA, 2014)

There is a huge variability in nutrient content among species from aquatic environment, belonging to the origin (sea or freshwater), the life stage and the season. Except for protein profile, these parameters deeply influence the nutritional composition, especially in the amount and the type of fats. Sex and reproductive stage strongly determines fat distribution: prior to spawning fat stocks are transferred from tissues and organs to the eggs. Water temperature also conditions the composition of cellular membrane of fish, increasing the percentage of unsaturated fatty acids at low water temperature to maintain membrane fluidity (Weichselbaum *et al.*, 2013; EFSA, 2014). Moreover, a seasonal variability in lipid composition is demonstrated by the measurement of higher content in n-3 LCPUFA in summer than in winter, after the period of starvation and spawning (EFSA, 2005a).

The fatty acid profile also depends on the eating habits of fish, because it is strictly related to the type of lipids present in the diet. Phospholipids and triglycerides content is controlled in farmed fish, but in wild species it is related to the seasonal feed availability (abundant in summer, lower in winter): n-3 LCPUFA are produced by algae and then they spread and accumulate through the food chain (Lund, 2013; EFSA, 2014).

1.2.2 Health benefits of seafood

Seafood was soon appreciated for its low-fat protein composition and then considered by the experts a healthy food choice (Hellberg, 2012). The beneficial effects of fish consumption have been investigated since the 80's, after the observation of the low occurrence of cardiovascular diseases in Greenland Inuit. This population, whose diet is rich in fish, presented an uncommon plasma lipid profile with reduced cholesterol rates. Despite data from those studies suggested a strong genetic influence on the development of this pathology, a positive correlation between high seafood consumption and reduction in important chronic diseases (as diabetes, obesity,

coronary heart disease and some cancers) have been supposed and lately studied (Lund, 2013). This connection was suggested by the findings of some investigations which compared Inuit and Danish diets and measured an evident higher rate of marine n-3 long chain polyunsaturated acids in Inuit diet compared to the Danish one (Lund, 2013). At the beginning of the 21th century, the American Heart Association (AHA) confirmed the scientific evidence of a beneficial activity of plant or marine n-3 LCPUFA on subjects at risk of coronary heart disease, although an ideal intake resulted impossible to calculate. In 2006 the American Institute of Medicine of the National Academies confirmed that seafood, as a low-fat protein source, contributed to support a correct child neurodevelopment (FAO/WHO, 2011).

Despite this, nowadays the mechanism of action of n-3 LCPUFA is not completely understood yet, due to the presence of conflicts in results found in literature (Domingo, 2014).

1.2.2.1 Seafood and cardiovascular diseases

Recent studies ascribe the protective properties of seafood against chronic sub-clinical inflammation to the ability in reducing markers of inflammation and blood pressure (Lund, 2013). Avoiding vascular damages provoked by hypertension and inflammatory response, nutrients contained in fish are thought to protect from the risk of mortality due to coronary heart disease (CHD) (Lund, 2013; EFSA, 2014). Some investigations also reported the reduction of the risk of thrombosis and arrhythmias (Domingo, 2014). On the base of results from scientific publications, the experts of European Food Safety Agency (EFSA) assumed that this outcome is realistically attributable to seafood content in n-3 LCPUFA, especially EPA, and selenium (EFSA, 2014).

In general, the most studied beneficial properties attributed to n-3 LCPUFA on cardiovascular system are: anti-arrhythmic effect, improved myocardial efficiency, reduction in blood triglyceride, regulation of endothelial function and anti-thrombotic action (Weichselbaum *et al.*, 2013). In its last report, EFSA analysed the available literature and found that cardiovascular occurrences investigated were disparate (e.g.

CHD mortality, stroke, arrhythmia and much more) and data were not uniform, being sometimes related to n-3 LCPUFA intake and sometimes to fish consumption in general (EFSA, 2014).

Incongruences between study results could be also due to the lack in harmonisation of study designs (Weichselbaum *et al.*, 2013), to the heterogeneous methods of reporting results, to the variability of EPA and DHA content in different species and to the method of preparation of the servings (Wang *et al.*, 2006). Different groups of populations have different CHD risk, so the variability in results could also be a consequence of the people in exam (in terms of age, type of illness and stage of heart disease at baseline) (Weichselbaum *et al.*, 2013).

Observational studies on healthy population consuming seafood (primary prevention) have been carried out, together with intervention studies on diseased population treated with n-3 LCPUFA supplementation (secondary prevention) (Weichselbaum *et al.*, 2013). Despite data from observational studies resulted contradictory, a general conclusion could be drawn, confirming that seafood intake lowers the risk of mortality for CHD compared to scenery where no seafood is consumed (EFSA, 2014).

These findings were also confirmed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) which concluded that the protective effects of n-3 LCPUFA on CHD mortality were supported by a strong evidence (FAO/WHO, 2011). The American Dietetic Association suggested a daily intake of 500 mg of EPA and DHA (obtained by two servings of fatty fish) for the primary prevention of coronary diseases. These finding are in line with the provisions promulgated by several Authorities worldwide, such as Australian and New Zealand National Health and Medical Research council, Agence Française de Sécurité Sanitaire des Aliments, Dutch Health Council, Superior Health Council of Belgium, International Society for the Study of Fatty Acids an Lipids and United Kingdom Scientific Advisory Committee in Nutrition (Kris-Etherton *et al.*, 2009) and with the results of some renowned publications (Mozaffarian and Rimm, 2006; Harris *et al.*, 2008). In case fish consumed is low in n-3 LCPUFA (that is containing less than 1 g of EPA plus DHA per 85 g of servings) a dietary supplementation is recommended (Harris *et al.*, 2008).

In 2010 EFSA declared that an EPA plus DHA daily intake between 250 and 500 mg (obtained by a daily serving of fatty fish or by n-3 LCPUFA supplements) reduced the risk of CHD mortality and sudden cardiac death. These daily amounts were considered sufficient for a primary prevention from CHD in healthy people and had been recommended for adult general population (EFSA, 2010). The FAO Expert Consultation was in agreement with the conclusions reported by EFSA (FAO, 2010) and, more recently, these findings have been confirmed also by Zhang *et al*., which translated the daily intake in a weekly value, of 29 mg/kg body weight (Zhang *et al.*, 2015).

For what about people affected by hypertriglyceridemia, AHA suggested a daily supplementation of EPA and DHA of about 2-4 g, in addition to a traditional medical therapy (Kris-Etherton *et al.*, 2002). However, such a high assumption of n-3 LCPUFA is reported to have adverse effect on gastrointestinal system; symptoms commonly arise after a daily intake of more than 3 g of EPA and DHA, but supplementations generally do not exceed 1 g/day (Wang *et al.*, 2006).

Usydus *et al.* published interesting data on EPA and DHA content in European fish species and fish products in the perspective to achieve the recommended amounts for CHD prevention. The fish species with highest n-3 levels resulted Baltic sprats, Mediterranean sardines, Atlantic mackerel, Baltic salmon, Atlantic herring, and farmed trout. The daily amounts for each fish product, necessary to reach the recommended doses of n-3 LCPUFA for CHD prevention, were also reported (Usydus and Szlinder-Richert, 2012). Salmon contains such EPA plus DHA (more than 3800 mg/100 g of tissue) that two servings per week of 100 g of Baltic salmon were calculated to be sufficient for the CHD prevention, while, to reach the same amounts of EPA and DHA from herrings, a daily intake of 100 g are necessary (Usydus *et al.*, 2011). Trout contains up to 1800 mg/100 g of tissue of n-3 LCPUFA and it was calculated that two servings of 200 mg per week can meet the requirements for people suffering from CHD; contrariwise, species like cod and sole are not suggested for these purposes, due to their low levels in fatty acid from the n-3 family (Usydus *et al.*, 2011).

While the protective action (primary prevention) of n-3 LCPUFA from CHD mortality is supported by scientific results (Wang *et al.*, 2006), the effects of a secondary

prevention are still subject of conflicting opinions. On the one hand, the American Heart Association reported the evidence of a protective effect on people affected by CHD from a daily EPA plus DHA intake between 500 and 1800 mg and promoted a constant and life-long dietary intake fatty fish (Kris-Etherton *et al.*, 2002); on the other hand, some authors sustained that, such levels are hardly reachable without diet supplements with fish oil capsules or functional foods (Weichselbaum *et al.*, 2013). While Wang *et al.*, by the analysis of scientific publications, assert that the evidence of benefits on cardiovascular outcomes from fish oil intake are stronger in secondary- than in primary prevention (Wang *et al.*, 2006), recent studies deny the beneficial action of n-3 LCPUFA supplementation on population affected by cardiovascular diseases, finding inconsistent results from studies of secondary prevention (Weichselbaum *et al.*, 2013).

Despite this, generally authors agree in suggesting additional studies to identify the mechanism of action of n-3 LCPUFA in the protection of the cardiovascular system (Kris-Etherton *et al.*, 2002; Weichselbaum *et al.*, 2013). For example, Kris-Etherton *et al.* (2002) suggested to take into consideration the safety and the efficacy of n-3 LCPUFA on CHD patient under pharmacological therapy or affected by type 2 diabetes, dyslipidemia an hypertension. Moreover, the great majority of dietary recommendations advise for EPA plus DHA intake without differentiate or specify each amount, for this reason Kris-Etherton *et al.* (2009) claim specific recommendation for both the fatty acid, because for some endpoints the have not an equivalent biological effects.

From a broader perspective, further analysis to consider the whole components of fish as contributors to the health outcomes (especially vitamins and minerals) are recommended (Weichselbaum *et al.*, 2013). For example, some studies reported vitamin B12 an glycine betaine can reduce plasma levels of homocysteine, a predictive biomarker of cardiovascular and cerebrovascular illnesses; while selenium is thought to potentially contrast the fish and humans accumulation of Hg (Hellberg, 2012; Lund, 2013).

In conclusion, seafood intake is associated with positive dietary and lifestyle habits (e.g. higher fruits and vegetables intakes, physical activity). The positive effects of frequent seafood intake could be related to the simply substitution of other foodstuffs containing higher levels of saturated fatty acid and cholesterol (as meat and dairy products) which have putative adverse effects on cardiovascular system if consumed in excess (Weichselbaum *et al.*, 2013; EFSA, 2014).

1.2.2.2 Seafood and child neurodevelopment

With regard to young people, recent investigations have highlighted the importance of fish consumption during pregnancy for foetus neurodevelopment. DHA seems to be the main responsible of this outcome being a structural component of cell membrane phospholipids, especially in brain neurons and retina (EFSA, 2010; EFSA, 2014).

Starting from the third trimester of pregnancy, the baby gets DHA from the placenta and quickly accumulates it in the brain. Although the foetal ability to synthesise DHA in the brain increases during pregnancy, the large demand continues after the first two years of life as a result of the brain's growth spurt. Thus, the DHA requirement has to be satisfied throughout the breastfeeding (EFSA, 2010; EFSA, 2014), in fact the normal development of foetal nervous system can be obtained whether, during pregnancy, mother's requirements of DHA are respected. The obvious consequence of a low maternal DHA status is an insufficient accumulation of this substance in the body and in the brain of the new-born (EFSA, 2014). The fact that most of worldwide populations are not meeting the recommended EPA plus DHA intake is a cause of public concern (Hellberg, 2012).

In 2010 JECFA confirmed the clear evidence of an improved neurodevelopment in infants and young children in case mothers consumed seafood before and during pregnancy (FAO/WHO, 2011). Meanwhile, EFSA recommended pregnant and lactating women to introduce 100-200 mg/day of DHA in addition to the n-3 LCPUFA advised for adult people, in order to compensate maternal losses and ensure the needs of their infants, especially the last months of pregnancy and throughout the breastfeeding. For

children under two years old EFSA suggested a DHA daily intake of 100 mg, an amount found effective to improve visual function (EFSA, 2010). The FAO expert consultation suggests a minimum daily intake of 300 mg of n-3 LCPUFA (200 mg of them should be DHA) for pregnant and lactating women (FAO, 2010), while the Word Association of Perinatal Medicine and other health Authorities only recommend a supplementation of 200-300 mg of DHA to the same categories of women (Kris-Etherton *et al.*, 2009). Higher intake are recommended by Harris *et al.*, who state that up to 400-500 mg per day of EPA plus DHA from seafood can be even considered safe from hazardous contaminants (Harris *et al.*, 2008).

Some authors sustain that, while fish intake in early life is recognised to improve cognitive development, the supplementation with EPA and DHA does not seem to have the same beneficial effect. Pregnant and breastfeeding women are then advised to achieve the suggested intake of DHA by the weekly consumption of one or two portions of oil-rich fish (Weichselbaum *et al.*, 2013).

Moreover, another important element for infant neurodevelopment is iodine; a foetal iodine deficiency, due to a maternal insufficient intake during pregnancy results in an incomplete early brain development, which leads to lower cognitive and motor performance during the growth. According to EFSA, 200 µg/day of iodine are necessary for pregnant women to avoid mental retardation and lower motor performance due to disorders in the early brain development. In situations where alternative sources of iodine are not available, seafood consumption during pregnancy could be fundamental for the correct development of the new born (EFSA, 2014).

Further investigations on the effects of maternal fish intake on the health of the new born suggested a possible reduction in the occurrence in eczema and asthma in babies whose mother consumed fish during pregnancy. However, deeper investigations in this field are needed due to the inconsistence of data (Weichselbaum *et al.*, 2013).

1.2.2.3 Further beneficial effects on human health

The association of fish consumption with type 2 diabetes have recently been discussed: some authors disagree in finding a positive or a negative correlation. The uncertainty that other factors (as cooking methods) can influence study results still lasts (Weichselbaum *et al.*, 2013).

Investigations trying to explain why seafood consumption is often associated with a reduced risk of obesity provided heterogeneous results. Fish intake per se does not seem to induce significant metabolic modifications, whereas n-3 LCPUFA showed specific properties addressed to the decrease in adipocytes' size and to the reduction in body fat. A more accredited theory is supported by the awareness that fish brings an important amount of protein in the diet and that proteins have a stronger satiating effect than carbohydrates and fat. This can lead to an increased feeling of satiety and a consequent loss of weight (Weichselbaum *et al.*, 2013).

Some publications suggested other beneficial properties of EPA and especially DHA on human health, such as improved visual development in children and a reduced cognitive decline, dementia, incidence of Alzheimer's disease and depression in elder people (Hellberg, 2012; Weichselbaum *et al.*, 2013).

Pilkington *et al.*, in addition, published scientific data demonstrating the protective effect of n-3 LCPUFA on the skin from ultraviolet radiation injuries (Domingo, 2014).

Moreover, alleviation of colitis and rheumatoid arthritis was also scientifically proved (Hellberg, 2012). Further studies are needed to ascertain the correlation between fish and an increased bone mineral density and reduced osteoporosis; fatty fish is known to provide vitamin D, which is a promotor of bone health, likewise seafood eaten with soft bones represents an important source of calcium (Weichselbaum *et al.*, 2013).

The role of fish intake in cancer prevention is still under investigation. According to what stated by the Expert Consultation of JECFA in 2011, the evidence of positive outcomes due to seafood intake is insufficient (FAO/WHO, 2011). On the other hand, recent studies strongly support the hypothesis that consuming fish more than twice a week could protect against colorectal cancer (CRC). Less evidence links such a

beneficial effect towards prostate and thyroid cancer, supported only by case-control studies and not by cohort ones (Lund, 2013; Weichselbaum *et al.*, 2013). Substances ascribed to have preventive actions had not been identified yet, but it seems that n-3 LCPUFA, vitamin D and selenium could contribute in reducing tumour promotion in CRC triggered by chronic inflammation (Lund, 2013).

However, scientific investigations have not clarified yet the specific effects of each nutrients on human health. At the base of these uncertainties there is the awareness that bioactive compounds, administrated simultaneously, could work synergistically; this topic complicates the association of a nutrient with a health outcome (Lund, 2013).

In the last 10 years, scientific cohort studies confirmed the positive correlation between seafood consumption and the beneficial effects on children's and adults' health, considering seafood as the whole combination of each nutrients (EFSA, 2014). Although it is proved that these beneficial outcomes are most likely due to n-3 LCPUFA intake, even JECFA stated that the health effect of seafood consumption reflects the sum of positive and negative contributions from all of the constituents, resulting in a greater beneficial action than the sum of the single constituents introduced individually (FAO/WHO, 2011).

Unfortunately the quantification of the benefits linked to n-3 LCPUFA assumption is a challenging task, due to the heterogeneity of methodologies used in the investigations carried out until now (EFSA, 2014).

1.2.3 n-3/n-6 polyunsaturated fatty acid ratio

Together with EPA and DHA content, one of the best nutritional indexes to evaluate fish quality is n-3/n-6 polyunsaturated fatty acid (PUFA) ratio (Hosseini *et al.*, 2014). This is an important factor in human diet, considered predictive for coronary artery events (Usydus *et al.*, 2009). For this reason, dietary recommendations generally include guidelines, not only for the adequate EPA and DHA intake, but also for the achievement of the correct n-3/n-6 ratio in the diet (EFSA, 2010).

It has been estimated that the ratio of these PUFA in the human diet have increased over the centuries from approximately 1:1 to 1:20–25. This huge shift is thought to be a consequence of the reduction in fish consumption (Usydus *et al.*, 2009) and of the abundant presence of vegetable oils and animal fats in the diet (Usydus *et al.*, 2011). An appropriate n-3/n-6 ratio for the prevention of CHD has not been already set (Usydus *et al.*, 2009) but should stay around 1:5 (Usydus *et al.*, 2011).

Some authors suggest two strategies for lowering CHD risk: the replacement of saturated and trans fatty acids with monounsaturated fatty acids, and the increment of n-3 LCPUFA dietary intake from plant sources and fish (Usydus *et al.*, 2009). In practice, an increment in n-3/n-6 ratio can be only achieved by increasing the intake of n-3 LCPUFA and not by lowering n-6 PUFA in the diet (Mozaffarian and Rimm, 2006).

Among the most consumed fish species in Europe, n-3/n-6 ratio ranges from 20:1 in the herrings to 3:1 in the carp (Usydus *et al.*, 2011); generally marine fishes have a n-3/n-6 PUFA ratio content nearly 10-fold higher than freshwater species (Schneedorferová *et al.*, 2015).

In conclusion, the increment of seafood consumption, with a correct selection of fish species, could represent a further dietary strategy to modify the n-3/n-6 ratio from dietary intake, in favour of n-3 PUFA.

1.3 Seafood and contamination

Certain dietary habits can contribute to compromise human health by being a source of exposure to environmental toxic contaminants (Lund, 2013). Benefits derived from seafood consumption are ascertained, but cannot avoid the concern for the presence of several potential hazards, including microbiological pathogens, marine toxins, seafood allergens, heavy metals and environmental pollutants (Hellberg, 2012).

The elimination of pathogen from the foodstuff is easily obtained by cooking treatments or by properly handling and storage. What generally represents a health risk for seafood consumers are chemical pollutants, since the risk associated with allergens and toxins are more manageable (Hellberg, 2012).

For what about some emerging pollutants, such as pharmaceuticals, personal care products, nanomaterials, awareness arisen only recently and their potential effects on human health are still incompletely understood (EFSA, 2011b).

Human activities made marine environment (water, sediment, biota) an ultimate repository for a considerable number of natural and anthropogenic contaminants, that can concentrate in fish and shellfish tissues for human consumption (EFSA, 2005a). In the same way, river and coastal waters are well-recognised sources of environmental contaminants, which reach these ecosystems as a consequence of agricultural development, industrialization, urbanization and transportation pollution (Weichselbaum *et al.*, 2013; Domingo, 2014; Zhang *et al.*, 2015).

As a consequence, the contamination of aquatic food sources represents a concern for European consumers, since adverse health effects have been associated with the exposure to such compounds. The European Directive 2008/56/EC (Marine Strategy Framework Directive) was issued to guide Member States in taking the necessary measures to protect marine environment within 2020, by means the identification of "qualitative descriptors" for the determination of the environmental status (Directive 2008/56/EC).

The Aquatic ecosystem is extremely complex and environmental contamination, in association with climatic changes (for example, intense rainfall, excessive rising water temperatures during summer and ocean acidification) may be deleterious for fish, molluscs and crustaceans (EFSA, 2011b).

According to several investigations, the vast majority of environmental pollutants contaminates human body through the diet and the most involved foods are of animal origin. Particular attention must be paid to seafood, which is considered one of main source of exposure to chemical agents through the diet (Domingo, 2014). Since cooking methods do not reduce pollutants content in fish (WHO, 2007), the issue that frequent seafood consumption potentially exposes consumers to contaminants is of notable concern for the Authorities and scientific panels, especially if high intakes of specific marine species are recognised to be dangerous for human health (Domingo, 2014; Cano-Sancho *et al.*, 2015). Chemical contaminants frequently found in seafood are heavy metals and persistent organic pollutants (POPs) (Domingo, 2014; Cano-Sancho *et al.*, 2015). These toxic compounds, due to their chemical properties, have in common the ability to persist in the environment for long periods and in different media. Removal operations of these chemicals from the environment are extremely expensive and complicated (Hennig *et al.*, 2012; Domingo, 2014). Many of them are fat soluble and can accumulate in the organism; this means that in marine environment both heavy metals and POPs can easily biomagnificate through the food web, due to prey-predator relationships, and larger species of fatty fish could turn out to be highly contaminated (Domingo, 2014; EU, 2016). For example, when high consumption of salmon is recommended for cardiovascular diseases prevention (200 g per week), the issue regarding contamination by environmental pollutant is of notable concern (Usydus *et al.*, 2011).

1.3.1 Heavy metals

Among heavy metals, mercury (Hg), arsenic (As), cadmium (Cd) and lead (Pb) are inorganic elements without biological purpose or homeostasis mechanism in human body, but known toxic effects. Their potential toxicity is a function of the concentration and the duration of the exposure; chronic exposure to even low levels of As, Cd, Hg and Pb can provoke adverse consequences for consumers (EFSA, 2004; Domingo, 2014).

The opinion of most of scientific expert is unanimous in considering MeHg the most concerning contaminant among seafood chemical pollutants, due to its adverse health effects on human (Hellberg, 2012). The presence of Hg in the environment derives from the release in the atmosphere after natural (volcanic eruptions) and anthropogenic emissions. Through rain-water Hg reaches lakes and streams and, once in aquatic medium, the inorganic Hg is converted by microbial activity in the organic form (methylmercury) (Mozaffarian and Rimm, 2006; WHO, 2007). This step is crucial because increases the bioavailability and the potential toxicity of this pollutant (Mozaffarian and Rimm, 2006); the organic Hg is in fact the most toxic form (EFSA, 2005a). While the elemental and inorganic form hardly cross tissue barriers, methylmercury (MeHg) is rapidly adsorbed by phytoplankton and aquatic organisms (Mozaffarian and Rimm, 2006).

Due to its chemical stability and long half-life elimination, fish can eat contaminated preys and consequently accumulate MeHg in the body; then it concentrates through the food chain, in a process called "bioaccumulation". MeHg levels in aquatic species depend on many factors, including water contamination, chemical characteristics of the aquatic habitat, predatory nature of the species and lifespan of the animal (Mozaffarian and Rimm, 2006; US EPA, 2014). The higher accumulator species are the larger and long living predators (e.g. swordfish, tuna and sharks), while smaller or shorter-lived fish (e.g. salmon or shellfish) have very low levels of contamination (Mozaffarian and Rimm, 2006).

Zhang *et al.* (2015), in their study conducted in China, found sea species more contaminated than freshwater ones, and justified the results with the broader diet and larger body sizes of marine fish.

In foodstuff other than fish products Hg is generally present in the inorganic form; outside the occupational exposure, seafood consumption is considered the primary MeHg source of contamination for human body (EFSA, 2004; WHO, 2007; Hellberg, 2012; Zhang *et al.*, 2015).

The toxic action of MeHg occurs at low exposure levels and seems to be related to its affinity for active site of enzymes (thiol groups), ion channels and receptors, which leads to the inhibition of antioxidant patterns and the consequent releasing of free radicals and reactive oxygen species (EFSA, 2005a; Mozaffarian and Rimm, 2006). MeHg is a neurotoxin (US FDA, 2014) which exert its harmful action especially on nervous system during brain development in foetus (EFSA, 2004). It can easily cross the placenta, so foetal exposure is directly correlated to maternal contamination during gestation. In case of a very high gestational exposure, for example for maternal consumption of highly contaminated seafood, children may show serious neurodevelopmental outcomes, such as lower visual response memory (Mozaffarian and Rimm, 2006; Zhang *et al.*, 2015). The correspondence between MeHg exposure and the aforementioned adverse neurological outcomes is also supported by the last report of JECFA (FAO/WHO, 2011).

In adult population, intoxication symptoms arise at high MeHg exposure and can occur in case of accidents (Minamata Bay) or frequent and prolonged consumption of highly contaminated species (1-2 servings per day for more than 10 years) (Mozaffarian and Rimm, 2006). Neurological outcomes are the most common: in general occurring as paresthesia, but in the last few years cognitive decline and dementia have been observed, together with immunological and reproductive alterations. The neurological symptoms are frequently reversible when the exposure is reduced (Mozaffarian and Rimm, 2006). MeHg is also thought to promote myocardial infarction and coronary heart disease, although there is still an absence of convincing evidence (EFSA, 2004; Mozaffarian and Rimm, 2006; FAO/WHO, 2011).

The MeHg poisoning outbreaks occurred in Minamata Bay (Japan) and in Iraq in 1950s attracted the attention of health Authorities worldwide, which recognized for the first time MeHg in seafood as a hazard. Large-scale epidemiological studies were later conducted on population known for their high-fish diet, as Faroe Islands and Seychelles (Hellberg, 2012). With the aim of protecting the consumers' health, in 2001 the European Commission Regulation 466/2001 set a maximum level for total Hg in fisheries products of 0.5 mg/kg, with the exception for certain predatory species (e.g. tuna, shark and swordfish) which could reach 1 mg/Kg (Commission Regulation 466/2001). In the same year the Environmental Protection Agency of United States (US EPA) calculated a reference dose level for MeHg and estimated a maximum acceptable daily intake corresponding to 0.1 µg/kg (Mozaffarian and Rimm, 2006; US EPA, 2014).

In June 2003 the JECFA Committee reduced the existent provisional tolerable weekly intake (PTWI) for MeHg (3.3 µg/kg) to 1.6 µg/kg body weight, on the base of epidemiological studies on the exposition of the most sensitive target population (women of childbearing age) (EFSA, 2004).

Almost no one in the world can avoid the assumption of Hg and its consequent accumulation, although the majority of the population shows contaminations below the set limits; this fact reflects the pervasive presence of this pollutant in the environment (US EPA, 2014).

On the other hand, EFSA declared that some European populations (for instance the Portuguese) could easily reach the PTWI, being frequent consumer of predatory species; moreover, studies conducted in France stated that French children are near to exceed the PTWI (EFSA, 2004).

Then, reliable data MeHg intake from seafood must be collected, in order to minimise population exposure to this compound (EFSA, 2004). In 2011, the European Commission asked the CONTAM Panel of EFSA to evaluate whether the JECFA PTWI for MeHg was still appropriate and, after a depth epidemiological study on Seychelles child, the Panel choose to reduce the value to 1.3 µg/kg (EFSA, 2014).

The experts also highlighted the lack of evidence of adverse effects on heart and cardiovascular system, which are likely to be counteracted by the presence of n-3

LCPUFA in fish (EFSA, 2014). The Food and Drug Administration declared that the whole adverse effects of MeHg overcome the benefits from seafood consumption only when the cumulative exposure is high enough (US FDA, 2014).

For what about As, it is a well-known toxic element for human and other living being; likewise MeHg, the inorganic form present in water is metabolised by aquatic organisms in an organic compound (arsenobetaine and arsenocholine). In this case the organic form is characterised by less toxicity (EFSA, 2005a).

The International Agency for Research on Cancer (IARC) classified the inorganic As as a substance "carcinogenic to humans", on the basis of the observation of an increased incidence of cancer of the urinary bladder, skin and lung, related to its exposure. On the other hand organic As is not genotoxic in mammalian cells (EFSA, 2005a).

Marine seafood is the main source of dietary exposure to As for humans, especially in the organic form: more of 90% is present as arsenobetaine, the reminder is arsenocholine; therefore arsenobetaine represent the predominant form of As from the diet. The inorganic form is generally detectable in fish only in percentages below 1% (EFSA, 2005a).

A PTWI for this pollutant was established by JECFA in 1988 an set to 15 µg/kg; anyway, EFSA in 2005 declared that, regarding As contamination, seafood consumption is not considered harmful for human health (EFSA, 2005a).

Cd is another heavy metal known for its adverse effects on human health, such as carcinogenicity, genotoxicity, teratogenicity, neurotoxicity, nephrotoxicity and endocrine disruption and reproduction impairment (EFSA, 2005a). Cd shows an high affinity for metallothionein, which is a cysteine rich protein responsible for Cd retention in tissue and for protection from its acute toxicity (Klaassen *et al.*, 2009); in fact, the free Cd fraction is the only responsible for the toxic effects of this compound, which are linked to the interference on calcium homeostasis; kidney is the most sensible target organ (EFSA, 2005a).

Due to the binding to metallothionein and the consequent accumulation in human tissues (Klaassen *et al.*, 2009), Cd shows an extremely long half-life in human body, of about 40 years. A PTWI of 7 µg/kg has been set for this pollutants, a dose level

considered not completely protective for those people at risk of tubular dysfunction (EFSA, 2005a).

Pb is another inorganic pollutant, mainly known for cases of intoxication due to occupational exposure. The adverse effects of Pb on human health are anaemia, cardiovascular and renal diseases, carcinogenicity and reproduction impairment. Pb can alter the mitochondrial function in endothelial cells and, since it crosses the blood brain barrier, it could be also responsible for neurotoxicity. This pollutant is not genotoxic but it was classified by IARC as "probably carcinogenic for humans" and a PTWI of 25 µg/kg was set by JECFA in 1986 (EFSA, 2005a).

1.3.2 Persistent organic pollutants

As well as inorganic contaminants, even organic pollutants represent an important risk factor for the health of fish consumers. POPs is a group of synthetic halogenated organic compounds with lipophilic properties, which can persist in the environment likewise heavy metals (Mozaffarian and Rimm, 2006).

Due to their chemical stability and semi-volatile structure, these organic pollutants represent a significant environment and health problem: they can easily spread through the worldwide ecosystem and accumulate in the living organisms. Their presence in the environment is a consequence of human activities: some of them are widely synthetized for several commercial and industrial applications (additives, pesticides, etc.), while others are accidentally produced during industrial processes (e.g. waste combustion, paper bleaching and plastic production) (EU, 2016).

The list of POPs is extensive, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/PCDFs), polychlorinated biphenyls (PCBs), polychlorinated diphenyl ethers (PCDEs) and other organohalogenated compounds, such as polybrominated diphenyl ethers (PBDEs),

polychlorinated naphthalenes (PCNs) and perfluoroalkyated substances (PFAS) (Cano-Sancho *et al.*, 2015).

The most studied and alerting POPs are PCDD/PCDFs and PCBs, that had been grouped under the collective term of dioxins by the Unite States Environmental Protection Agency (US EPA). Various congeners with similar chemical structures belong to this group and show the same mechanism of toxicity, except for 209 congeners of PCBs called “non-dioxin-like PCBs” (NDL PCBs) (Hellberg, 2012).

POPs are characterised by two or three rings that can be more or less chlorinated in different positions; dioxins can have up to 8 chlorine atoms substitutions, while PCBs to 10. The higher chlorinated compounds, or those with chlorination in specific molecular sites, are the most persistent in the environment and the morst bioaccumulative. In addition, the level of chlorination and the specific chlorination pattern influence also their toxicity (WHO, 2010).

The acute exposure to high levels of dioxins and dioxin-like PCBs (DL PCBs), which occurs only in case of occupational settings or industrial accidents, can cause persistent skin lesions, as chloracne. A long-term exposure, even though at low doses, can cause immunotoxicity, suboptimal development and neurodevelopment, hormonal and reproductive alterations. Foetus, and in particular breastfed infants, are the most sensible target population (WHO, 2010). Conflicting results suggested the prenatal toxicity of dioxins and DL PCBs on neurodevelopment, resulting in child neurologic outcomes (Mozaffarian and Rimm, 2006), which had been subsequently supported by several experimental animal data (FAO/WHO, 2011).

Certain POPs have adverse action against reproductive and hormonal homeostasis at levels of exposure 10 times lower than the normal background (Domingo, 2014). This group of emerging pollutants are known as “endocrine disruptors” and are considered a priority by the Marine Strategy Framework Directive (MSFD) (Cano-Sancho *et al.*, 2015).

Scientific investigations suggest that several environmental pollutants can also induce cellular oxidative stress, which can represent the aetiology of many chronic diseases (Domingo, 2014). POPs, in particular, alarm for their ability to generate free radicals

and to trigger inflammatory promoters, considered responsible for several inflammatory-related diseases, as diabetes, atherosclerosis, hypertension, arthritis, osteoporosis, and cancer (Hennig *et al.*, 2012).

Some scientific studies on human exposure, supported by experiments on animal models, highlighted the carcinogenic activity of dioxins and DL PCBs, probably due to the alteration of a transcription factor modulating the cellular gene expression (Mozaffarian and Rimm, 2006). Dioxin and NDL PCBs are considered human carcinogenic agents by US EPA. In particular, the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is considered the most toxic compound and in 1997 was classified as "human carcinogen" by IARC; recently the 2,3,4,7,8-pentachlorodibenzofuran and the 3,3',4,4'5-pentachlorobiphenyl had been added at this list. NDL PCBs were instead classified as "probably carcinogenic to humans" (WHO, 2010; Hellberg, 2012).

In 1977 the production and utilization of PCBs in industrial processes was prohibited under the Stockholm Convention on POPs and the emissions of dioxins have been strongly reduced (more than 90%) from 1987, with adequate remedial actions. Nevertheless dioxin and PCBs continue to be released accidently in the environment and to be found, even though in low concentrations, in many foodstuffs (Mozaffarian and Rimm, 2006; WHO, 2010).

Generally, the environment contamination from dioxins is represented by a mixture of polychlorinated compounds, characterised by different halogenation degree, in function of the source material incinerated and the amount of chlorine available during the combustion (WHO, 2010).

As lipophilic substances, dioxin and PCBs levels in drinking and surface water are very low; inadequate waste incineration makes these substances released in the soil and aquatic sediments, where they reach biological organisms and then bioaccumulate. As for heavy metals, except for occupational exposure, diet is considered the main route of contamination by POPs for humans. The half-lives of these substances in human body can overweight 7 years (WHO, 2010; Domingo, 2014).

Although the main dietary source of dioxin and PCBs are fatty food (in order, meat, dairy products and vegetables), considerable attention should be paid to fish, since

their persistent lipophilic nature causes their accumulation in lipids and their diffusion through the food chain (Mozaffarian and Rimm, 2006; Weichselbaum *et al.*, 2013).

Dioxin and PCBs are a group of congeners with different levels of toxicity and a cumulative mechanism of action (WHO, 2010). Because of the difficulties in differentiating their relative contribution to food contamination, in 1998 the World Health Organization (WHO) established that a toxic equivalent (TEQ) should be calculated for these compounds, assigning a toxic equivalency factor (TEF) to each congener (FAO/WHO, 2011). This value had been obtained comparing its degree of toxicity to the reference compound, 2,3,7,8-TCDD, which is the most toxic one, with a TEF equal to 1. Subsequently, a TEQ for each congener is calculated multiplying the pollutant concentration with its specific TEF; the sum of all TEQs estimates the level of contamination of a target foodstuff (Mozaffarian and Rimm, 2006; FAO/WHO, 2011).

The long half-lives of dioxins in human body make their accumulation higher as more the exposure lasts; therefore chronic consumption patterns should be avoided to preserve human health (FAO/WHO, 2011).

To protect consumers from POPs carcinogenicity, the Scientific Committee on Food (SCF), designated by the European Commission, in 2001 set a tolerable weekly intake (TWI) of 14 pg TEQs/kg body weight (b.w.) for 2,3,7,8-TCDD, and extended this limit to the other congeners, together with PCDFs and DL PCBs (EU, 2001).

In 2002 JECFA committee established a provisional tolerable monthly intake of 70 pg/kg body weight for dioxin (PCDD/PCDFs and DL PCBs) expressed as TEFs. This value had been formulated in “monthly intake” to emphasise the typical cumulative and chronic exposure to these compounds (WHO, 2010).

Stockholm Convention required regulatory actions from the bounded States, aimed to reduce emissions of this contaminants in the environment, in order to low human exposure, in particular that of infants. WHO recommended the Member States to develop new strategies to monitor dioxins and furan releases, including the improvement of suitable waste incineration technologies, with the aim of reducing food contamination. In addition, WHO advised to monitoring dioxins and DL PCBs levels in foodstuff and human milk (WHO, 2010)

PCNs are other poly-halogenated POPs with carcinogenic toxicity comparable to that of TCDD; PBDEs are a class of brominated flame retardants, alarming for its increasing concentrations in human tissues and fluids. Their target organ is the liver, but some of them are also endocrine disruptors compounds (EDCs) (Domingo, 2014).

A recent monitoring conducted in Scotland measured POPs levels in freshwater and marine fish and shellfish, and did not find any concentrations above the regulated limits; nevertheless, this investigation also revealed a contamination from a wide range of environmental pollutants, which concerns for a possible synergic toxic action from each compound, although present in low levels (Weichselbaum *et al.*, 2013).

A Dutch study reported that consumers' exposure to dioxins could exceed the specific tolerable weekly intake set by SCF (14 pg TEQ/b.w.) in case of high seafood intake (van der Voet *et al.*, 2007). The same conclusion was drown by Sioen *et al.*, who calculated exposures and intake levels worldwide, and found that Countries with the highest fish consumption exceeded PTWIs both for dioxins and MeHg (Sioen *et al.*, 2009).

The real risk for human health from POPs occurs when fish is captured from high contaminated waters and largely consumed by sensible population groups, such as pregnant women and young children (Hellberg, 2012). POPs rather accumulate in fatty fish species, therefore caution in the consumption of predatory fish has been also suggested (Weichselbaum *et al.*, 2013; Domingo, 2014). Moreover, the aforementioned Scottish study revealed higher levels of organic pollutants in freshwater species than marine ones, in contrast to what happen for the heavy metals (Weichselbaum *et al.*, 2013).

The age of the fish can influence its level of contamination, since wild older subjects, generally larger, eat bigger prey species and can accumulate higher levels of contaminants; moreover older fish store chemical pollutants for a longer period of time compared to younger, smaller subjects (EFSA, 2005a).

Further parameters can influence contaminant levels in seafood are season and life stage, since the amount of storage lipids, which are the main repository tissue for lipophilic pollutants, varies widely during the different periods of the year and of the life of the subjects. For example, fish usually store lipids prior to sexual maturation,

ready to be transferred to the developing ovaries before the spawning; therefore, fish captured or harvested in the early stages of maturation are expected to have higher lipid contents in tissues and organs than those captured after spawning (EFSA, 2005a). In general, even the distribution of adipose tissue is not uniform among fish fillets: it usually decreases from head to tail and from dorsal to ventral, with accumulation sites below the skin and in red muscle (EFSA, 2014).

For what about intra-species variability, differences in lipid storage among species justify the variable lipophilic contaminants accumulation observed: cod fat is stocked in the liver, salmon instead stores most of fat in the peritoneal tissue and particularly in the dermis of the skin, and not in the liver, while mackerel and tuna are likely to stock fat in skeletal muscle. Thus, the lipid content measured in fish is critically dependent on which tissue is sampled and in which period of year; this leads to significant variations in measured levels of pollutants, depending on species, age and tissue sampled. Therefore there is a strong need for standardization of sampling procedures to make reliable comparisons between different fish species and between farmed and wild subjects (EFSA, 2005a).

Moreover, the lack of an appropriate international control network on contaminants made the available databases on seafood unreliable. For a more efficient future monitoring, cooperative and programs between Countries are necessary to create complete databases on contaminants and nutrients. To permit the exclusion of unsafe food from the food chain it should be necessary to make appropriate inspections. Government efforts and awareness programs are needed to implement seafood control and to obtain safe aquatic food (Domingo, 2014; FAO, 2014).

1.4 Risk/benefit analysis

Seafood is known for its beneficial properties on human health, being a good source of protein with high biological value, vitamins, minerals and n-3 LCPUFA (EFSA, 2014). Since diet can influence negatively or positively the occurrence of chronic diseases, the unavoidable presence of environmental toxicants, such as heavy metals and POPs, can contribute to lower the levels of antioxidants and aggravate pre-existing inflammatory states (Hennig *et al.*, 2012).

Risks associated with contaminants in food can be managed by reducing the pollution of the sources, otherwise by reducing the intake of contaminated food. For what about MeHg, the application of the first option seems to be impossible (Abbott, 2014), therefore in the last years the debate concerning the benefits and risks of seafood intake have resulted in increasing confusion about how frequent, or even if, fish should be consumed. Food safety agencies worldwide felt the need to provide consumers simple and clear advice about fish consumption (FAO/WHO, 2011).

The risk-benefit analysis is a science in constant evolution and helps government Agencies and Authorities in developing food policy and consumption guidelines for the citizens (Hellberg, 2012). One of the Authorities' challenges is the establishment of balanced policies in this trade, with the priority to preserve safe seafood across the food chain (FAO, 2014; Cano-Sancho *et al.*, 2015).

A risk-benefit assessment method, proposed by EFSA in 2006, included the separated analysis of risks and benefits, and a final risk-benefit comparison. Both benefit and risk assessments are composed by 4 steps: identification of positive health effects/reduced adverse effects, characterization of those effects with a dose-response assessment, exposure assessment, and characterization of benefits. Some authors suggest the implementation of the exposure assessment before the characterization step, in order to avoid an useless research in case the levels of exposure are not of concern (EFSA, 2006; Hellberg, 2012).

The identification of risks or benefits elements involved the collection of all the information about the adverse or positive health effects of food or single components of food. The exposure assessment was carried out estimating the intake of these compounds, then the adverse/health effect characterization was conducted developing a dose-response curve that matches these effects with the intake of the foodstuff or the specific food component. This procedure generally establishes a tolerable daily intake (TDI) for a hazardous compound or a reference daily intake (RDI) for a beneficial one. Lastly, the risks or the benefits are characterized comparing the exposure of a specific food or food compound with the dose-response relationship, to estimate the probability of a health risk or a positive outcome to occur (EFSA, 2006; Hellberg, 2012). In the figure below (Figure 2) it is representing a scheme of the major steps in risk-benefit assessment, as recommended by EFSA in 2006.

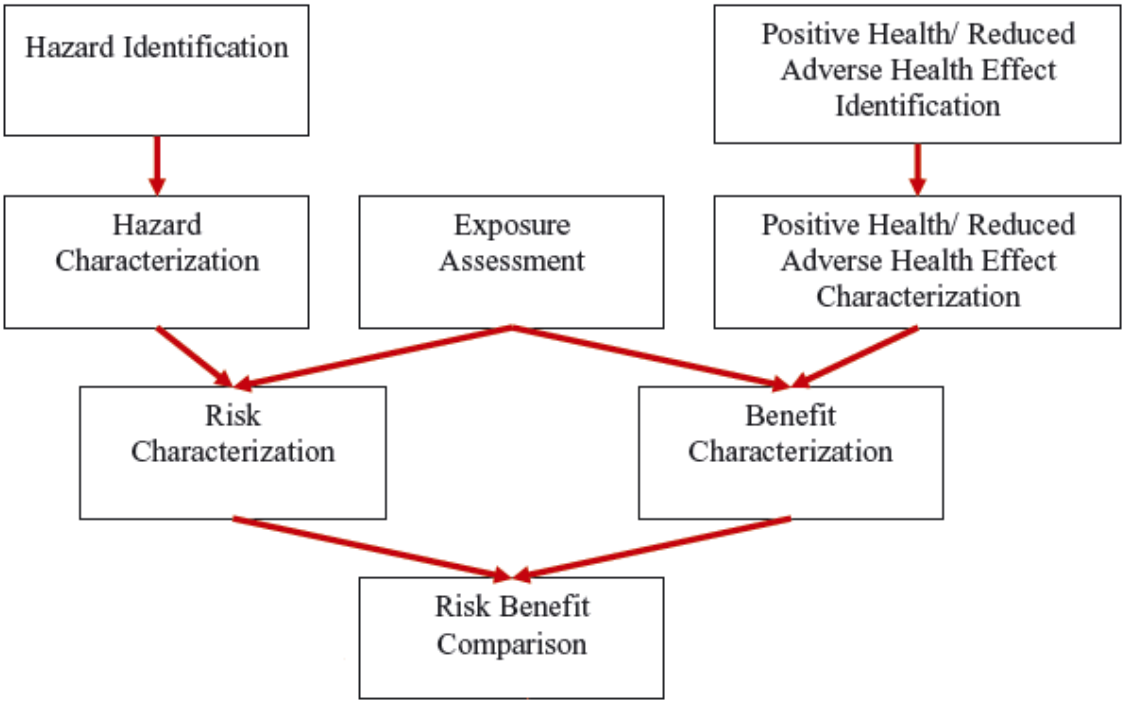


Figure 2: Risks/benefits assessment scheme proposed by EFSA (EFSA, 2006). Risks and benefits are first assessed separately and then the results are compared in the final step (risk/benefit comparison (Hellberg, 2012).

The toxicological risks and health benefits from fish consumption may vary through different life stages then the data are not easily comparable; moreover the issues are multidisciplinary and cross different branches of science, making them difficult to be interpreted by the public Authorities (FAO/WHO, 2011).

Numerous large-scale qualitative studies have been conducted in the last ten years, with the aim of providing specific seafood intake recommendations for general or target population.

In 2002 the American Heart Association (AHA) published dietary recommendations for cardiovascular diseases prevention, stating that fish intake must be balanced with concern about the presence of environmental pollutants, in particular MeHg and POPs; therefore, to achieve the desired cardiovascular positive outcomes and to minimize any potentially adverse effects due to environmental pollutants, AHA advices the consumption of two servings per week of a variety of fish species, in particular fatty fish (Kris-Etherton *et al.*, 2002).

In 2004, the United States Environmental Protection Agency (US EPA), together with the United States Food and Drug Administration (US FDA) published an advisory directed to pregnant or nursing mothers and women of childbearing age, with the aim of informing and protect from MeHg contamination (US EPA/US FDA, 2004).

The experts recommended the overall consumption of 340 g of seafood divided in two servings per week, and a maximal weekly intake of 170 g of albacore tuna (a species recognised more contaminated than canned light tuna) and the avoidance of highly polluted fish species: as shark, swordfish, king mackerel and tilefish; they then recommended the consumption of low contaminated fish species, as shrimp, light canned tuna, salmon, pollock, cod and catfish. For what about the local freshwater or marine fish caught from the wild, the experts suggested the consultation of specific regional advisories while, in case of lack of local directives, the reduction in local fish consumption (US EPA/US FDA, 2004).

The even more proved presence of contaminants in seafood induced, in the same year, the necessity to set an upper limit for fish intake in order to avoid any potential negative effects on human health (Weichselbaum *et al.*, 2013). The English Scientific

Advisory Committee on Nutrition (SACN) found that adult population generally have dietary MeHg intakes below the provisional tolerable weekly intake (PTWI) of 1.6 µg/kg body weight set by JECFA, and assumed that the threshold was sufficient to protect foetus against detrimental effects of nervous system (SACN, 2004).

For what about dioxins and DL PCBs, adult people were recommended to weekly consume till 4 portions of oil-rich fish per week., because there is not a threshold for risk, since general people could tolerate even higher intakes maintaining dioxins and DL PCBs intake below the guideline value of 8 pg TEQ/kg b.w. per day (SACN, 2004).

For the most sensitive subgroups of population (women of reproductive age, infants and pregnant women), the experts advised the application of similar measures to those published by EPA and FDA: one or two portions, of 140 g each, of fatty fish per week and restriction on shark, marlin and swordfish consumption. The intake of tuna was also restricted, up to 2 servings per week for young or pregnant women, in order to maintain dioxins and DL PCBs intake below the TDI of 2 pg TEQ/kg b.w. per day (SACN, 2004).

The Committee concluded that even sensitive individuals could exceed the tolerable intake ranges for a short time, but not in the long-term because negative consequences on health could occur (Weichselbaum *et al.*, 2013) Finally scientists recommended further investigation to identify more sensible population groups and to carry out risks-benefits assessments of fish consumption (SACN, 2004). This is in accordance with what published by Zhang *et al.* (2015), who sustain that specific guidelines have to be delivered with particular considerations for vulnerable population groups, since there are still many doubts regarding the optimal fish intake for the most sensible consumers.

In 2005, the scientific experts of the Institute of Medicine (IOM) of the National Academies compared benefits from EPA plus DHA intake from seafood with the related risk of heavy metals, POPs, microbes, allergens and biotoxins contamination. They found a net positive effect, measuring a reduced incidence of cardiovascular disease (CVD) in adult people with regular seafood consumption, and improved

children visual and cognitive performances associated with maternal n-3 LCPUFA intake (Hellberg, 2012).

IOM also evaluated the adverse effects of MeHg, cadmium and persistent organic pollutants (POPs) and drawn the conclusion that MeHg still represented the grater concerning pollutant because the toxicity of POPs was not ascertained yet. The experts then published specific consumption guidelines for different target populations: for healthy young and adult people, the selection of a variety of different seafood species consumed more than twice a week was considered protective from CVD risk; for what about people at risk of CVD, instead, the selection of fatty fish species was strongly recommended; for women near to become pregnant or during lactation and children up to 12 years old, the experts suggested to follow the same guidelines given by US EPA and US FDA (Hellberg, 2012).

Meanwhile, the European Parliament asked the Panel of Contaminants in the Food Chain (CONTAM) of EFSA to publish a Scientific Opinion on the health risk related to wild and farmed fish consumption. The assessment focused on the most marketed and consumed finfish species in the European Union (tuna, anchovies, herring, salmon, rainbow trout, carp, pilchards and mackerel), with a special attention paid to Baltic herrings (EFSA, 2005a).

The Panel confirmed the evident connection between health benefits on cardiovascular system and regular fish consumption (once or twice a week), and promoted seafood intake for primary prevention of CHD; such amount of fish is considered safe for pregnant women from MeHg contamination, especially if wild subjects of Baltic herrings and Baltic salmon, and other fish species on the top of the food chain are avoided. (EFSA, 2005a).

An important and alarming conclusion drawn by the Panel was that the high consumption of certain species (herrings and salmon from Baltic Sea) may lead to the excess of the PTWI for MeHg, dioxins and DL PCBs, even without consider other possible sources of dietary exposure; in fact, the substitution of fish meat with cuts from terrestrial species would not decrease the risk of exposure to dioxins and DL PCBs. Moreover, possible measures to decrease women body burden are only feasible

towards MeHg, by lowering the dietary intake of contaminated food; not towards dioxins and DL PCBs, since they have longer half-lives (EFSA, 2005a).

The Panel concludes that advices on seafood consumption should necessarily consider total contribution of relevant pollutants on human exposure, by referring to specific dietary patterns of each Country (EFSA, 2005a).

One of the most relevant risk-benefit assessment on seafood intake ever published was elaborated by Mozaffarian and Rimm in 2006. They examined Government Advisories and Scientific Publications and conduced meta analyses on available studies, with the aim of comparing neurologic and carcinogenic effects of MeHg, dioxins and DL PCB against the beneficial effects of EPA plus DHA (Hellberg, 2012).

Their evaluation stated that consuming fatty fish at least once or twice a week can guarantee a daily intake of about 250 mg of eicosapentaenoic acid (EPA) plus docosahexaenoic acid (DHA), considered the optimal integration for the reduction of risk for CHD mortality (Mozaffarian and Rimm, 2006).

The introduction in the diet of 170 g of oily fish species (salmon, anchovies, herrings) per week could lead to a weekly intake of EPA plus DHA up to 2000 mg, thanks to the persistence of n-3 LCPUFA in cellular membranes for long time. These findings are in line with those of the American Heart Association (AHA), who declared that the consumption of at least two portions of 140 g each per week of fatty fish (salmon, mackerel, herring, lake trout, sardines and albacore tuna) would provide 400-500 mg of EPA plus DHA per day or 3.2 g per week (Weichselbaum *et al.*, 2013; AHA, 2015).

Since MeHg was suspected to bring cardiovascular negative outcomes, the Authors suggested the consumption of a variety of fish species and, in case of high seafood intake (more than 5 servings a week), it is advisable to avoid fish species with high MeHg levels (Mozaffarian and Rimm, 2006).

The assessment also emphasised the importance of DHA for a correct neurodevelopment, without underestimate the potential negative effects of MeHg, even if at low exposures. To obtain an optimal cognitive and visual development in infants, the Authors suggested to follow the advices published by US EPA and US FDA, taking care to limit the consumption of high contaminated species such as shark,

swordfish, king mackerel and golden bass, estimated to contain >50 µg MeHg per serving (Mozaffarian and Rimm, 2006).

For what about POPs, data collected until that time on PCBs and dioxins indicated a rather low seafood contamination therefore the possible health risks in adults are greatly exceeded by benefits of seafood intake (Mozaffarian and Rimm, 2006).

Mozaffarian and Rimm concluded that, for general population, the benefits of EPA plus DHA, provided by seafood consumption, overweight the potential MeHg, dioxins and DL PCBs negative outcomes; moreover the synergic action of the nutrients present in fish is supposed to contrast the negative effects of contaminants on consumers' health. Women of childbearing age and nursing mothers, instead, can benefit from modest fish intake by selecting specific species. The authors advised this sensitive category to follow the regional Authority recommendations about the consumption of local freshwater fish caught from the wild. They also highlighted that no fish intake through the diet could cause detrimental effects on children neurodevelopment and increase death rates for CHD (Mozaffarian and Rimm, 2006).

In January 2010, the Codex Committee on Food Additives and Contaminants requested JECFA to compare the risks and benefits of seafood consumption, with the aim of divulgating scientific advices both for general and for target populations, and to identify data gaps. The experts therefore developed a proper risk–benefit evaluation, by analysing available data published on levels of n-3 LCPUFA, MeHg and dioxins in a range of fish species, and by reviewing the existing assessments models. They focalized on specific health outcomes for target populations (foetuses, infants and young children, pregnant and breast-feeding women, women of child-bearing age and high fish consumers), comparing the beneficial effects of fish intake for children neurodevelopment and for adult cardiovascular system with the risks of MeHg and dioxins contamination (FAO/WHO, 2011).

JECFA, first of all, highlighted the difficulties in analysing data from different quantification methods and in comparing health effects and negative outcomes for a specific dietary pattern. For example, they found that most of studies focalized only finfish consumption to determine n-3 LCPUFA intake, while others also included

shellfish, which generally contains less n-3 LCPUFA; therefore data resulted non-homogeneous (FAO/WHO, 2011). In addition, seasonal variability in lipid composition of fish fillets (higher content in n-3 LCPUFA in summer then in winter) could be another cause of the divergence of the data (EFSA, 2005a).

Among the most important considerations made by the Consultation there is the assumption that dietary patterns established in childhood influence dietary habits during adult life. Furthermore, the effect of fish consumption on human health reflects the sum of the contributions from the nutrients and toxic substances assumed, which are likely to vary according to the fish species, fish size, but also to the harvesting and cultivation practices, as well as the amount of fish consumed and the cooking techniques. For example, JECFA found dioxin levels in seafood strongly correlated to fish lipid content and with EPA and DHA concentrations (FAO/WHO, 2011).

On the base of the strength of evidence, the Consultation confirmed the relationship between fish consumption and the reduction of mortality rates for cardiac death, together with the promotion of the optimal child neurodevelopment in case of maternal seafood intake before and during pregnancy. There is also a probable reduction in ischaemic stroke and a possible beneficial activity against depression. An anticancer effect of seafood consumption is not already proved instead (FAO/WHO, 2011).

In specific, regarding MeHg, the Consultation found a strong relationship between neurodevelopment delays in infants and prenatal exposure due to maternal contamination; on the other hand, the increased risk of CHD due to prolonged low-level MeHg exposure, cannot be proved (FAO/WHO, 2011).

For what concerns dioxins and PCBs, there is a probable evidence for children suboptimal neurodevelopment in case of high maternal contamination during pregnancy. In fact, from this assessment, the PTMI of 70 pg/kg b.w. for dioxins was found to be exceeded by more than 10 times in case of consumption of highly contaminated seafood (WHO, 2010; FAO/WHO, 2011). Therefore, the risk for children neurodevelopment can be considered negligible only in case maternal intake, from every dietary source, remains below the PTMI during pregnancy (FAO/WHO, 2011).

For what about CHD risk due to dioxin exposure, it can overweight cardiovascular benefits from EPA and DHA only in case of frequent consumption (more than 7 servings/week) of high contaminated fish (>8 pg/g) (FAO/WHO, 2011).

Besides, benefits of n-3 LCPUFA on heart health largely exceed cancer risk even in case of high dioxins contamination. It is expected to occur only in case of accidental or occupational exposure (FAO/WHO, 2011). According to this, an American study on general population reported that the daily assumption of 1 g of EPA plus DHA would save 300 times the number of lives from CHD mortality that those lost for cancer from exposure to organic contaminants (Foran *et al.,* 2005).

Moreover, the development of endocrine disorders, together with immunological and neurodevelopmental outcomes, related to dioxin contamination from fish, have not been demonstrated yet (FAO/WHO, 2011). In conclusion, JECFA Committee, in accordance to the SACN, declared that there are still insufficient data to positive correlate the adverse health outcomes due to the dioxin exposure with seafood consumption (FAO/WHO, 2011; Weichselbaum *et al.*, 2013).

Similarly to what reported by Mozaffarian and Rimm, the consultation concluded that risks of CHD mortality significantly increases in case of not eating fish; although maternal MeHg exposure is proved to increase the risk for child neurodevelopmental delays, nutrients contained in fish (especially DHA) promote e better brain development in those children whose mother ate fish during pregnancy and lactation, compared to those cases in which mothers did not eat seafood (FAO/WHO, 2011).

A French study estimated the EPA and DHA intake of pregnant women and found that only the 50% of them achieved the recommended value of 500 mg per day, and the 5% of them was even exposed to levels of MeHg that exceeded the PTWI (Pouzaud *et al.*, 2009). For this reason JECFA declared that selecting fatty species and implementing strategies to reduce MeHg contamination in fish, would lead to even more beneficial effects for children health (FAO/WHO, 2011).

In general, greater health benefits from fish are obtained maximising the n-3 LCPUFA intake (>15 mg/g) consuming fatty-fish with moderate frequency (1 or 2 serving/week) to minimize pollutants exposure (FAO/WHO, 2011). This findings are in according to

the dietary recommendations published by EFSA in 2010, where the adult population is advised to consume 1 or 2 fatty fish meals per week or 250-500 mg of EPA plus DHA per day for a primary prevention for CHD (EFSA, 2010).

With the aim of reducing the risk from contamination and to improve the health benefits, JECFA experts advised Member States to develop risk management and communication strategies to the citizens (FAO/WHO, 2011). The committee proposed the diffusion of two tables (Figures 3 and 4), considered effective risk-benefits tools to advice consumers about nutritional and safety perspectives.

For this purpose they classified fish species on the base of typical concentration of EPA plus DHA and existing data levels for MeHg (in the table indicated as total Hg) and dioxins, with the aim of giving health-based guidance on fish consumption.

		EPA + DHA			
		$x \leq 3$ mg/g	$3 < x \leq 8$ mg/g	$8 < x \leq 15$ mg/g	$x > 15$ mg/g
Mercury	$x \leq 0.1$ µg/g	**Fish:** butterfish; catfish; cod, Atlantic; cod, Pacific; croaker, Atlantic; haddock; pike; plaice, European; pollock; saithe; sole; tilapia **Shellfish:** clams; cockle; crawfish; cuttlefish; oysters; periwinkle; scallops; scampi; sea urchin; whelk	**Fish:** flatfish; John Dory; perch, ocean and mullet; sweetfish; wolf fish **Shellfish:** mussels; squid	**Fish:** redfish; salmon, Atlantic (wild); salmon, Pacific (wild); smelt **Shellfish:** crab, spider; swimcrab	**Fish:** anchovy; herring; mackerel; rainbow trout; salmon, Atlantic (farmed); sardines; sprat **Fish liver:** cod, Atlantic (liver); saithe (liver) **Shellfish:** crab (brown meat)
	$0.1 < x \leq 0.5$ µg/g	**Fish:** anglerfish; catshark; dab; grenadier; grouper; gurnard; hake; ling; lingcod and scorpionfish; Nile perch; pout; skate/ray; snapper, porgy and sheepshead; tuna, yellowfin; tusk; whiting **Shellfish:** lobster; lobster, American	**Fish:** bass, freshwater; carp; perch, freshwater; scorpion fish; tuna; tuna, albacore **Shellfish:** crab; lobster, Norway; lobsters, spiny	**Fish:** bass, saltwater; bluefish; goatfish; halibut, Atlantic (farmed); halibut, Greenland; mackerel, horse; mackerel, Spanish; seabass; seabream; tilefish, Atlantic; tuna, skipjack	**Fish:** eel; mackerel, Pacific; sablefish
	$0.5 < x \leq 1$ µg/g	**Fish:** marlin; orange roughy; tuna, bigeye	**Fish:** mackerel, king; shark	**Fish:** alfonsino	**Fish:** tuna, Pacific bluefin
	$x > 1$ µg/g		**Fish:** swordfish		

Figure 3: Classification of the content of n-3 LCPUFA by total Hg content (for this purpose all Hg present in fish is considered to be in the methylated form-MeHg) (FAO/WHO, 2011)

		EPA + DHA			
		$x \leq 3$ mg/g	$3 < x \leq 8$ mg/g	$8 < x \leq 15$ mg/g	$x > 15$ mg/g
Dioxins	$x \leq 0.5$ pg TEQ/g	**Fish:** anglerfish; catshark; cod, Atlantic; grenadier; haddock; hake; ling; marlin; orange roughy; pollock; pout; saithe; skate/ray; sole; tilapia; tuna, bigeye; tuna, yellowfin; tusk; whiting **Shellfish:** cockle; clams; crawfish; cuttlefish; periwinkle; scallops; scampi; sea urchin	**Fish:** flatfish; John Dory; perch, ocean and mullet; shark; sweetfish; tuna, albacore	**Fish:** redfish; salmon, Pacific (wild); tuna, skipjack	
	$0.5 < x \leq 4$ pg TEQ/g	**Fish:** catfish; dab; gurnard; plaice, European **Shellfish:** lobster; oysters; scallops; whelk	**Fish:** scorpion fish; swordfish; tuna **Shellfish:** mussels; squid	**Fish:** alfonsino; goatfish; halibut, Atlantic (farmed); halibut, Greenland; mackerel, horse; salmon, Atlantic (wild); seabass; seabream	**Fish:** anchovy; herring; mackerel; mackerel, Pacific; rainbow trout (farmed); salmon, Atlantic (farmed); tuna, Pacific bluefin **Shellfish:** crab (brown meat)
	$4 < x \leq 8$ pg TEQ/g			**Shellfish:** crab, spider	**Fish:** sardines; sprat
	$x > 8$ pg TEQ/g			**Fish:** bluefish	**Fish:** eel **Fish liver:** cod, Atlantic (liver); saithe (liver)

Figure 4: Classification of the content of n-3 LCPUFA by dioxins content (FAO/WHO, 2011)

In conclusion JECFA experts promoted fish consumption emphasizing the aforementioned fish health benefits and incited Member States to communicate to children and pregnant or nursing women, including women who may become pregnant, that avoiding consuming fish could represent a risks factor for the optimal foetus neurodevelopment (FAO/WHO, 2011).

In response to the large regional variability of data collected, the experts recommended national and regional Authorities to develop or improve existing databases with representative information on specific nutrients (for example n-3 LCPUFA) and contaminants (in particularly MeHg and dioxins) from local seafood, analysed in the form it is consumed. They also advised scientists to focalize on beneficial activity of fish nutrients in different life stages and to elaborate healthy eating patterns for later life (FAO/WHO, 2011).

Meanwhile, in 2010 the Dietary Guidelines Advisory Committee (DGAC) conducted a similar risks-benefits assessment to that elaborated by JECFA and published guidelines regarding both n-3 LCPUFA (EPA + DHA) and seafood intake in general.

This report considered the same health outcomes took into account by JECFA: the preventive action of n-3 LCPUFA on CHD mortality in adult people and the prevention of neurological delays in infants, together with an overall comparison of risks and benefits of fish consumption (Hellberg, 2012).

The conclusions identified were in agreement with those of the previous assessments: two servings of about 113 g per week of fish (a daily intake of about 250 mg of n-3 LCPUFA) are supposed to reduce risk of CHD mortality both in healthy people and in patients affected by cardiovascular diseases. Furthermore, DGAC found a considerable association between maternal intake of the recommended doses of n-3 LCPUFA and elevated levels of DHA in breast milk, which leads to improved visual acuity and cognitive development in children (Hellberg, 2012).

From this assessment it can be assumed that the consumption of almost one serving per week of seafood during pregnancy promotes correct neurodevelopmental benefits and increased cognition scores in infants. Moreover, consistent with JECFA report, DGAC reported that 2 servings per week of cooked seafood provide health benefits that outweigh the potential risks derived from MeHg and POPs exposure, also for the most sensible population

Consumers are then exhorted to eat weekly at least 340 g of a variety of fish species, paying attention to the local regulations and limitations for large predatory fish (Hellberg, 2012).

More recently the European Commission requested the Panel on Dietetic Products, Nutrition and Allergies (NDA) of EFSA to set up and publish a risk-benefits analysis comparing health benefits of seafood consumption in relation to health risks associated with exposure to MeHg. Therefore in 2014 the NDA Panel of EFSA delivered a Scientific Opinion in which it was reviewed the role of fish in the diet, in the European contest, and carried out the risk-benefits assessment in relation to the same

health outcomes and population subgroups evaluated by the previous studies (children's neurodevelopment and risk of cardiovascular disease in adults).

EFSA also deepened the investigation to identify the most beneficial nutrients to the aforementioned positive outcomes (EFSA, 2014).

The NDA Panel draw the same conclusion of JECFA Committee, confirming that 1 or 2 servings per week (up to 4 servings) are associated with a lower risk of CHD mortality in adults and with a better children neurodevelopment in comparison with no mother seafood consumption during pregnancy. EFSA also specified that no additional benefits might be expected at very high fish daily intakes (more than 1 or 2 servings) (EFSA, 2014).

The Panel also identified n-3 LCPUFA as the main beneficial nutrients in reducing the risk of CHD mortality, but a quantification of their health effects resulted a challenging task, due to the heterogeneity of methodologies used in the investigations carried out until now, in terms of tools used to estimate fish consumption and to measure the outcomes (EFSA, 2014).

The big variation in the amount of seafood consumed across European Countries and age groups, makes data from surveys difficult to compare; besides the different seafood species consumed are largely unknown in some Countries, and data were particularly scarce for infants (EFSA, 2014).

In conclusion, EFSA exhorted the need to deliver food based dietary guidance on the fish species to be consumed with the purpose to ensure the provision of sufficient amounts of n-3 LCPUFA, vitamin D, iodine and selenium (EFSA, 2014).

Meanwhile, US FDA and US EPA updated the previous advice, aimed by the awareness that general population don not currently achieve the recommended fish intake. In June 2014 the expert Committee published the report with clear instruction designed to guarantee a correct child neurodevelopment. The guidelines are addressed to pregnant or breastfeeding women, or anyone who handle the nutrition of young children, and exhort the assumption of nutrients belonging form fish during pregnancy and the whole childhood. The experts finally recommend the same measures published ten year before, with a more awareness on seafood consumption in terms of

selection of fish species and amount of seafood consumed, which could not exceed the calorie needs (US FDA/US EPA, 2014).

Hibbeln *et al.* (2007) declared that children whose mother do not eat fish result more at risk, but he went so far sustain that higher benefits on children neurodevelopment were expected in case of maternal intake greater than 340 g per week. This is not completely in accordance to what recently published by US EPA and US FDA report, who considers the MeHg content of fish more critical than n-3 LCPUFA deficiency in determining the net balance of child neurodevelopment. The report in fact stated that the amount of fish recommended varied according to its contamination and estimated 340 g of a variety of seafood per week the optimal intake for mothers (US FDA, 2014).

A general conclusion of the aforementioned risk/benefits assessments suggests that benefits of fish consumption far outweigh any risk of contamination, except for few extreme examples (Lund, 2013).

From another prospective, Domingo (2014) strongly supports the need to extend the monitoring to emerging chemical pollutants. He points out that most of investigations only focused on MeHg, dioxins and PCBs and suggests that, avoiding the inclusion of other toxic elements, such as As, Cd, Pb, PAHs, PBDEs, PCNs, PCDEs and PFAS, the conclusions made by previous assessments cannot express the status of the real seafood contamination. Moreover, in the great majority of reports available there are no information on the variability of pollutants concentrations during the fish life-time (Domingo, 2014).

For what about heavy metals other than MeHg, such as lead, chromium, manganese, As and Cd can be frequently found in seafood, although fish does not seem to be a main route of exposure to these contaminants (Hellberg, 2012). Sirot *et al.* (2012) calculated an optimum intake for adult people in order to minimize inorganic As exposure and to increase vitamin D intake: a weekly intake of about 200 g of fatty fish species, and approximately 50 g of lean fish, molluscs and crustaceans, was considered adequate to guarantee the recommended intake of n-3 LCPUFA, selenium and iodine, without overcome the PTMI for MeHg, cadmium, dioxins and PCBs (Domingo, 2014).

Cano-Sancho *et al.* (2015) built a risk index with the aim of drawing a map of risk related to several chemicals pollutants (heavy metals, PAHs, PCBs, dioxins, PBDEs, PCNs, and EDCs) in target fish species (sardines, canned tuna, salmon and mussels) provided by selected European Countries (Belgium, Ireland, Italy, Portugal and Spain).

Results revealed that the different diet patterns influence risk of contamination among population: Belgium and Ireland resulted Countries at lower risk compared to the others. Canned tuna was find to be the higher contributor in EDCs, mussels in PAHs and heavy metals, and sardine and salmon in dioxins and PCBs. Heavy metals resulted the main contributors to risk index calculated in this work in every Country, and sardine the fish species with the main impact on human exposure, due to its high levels of PCBs and MeHg, and rather high consumption frequency.

Hellberg *et al.* in their review stated that the geographic source of fish represents a critical factor for its contamination and that consumption of a variety of seafood species was a suitable way to reduce the exposure to heavy metals (Hellberg, 2012). On the other hand, Cano-Sancho *et al.* (2015) found uniform levels of contamination in seafood throughout European Countries and concluded that the most determinant parameter for exposure estimation is the frequency of consumption. However, being the study focalized on few fish species, the authors suggested to investigate further fish species, which could result more influenced by geographical factors (Cano-Sancho *et al.*, 2015).

Maulvault *et al.* (2011) noted that the current regulations do not consider the consumption of other edible tissue than fish fillets (e.g. livers or crustaceans brown meat), measured the bioavailability of inorganic contaminants in raw and cooked black scabbard fish (*Aphanopus carbo*) and edible crab (*Cancer pagurus*).

These species represent two of the most important gastronomic and economic marine sources in Southern European Countries, often reporting levels of Hg, Cd and As over the limits set by International Agencies. The toxicological risk assessment conducted in this study revealed that children should limit the consumption of edible crab brown meat and grilled black scabbard fish for their hazardous cadmium and MeHg content (Maulvault *et al.*, 2011).

The optimum fish intake, calculated for minimise inorganic As exposure and for increase vitamin D intake in general adult population, was proposed by Sirot *et al.*, (2012) with the aim of inspiring food consumption recommendations in a public health perspective. The weekly consumption of 200 g of certain fish species (swordfish, herring, halibut, salmon and mackerel), together with approximately 50 g of lean fish, molluscs and crustaceans, have been established to guarantee the recommended intake for n-3 LCPUFA, selenium and iodine, as vitamin D appeared to be the limiting factor; besides these amounts also allowed to remain below the tolerable upper intakes for MeHg, cadmium, dioxins, PCBs, zinc, calcium and copper.

Marine Framework Directive (MFD) divulged a list of emerging contaminants of priority importance in seafood, considered a potential risk for public health for their carcinogenesis neurotoxicity, nephrotoxicity, hepatotoxicity, impairment on the of immune and endocrine systems (Directive 2000/60/EU). Ongoing investigations are currently collecting data on priority contaminants, including NDL PCBs, brominated flame retardants (BFRs), PFAS, organotin compounds, organochlorine pesticides and phthalates, in high risk seafood species of European background (Cano-Sancho *et al.*, 2015)

The conclusion drawn by Mozaffarian and Rimm, stating that the benefits of fish intake exceed the potential risks from MeHg, dioxins and DL PCB contamination, is not shared by Stern (2007), who defines those statements “not supportable” because “based on an inaccurate and insufficiently critical analysis of the literature”. In accordance to these findings, recent study conducted by Zellmaker *et al.* reported that, for most seafood species, the toxic effects of MeHg exposure exceeded the beneficial effects of n-3 LCPUFA (Abbott, 2014).

Therefore, Stern promoted what lately advised by JECFA and EFSA Committees, that is the divulgation of appropriate public health message to allow consumers the aware selection of those fish species that offer both high n-3 LCPUFA and low MeHg (Stern, 2007).

Planktivorous species seem to be the best choice for consumer in order to maximize the health benefits and minimizing the risks by MeHg (Zhang *et al.*, 2015).

The topic of which species is better to consume becomes more complex when other contaminants, such as PCBs, or other nutrients, has been discussed by many authors (Mahaffey *et al.*, 2011); for example, smoked Baltic salmon and smoked sprat are reported to represent a source of threating levels of dioxins and DL PCBs for sensible population (Usydus *et al.*, 2009). It is in fact necessary to deliver, on a national level, a list of seafood species to choose, and those to avoid (Oken *et al.*, 2012).

In addition, fish consumption advice should be as clear and simple as possible to have an impact on general population and protect public and global health (Oken *et al.*, 2012). Citizen should be informed not only on which species to be selected and in how much it consume, but also on the fish size to choose, in order to obtain maximum benefits from n-3 LCPUFA and minimal risk from contaminants. The fish size could be a very predictive and useful parameter to optimize future consumption guidelines for specific consumers (Zhang *et al.*, 2015).

International organizations, together with Governments and Agencies, are exhorted to focus on eliminate sources of fish contamination and, where it is not possible, to find remediations. Moreover, they are incited to establish policies for sustainable and economically viable fishing practices, so that fish could continue represent a healthy dietary choice for future generations (Oken *et al.*, 2012).

1.5 Aquaculture

As hinted in the previous section (chapter 1.1), a global increment in seafood consumption and the strong growth of aquaculture production had been observed in the last decade (FAO, 2007). Since all the main world stocks of fish have been already exploited (FAO, 2007), the sustainability of increased fish consumption represented a concern in the perspective of an adequate n-3 LCPUFA human intake (Lund, 2013). The role of fishing in addressing the challenge of eradication of hunger and malnutrition is clearly recognised, being fish a cheaper source of protein compared to meat for populations from poor Countries. Many Authorities promote aquaculture as a strategy to face these problems (FAO, 2014).

Nowadays almost two-thirds of seafood consumed in European Countries is caught from the wild (EFSA, 2005a). In a future prevision made by FAO, white fish species consumed over the next fifteen years will be nearly the same as today, with a strong prevalence of consumption of demersal marine seafood, such as cod, Alaskan pollock and hake. Some of these species may be produced by aquaculture, but it would rather be more probable an innovation in technologies for the breeding of the typical farmed species, as salmon and trout (FAO, 2007).

FAO predicts no growth in capture fishery while a further aquaculture increment is expected, mainly focused on the feeding of diadromous species and molluscs. Fish farmers are urged to innovate the commercial value of their products, because the strategy of reducing prices cannot longer be applicable. The main contributors to the growth will be Norway and United Kingdom, while the southern Countries, like Greece, Italy and Spain are prompted by FAO to cooperate with the sea bream and sea bass production (FAO, 2007).

From a nutritional point of view, farmed fish differs from captured wild fish by a more constant nutrient composition due to more stable environmental conditions and feed, monitored and managed with the aim of optimising the final product (FAO, 2014). A

correct feed formulation is required for the optimal fish growth and the promotion of consumer's health (Lund, 2013).

Marine fish, for example, need EPA, DHA and ALA for their physiological development (EFSA, 2005a), since fish are not generally able to independently synthesize n-3 LCPUFA; therefore the ingestion of phytoplankton and zooplankton provides their supply in wild subjects (Zhang *et al.*, 2015).

With the aim of simulating the natural diet pattern, farmed fish are traditionally fed with high percentage of fish meal, enriched with fish oil rich in n-3 LCPUFA of marine origin, to achieve the dietary requirements. However, the limited availability of fish oil and fish meal makes the aquaculture sector trying to replace them with vegetal products, without obtaining any negative effects on fish health (Lund, 2013; Weichselbaum *et al.*, 2013) and do not affecting the nutritional composition of fish products (EFSA, 2005a). Substituting fish products containing EPA and DHA with vegetable oils rich in ALA can substantially change the lipid composition of reared fish (EFSA, 2005). Fish fed by plant oils instead of fish oil may probably contain lower levels of n-3 LCPUFA (Weichselbaum *et al.*, 2013).

An American study conducted on sensitive female population (child-bearing age) stated that two weekly meals of 180 g of farmed salmon or trout would be expected to provide the recommended 440 mg of EPA and DHA per day. This amount results also protective for this target population because do not exceed the Canadian PTWIs for MeHg, dioxins and DL PCBs (Dewailly *et al.*, 2007).

Vitamin D content in farmed fish also depends on the levels in feed and, for what about selenium, its bioavailability of is contingent upon which form is present in the formulation (Lund, 2013).

FAO sustain that both wild-caught and farm-raised seafood are "a healthier alternative to almost any other meats" (FAO, 2014); the main difference in composition stays in the quantity and quality of fats (FAO, 2013). Farmed fish generally have higher total lipid levels and a lower percentage of n-3 LCPUFA than wild-caught; this means that the amount of n-3 LCPUFA per portion is similar between farmed and wild fish (EFSA, 2005a).

A recent study conducted on salmon from Norway confirmed these findings, having found a two-fold higher content of total fat in farmed fish compared to that found in wild-caught, but similar levels of n-3 LCPUFA (Weichselbaum *et al.*, 2013).

Concluding, wild and farmed fish proximate composition differs mainly in total lipid content and in fatty acid composition, but if farmed are reared with appropriate diets, their nutritional value would be comparable to that of wild, or even higher in terms of n-3 LCPUFA supply (EFSA, 2005a).

Further studies are needed to understand the desirable level of n-3 LCPUFA in farmed fish with the purpose to balance the amount of fish meal and oil necessary for their breeding, to obtain fish products in a more sustainable way (Weichselbaum *et al.*, 2013).

For what about contaminants, both farm and wild fish seem to show similar levels of contamination (Abbott, 2014), end equally contribute to the consumer body burden (EFSA, 2005a).

In general carnivorous species are found to be more contaminated than omnivorous ones, whether farmed or wild. Diet is the main source of exposure to contaminants in fish, although uptake also occurs via gills. Levels of contaminants with bioaccumulative properties result higher in species on the top of the food chain. In farmed fish, the contamination of feed materials (fish oil and fish meat) can be monitored and controlled, whereas in wild fish the exposure remains unknown and will vary considerably in different geographical regions (EFSA, 2005a).

The highest levels of MeHg are found in wild-caught tuna, while the highest concentrations of dioxins were measured in fatty species, such as wild herrings and wild salmon. Particular attention must be paid to wild fish from the Baltic sea, which are found to be particularly contaminated in dioxins. (EFSA, 2005a).

The substitution of fish products in fish breeding by vegetable oils and proteins can represent a possible way to reduce the levels of contamination of farmed seafood products, while for wild-caught fish the only way to minimise the contamination level is the reduction of the pollutant emissions in the environment (EFSA, 2005a).

The Directive 2002/32/EC, which lists the undesirable substances in animal feed and specifies their maximum limits, regulate the presence certain heavy metals (like lead and cadmium), dioxin and some pesticides, and plans the inclusion of NDL PCBs in order to promote the reduction of levels of contaminants in food products. This Directive is regularly updated according to the improvement of scientific and technical knowledge (Directive 2002/32/EC; EFSA, 2005a).

The choice of eating fish not only provides health benefits for humans but also contributes to help sustainable rural livelihood (FAO, 2014). Consumers are not only concerned by possible contamination affecting directly their health and well-being, but they also request food producers to assure that their ethical and environmental perceptions would be reflected in the products available (FAO/WHO, 2011).

Besides, in order to ensure the sufficient supply of fish for future generations, novel strategies are required, as current fishing practices are unsustainable (Weichselbaum *et al.*, 2013). New policies aimed to increase sustainability in fishing field, together with the introduction of eco-labelled seafood products and specific consumers guides, could strongly contribute to minimise damage to wildlife and habitats (Weichselbaum *et al.*, 2013). For this purpose, in 2008 was issued the Marine Strategy Framework Directive which requires Member States to draw measures with the aim of improving the status and functioning of their marine ecosystems by 2020. Finally, the Directive requires the sustainable use of marine resources (Weichselbaum *et al.*, 2013).

1.6 Cooking treatments

Seafood is usually subjected to cooking treatments, to ensure the microbiological safety of the product and to improve its digestibility, together with the enhancement of its flavour and taste (Costa *et al.*, 2013).

It is important to highlight that, even though it is evident that seafood is generally consumed after cooking processes (Mozaffarian and Rimm, 2006), available data on contamination and nutritional contents of fish are generally obtained by unprocessed products (Domingo, 2014), and even current safety limits from International Agencies are set on raw products (Maulvault *et al.*, 2011).

This means that the regular consumption of fish, with levels of contamination close to the tolerability, may expose consumers to health risks even greater than those estimated by the Authorities (Maulvault *et al.*, 2011). Furthermore, some authors claim that data provided on EPA and DHA contents in raw fish may not give reliable information about the nutritive value of cooked fish (Gladyshev *et al.*, 2007).

For these reasons, cooking procedures frequently used in domestic field (e.g. frying, grilling, roasting, microwave cooking, baking or boiling) have been tested with the aim of measuring their influence on nutrients and chemical pollutants content in fish samples (Costa *et al.*, 2013; Domingo, 2014).

Cooking processes cause physical and chemical changes on fish muscle, such as protein denaturation and water loss; therefore, alterations in proximate composition and moisture content of fish are frequently observed. Culinary treatments, as frying, grilling and roasting generally increase nutrients rates, expressed in percentage terms. This applies for the fatty acid profile, including EPA and DHA, and most of minerals as consequence of the dehydration (Costa *et al.*, 2013).

As consequence of the dehydration of the food, it occurs an increment in proteins (Turkkan *et al.*, 2008), total amino acid (Oluwaniyi *et al.*, 2010), ash (Hosseini *et al.*, 2014), lipid, macro and ultra-trace elements content, especially after grill and roast treatments (Costa *et al.*, 2013). On the other hand, boiling generally causes the

reduction in essential amino acid percentage (Oluwaniyi *et al.*, 2010), a great loss in mineral contents (sodium, potassium, phosphorus and zinc), together with a decrease in vitamins (B_1, A and D) and n-3 PUFA (Hosseini *et al.*, 2014).

A study conducted on kutum roach (*Rutilus frisii kutum*) demonstrated that boiling, frying and microwaving modify the fatty acid composition of fish, whilst baking on the contrary does not induce such nutritional alterations. For what about n-6 PUFA levels in particular, no significant effects of these cooking processes have been observed, except after frying treatments; the least had been found to even increase n-6 PUFA content and then to reduce the n-3/n-6 ratio (Hosseini *et al.*, 2014).

The effects of cooking treatments on the amount and composition of n-3 PUFA seem to be specie-related. For what about EPA and DHA, no degradation effects had been observed during culinary processes in farmed meagre (*Argyrosomus regius*) (Costa *et al.*, 2013), sea bass (*Dicentrarchus labrax*) (Turkkan *et al.*, 2008), trout (*Salmo trutta*), sole (*Lepidopsetta bilineata*) and in herrings (*Clupea harengus*) (Gladyshev *et al.*, 2007), while a particular loss in n-3 PUFA have been observed in cod (*Gadus morhua*) (Schneedorferová *et al.*, 2015). Another multi-species study confirmed the great EPA and DHA loss in cod after boiling treatments (Gladyshev *et al.*, 2007).

No appreciable effects of cooking on n-3 LCPUFA content were also reported in Chinook salmon (*Oncorhynchus tshawytscha*), common carp (*Cyprinus carpio carpio*), lake trout (*Salvelinus namaycush*) or walleye (*Sander vitreus*) from Canada (Neff *et al.*, 2014).

A study on New Zealand King Salmon (*Oncorhynchus tshawytscha*) confirmed the good preservation of n-3 PUFA after different cooking techniques, and highlighted the specific property of the fillets belonging this species in protecting PUFA from degradation (Larsen *et al.*, 2010). A slight reduction in EPA and DHA content was also observed in humpback (*Oncorhynchus gorbuscha*), demonstrating that muscle tissue of species from Salmonidae family are probably protected from heat treatment by the high level of natural antioxidants present in these subjects, as a consequence of an ecological adaptation (Gladyshev *et al.*, 2006).

Among cooking techniques, oven-baking (Hosseini *et al.*, 2014; Schneedorferová *et al.*, 2015), grilling (Ersoy and Ozeren, 2009) and broiling (Neff *et al.*, 2014) resulted the best cooking method for protein, PUFA, minerals and vitamins preservation. Furthermore, baking and broiling are considered a healthy choice also because these techniques generally do not increase the n-6 PUFA content, present in high amounts in the typical western diet (Neff *et al.*, 2014).

Frying and microwaving, on the contrary, are not recommendable methods for cooking fish, due to the considerable loss in n-3 PUFA, which decreases the nutritional value of the food (Weichselbaum *et al.*, 2013). Frying in particular increases the fat content of a meal, through lipid absorption during the procedure (Ersoy and Ozeren, 2009); therefore it increases the total energy of the product (Weichselbaum *et al.*, 2013). Since this cooking treatment causes also deleterious effect on total and essential amino acids content, it should be avoided in order to preserve the nutritional value of the fish meal (Oluwaniyi *et al.*, 2010).

Concerning inorganic contaminants, no losses in As, Hg and MeHg were observed after grilling and roasting, but even an increment in the risk of MeHg ingestion (Costa *et al.*, 2013). This trend was confirmed by Domingo (2014) who reported a notable increment in heavy metals concentrations in sardine, hake and tuna after cooking treatments. For these reasons, Costa *et al.* (2013) suggested an intake of lean fish not higher than two portions per week.

For what about organic pollutants, culinary processes are likely to variably influence the final contamination of the product. Since POPs mainly accumulate in storage lipids, all the procedures that release or remove fats from the food are expected to reduce the total content of the organic contaminants in the product (Domingo, 2014).

A study conducted on the levels of PCBs in fish reported that removing the skin and trimming belly and back fat during the filleting, reduce from the 12 to the 40% of pollutants content of the product (Mozaffarian and Rimm, 2006).

Furthermore, Domingo (2014) measured the variability of POPs concentration among raw and cooked fish products and found out that cooking treatments can either reduce

or increment the levels of organic pollutants in fish, depending on the fish species analysed.

For what about dioxins, cooking treatments reduced pollutants content in sardine, while an increment in dioxins concentration had been measured in hake and tuna. A similar trend was observed for PCBs: reduced concentrations were found in sardine and hake (especially after grilling), while a slight increment in PCBs levels was observed in tuna (Domingo, 2014).

This trend can also be observed for PCDE and other chlorine compounds, whose content in fish, after cooking treatments, varied among fish species: roasting, for example, increases the pollutants concentrations in hake samples, while quite no changes could be noted in tuna samples. Sardine resulted to be the most contaminated species by PCDE and HCB, but notable reductions in their levels were measured in cooked samples, especially in fried and grilled samples (Domingo, 2014).

For what about PAH, instead, no specie-related influences were noticed, but variations in concentration were found among different culinary treatments; for example, the highest levels were observed in fried and roasted samples (Domingo, 2014).

Regarding PFAS, Del Gobbo *et al.* (2008) conducted a monitoring on 18 fish species from Canadian markets and reported that all cooking methods, especially baking, reduced perfluoroctanoid acid (PFOS) concentrations. These findings are not in agreement with the results from the aforementioned study of Domingo, who found no sufficient data to conclude if cooking methods could significantly influence human exposure to PFAS (Domingo, 2014).

For what about food processing techniques, such as canning or freezing, they generally do not modify the proximate composition of a foodstuff, unless the product is previously cooked (Weichselbaum *et al.*, 2013). During the canning process, instead, much of the lipids contained in the fresh fillet are lost and then the nutritional value of the product decreases (EFSA, 2014). Comparing canned with fresh products, the firsts are generally richer in oleic, arachidonic or linoleic acids, derived from oils used for conservation (Sirot *et al.*, 2012).

The consumption of commercial fried fish products has been associated with the increment in cardiovascular risks reasonably related to the unfavourable balance between n-3 LCPUFA content vs the aforementioned fatty acids. In addition, the preparation of such products generally involves the use of white-meat fish, low in n-3 LCPUFA, and the reutilisation of the frying oil for multiple cycles, which adds oxidative and deteriorative agents to the meal (Mozaffarian and Rimm, 2006).

2. Objectives of the experiment

It is well known that seafood provides beneficial nutrients and is a healthier alternative to almost any kind of meat (FAO, 2014). As reported by a recent report of EFSA, fish is an optimal source of proteins with high biological value, together with other essential nutrients, such as vitamins A and D, selenium, calcium, and iodine (EFSA, 2014). Scientific research is increasingly oriented to the identification of the single nutrients of seafood and to the quantification of their beneficial properties. There is emerging evidence stating that the nutritional impact of fish consumption is higher than the sum of the benefits derived from the individual intake of nutrients; although it is well known that most of nutritional benefits on neurodevelopment and on cardiovascular system derive from n-3 LCPUFA assumption (FAO/WHO, 2011).

At the same time, scientific investigations demonstrated the unavoidable presence of organic and inorganic contaminants in fish and shellfish. Mercury and persistent organic pollutants (POPs), especially dioxins and polychlorinated biphenyls (PCBs) were the most concerning contaminants, alerting for their toxicity (FAO/WHO, 2011).

The debate regarding the benefits and risks of fish consumption resulted in doubts and confusion in how much, or even if, it should be consumed, especially for sensible groups of population as pregnant or nursing women, infants and young children (EFSA, 2014).

A general conclusion of the risk/benefit assessments recently conducted, suggests that benefits of fish consumption far outweigh any risk of contamination, except for few extreme examples. Several experts even promoted fish consumption emphasizing the aforementioned fish health benefits, and highlighting that an insufficient fish intake could itself represent a risk factor for the most sensible categories of consumers (EFSA, 2014).

However, several authors strongly support the need to extend the monitoring to emerging chemical pollutants other than MeHg, dioxins and PCBs, and declare that, avoiding the inclusion of other emerging contaminants, reliable assessments cannot be obtained (Stern, 2007; Domingo, 2014). Moreover, JECFA Committee (FAO/WHO, 2011) asserted that the contribution on cardioprotective and neuroprotective effects by single component of seafood should be investigated, with the aim to improve the reliability of benefits assessment of fish consumption. In conclusion, the Committee asked Member States for representative data with the aim of giving useful and clear advices to consumers, and to conduce them to the healthiest dietary choices (FAO/WHO, 2011).

The object of this thesis is to contribute to the assessment on the health risks and benefits of seafood consumption, by means of new and innovative analytical methods based on ultra-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). This work is divided in two sections: the first is the result of a partnership between two laboratories: the Laboratorio di Chimica Analitica e Bio-Agroalimentare (CABA-Lab) in the Department of Veterinary Medical Sciences of the University of Bologna (Italy) and the laboratory of the Department of Food Technology of the Instituto de Investigaciones Marinas (IIM) of Vigo, Pontevedra (Spain).

More in particular, an UHPLC-MS/MS method for the quantification of glycine betaine (GB) in *Tapes philippinarum* was developed and validated, taking the 2002/657 European Decision as guideline. This compound is a methyl-derivative of glycine and has two main physiologic roles: protect cells under stress, acting as organic osmolyte, and provide methyl groups in several vital biochemical pathways (Craig, 2004). Human GB supply is almost entirely from the diet and shellfish represent the richest animal sources (de Zwart *et al.*, 2003).

UHPLC-MS/MS is a technology of increasing interest for qualitative and quantitative assays in biomarkers analysis, although there are no agreed validation approaches in the field of endogenous compounds. Therefore, particular expedients have been

applied to achieve the validation of this analytical method, being GB an endogenous osmolyte present in very high concentrations in the target matrix.

The second part of the research has been carried out in CABA-Lab using a previously developed and validated UHPLC-MS/MS method for the quantification of the two most representative perfluoralkyl contaminants, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), in seven fish species (*Dicentrarchus labrax, Merluccius merluccius, Mugil cephalus, Oblada melanura, Pleuronectes platessa, Scomber scombrus* and *Sparus aurata*).

These emerging pollutants are anthropogenic substances characterised by chemical and thermal stability, which made them largely employed for industrial and consumer application during last sixty years. These properties made them global and persistent pollutants, with a declared toxicity on human body in terms of carcinogenicity, hepatotoxicity and immunotoxicity (EFSA, 2008).

As a result of its biomagnification properties, PFOS has been included in Annex B of the 2009 Stockholm Convention and its production has been restricted (Recommendation 161/2010/UE).

The good performances of the method allowed the fulfilment of a preliminary monitoring on seven different fish species from Italian markets; then the daily contribution to PFOS and PFOA assumption through seafood intake, has been calculated and the data compared to the corresponding Tolerable Daily Intakes.

Finally, a mono-specie monitoring on the levels of contamination of these pollutants was carried out in 140 sea bass samples, collected in different sites in the Mediterranean area, in order to characterise the main factors influencing fish contamination.

For what in our knowledge, no other mono-specie monitoring on the presence of PFAS was previously conducted on such a large number of fish of the same species, from wild or farmed sources.

3. Glycine betaine in seafood

The substances called “betaines” are small natural compounds produced by a wide variety of organisms (bacteria, plants, invertebrates and mammals). These molecules chemically derive from the full methylation of amino acids’ nitrogen atom, which forms a cationic functional group that cannot be deprotonated (de Zwart *et al.*, 2003; Chary *et al.*, 2012). This feature makes them highly polar but neutral zwitterionic compounds, able to retain their positive charge also at high pH (Lever and Slow, 2010).
In particular, glycine betaine (GB) is the methyl derivative of glycine (N,N,N-trimethylglycine) and was first isolated in the late nineteenth century from the juice of sugar beets (Beta vulgaris), hence the name “betaine” (de Zwart *et al.*, 2003; Craig, 2004). In the figure below the chemical structure is shown, with the positive amino group and the negative carboxylic group in evidence (Figure 5).

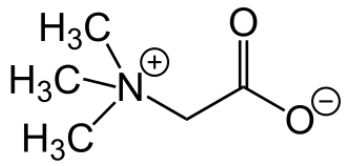


Figure 5: Glycine betaine (GB) chemical structure in the zwitterionic form

The sugar beet industry is the main source of commercial preparations of GB (Lever and Slow, 2010), available in European markets since 1982 (EFSA, 2005b). In USA it was commercialised since 1960’s, with recommended doses up to 1.5 g/day (EFSA, 2005b).
GB extraction from molasses occurs by water-based chromatography separation and following crystallisation (Craig, 2004), which can produce two chemical forms: anhydrous and monohydrate, both with the same metabolic and nutritional properties. GB preparations are highly hygroscopic and heat resistant (up to 245°C)

and their stability can last over 3 years at room temperature in an adequate packaging (EFSA, 2005b).

At the same time GB can also be naturally found in a variety of animal species, plants and microorganisms (de Zwart *et al.*, 2003; Craig, 2004).

GB is the only betaine with a demonstrated metabolic function in mammalians, together with carnitine (Lever and Slow, 2010), and it has two main physiologic roles in human body: protect cells under stress acting as organic osmolyte and provide methyl groups in several vital biochemical pathways (Craig, 2004).

3.1 Glycine betaine in diet

GB is abundant in plant foods such as cereals (especially wheat) and other vegetables belonging to the beet family, such as spinach, chard and beetroot (Lever and Slow, 2010; Hefni *et al.*, 2015), and the richest animal source is represented by shellfish. The main role for GB in plants and microorganisms is that to increase the water retention of cells and to protect intracellular enzymes from inactivation by osmotic or thermal stress. Exposure to drought, high salinity or extreme temperatures induces GB synthesis in mitochondria and its following accumulation in cells (Craig, 2004). Since it is also a major plant osmolyte, its content is variable and generally related to osmotic stress conditions during crops growth (Craig, 2004; Hefni *et al.*, 2015).

A recommended daily intake for GB has not been established yet (Ueland, 2011), but an average dietary intake was calculated to be almost 1 g per day, up to 2,5 g in case of diets high in cereals and seafood (Craig, 2004). More recent data reported much lower daily intakes, between 100 and 300 mg/day (Ueland, 2011), while some authors even consider a GB intake of 800 mg difficult to reach in a long-term acceptable diet (Lever and Slow, 2010). In general, high plasma levels of GB have been associated to the intake of foods rich in complex carbohydrates and fibre (such as high-fibre bread), while they have been negatively associated to Western diets characterized by high intakes of meat, sugar and fats (Ueland, 2011).

At the beginning of the Century, three large surveys of GB content in foodstuffs have been carried out in United States (Zeisel *et al.*, 2003) and New Zealand (de Zwart *et al.*; 2003 and Slow *et al*; 2005). In USA, the highest GB concentration had been found in wheat bran (15056 mg/kg), wheat germ (13949 mg/kg), cooked spinach (7255 mg/kg), pretzels (2659 mg/kg), shrimp (2459 mg/kg) and canned beets (3336 mg/kg) (Zeisel *et al.*, 2003). In New Zealand, high levels of GB were measured in foods containing grains or flour: concentrations up to 790 mg/kg were found in bread, 7200 mg/kg in cereal products and 1400 in pasta and crackers (Slow *et al.,* 2005). Data of this survey were consistent with those collected by de Zwart *et al.* in 2003, who reported 730 mg/kg in

flour and 820 mg/kg in pasta. Significant concentrations were found also in some vegetables (beetroot with 750 mg/kg, silverbeet with 910 mg/kg and spinach with 740 mg/kg) and shellfish: clams above all, with 2500 mg/kg and mussel with 1630 mg/kg. Finfish as cod, salmon and tuna, shown levels much lower, below 30 mg/kg, with an exception for Monkfish that was found with 500 mg/Kg of GB (de Zwart *et al.*, 2003; Slow *et al.*, 2005).

The amount of GB introduced in the diet can vary significantly, according to food source and to the type of cooking methods. Due to its osmoprotectant properties, GB content in food could vary depending on the stress level of the organism during its growth, as a response to environmental conditions.

Moreover, being a highly water-soluble compound, huge losses in GB levels are expected after cooking processes, especially boiling, (de Zwart *et al.*, 2003), since it is not bound to any matrix component which would prevent its leaching into water (Ross *et al.*, 2014). On the contrary, baking treatments are not expected to low the GB content of the meal, since the internal temperature (generally below 200°C) do not allow GB degradation (EFSA, 2005b). Cooking processes which do not involve the loss of water can increase GB content, since mechanisms of synthesis ex-novo can occur during heating processes; this phenomenon was reported by de Zwart *et al.*, (2003) during microwave cooking of spinach (Ross *et al.*, 2014). Ross *et al.* (2014) suggested to further investigate how cooking processes may influence GB content in food products, in order to better estimate GB intake for consumers. The investigations should be extended to other, less studied matrices, such as seafood.

As reported above, the main supply of GB in the normal western diet would derive from flour products (e.g. bread and pasta) and only a small contribution from shellfish, if not consumed regularly or in large amounts. As reported by de Zwart *et al. (2003)* significant increments in GB intake can be achieved through changes in dietary habits, even up to 500 mg/day; thus, for western countries consumers, GB intake would be widely enhanced by increasing shellfish consumption (de Zwart *et al.*, 2003).

3.2 Metabolism of glycine betaine

In humans, GB intake could derive directly from the diet or from endogenous synthesis through the oxidation of choline (de Zwart *et al.*, 2003; Craig, 2004) by a two-step enzymatic processes, involving choline dehydrogenase (or choline oxidase, CO) and betaine aldehyde dehydrogenase (BAD) (Lever and Slow, 2010, Katayama *et al.*, 2013). In mammalians these two enzymes are exclusively expressed in liver mitochondria and in kidney tissues (Lever and Slow, 2010).

After ingestion, GB is rapidly adsorbed by duodenum enterocytes and enters in portal circulation (Craig, 2004; EFSA, 2005b). Once in the bloodstream it is filtered by the kidney, reabsorbed and carried to the liver, where its catabolism takes place, or stored in tissues (EFSA, 2005b; Cholewa *et al.,* 2014). Plasma levels of GB increase rapidly after ingestion, due to its rapid absorption (t½ 0.3 hours) and distribution (t½ 0.6 hours). Blood concentration seems to be homeostatically controlled, at levels between 20 and 60 µmol/L. This compound is catabolised rather than excreted (Craig, 2004), with elimination half-life of about 14 hours (EFSA, 2005b). However, elevated urinary excretion have been observed in renal or diabetes patients (Craig, 2004).

GB is a vital methylating agent in primary biological processes through the action of the enzyme "betaine homocysteine methyl transferase" (BHMT) GB provides methyl groups for the remethylation of homocysteine to methionine (Craig, 2004), which would contribute to nucleic acid, protein and lipid synthesis (EFSA, 2005b). This reaction finally converts GB in dimethylglycine (DMG) (Craig, 2004). BHMT is a zinc metalloenzyme especially present in specific human tissue, such as liver, kidney and optic lens; GB catabolism mainly occurs in cytoplasm of hepatic and renal cells (Craig, 2004; Ueland, 2011; Hefni *et al.*, 2015).

The metabolic conversion of homocysteine in methionine is also sustained by folate-dependent enzymes (Holm *et al.*, 2003), although the 50% of the methylation capacity of the liver is carried out by BHMT (Lever and Slow, 2010). Therefore, GB formation closely interconnect the metabolic function of choline to folate-mediated one carbon-

metabolism (Ueland, 2011) in terms of regulation of homocysteine and methionine levels in human body (Holm *et al.*, 2003; Craig, 2004).

In Figure 6 it is graphically represented GB metabolic process.

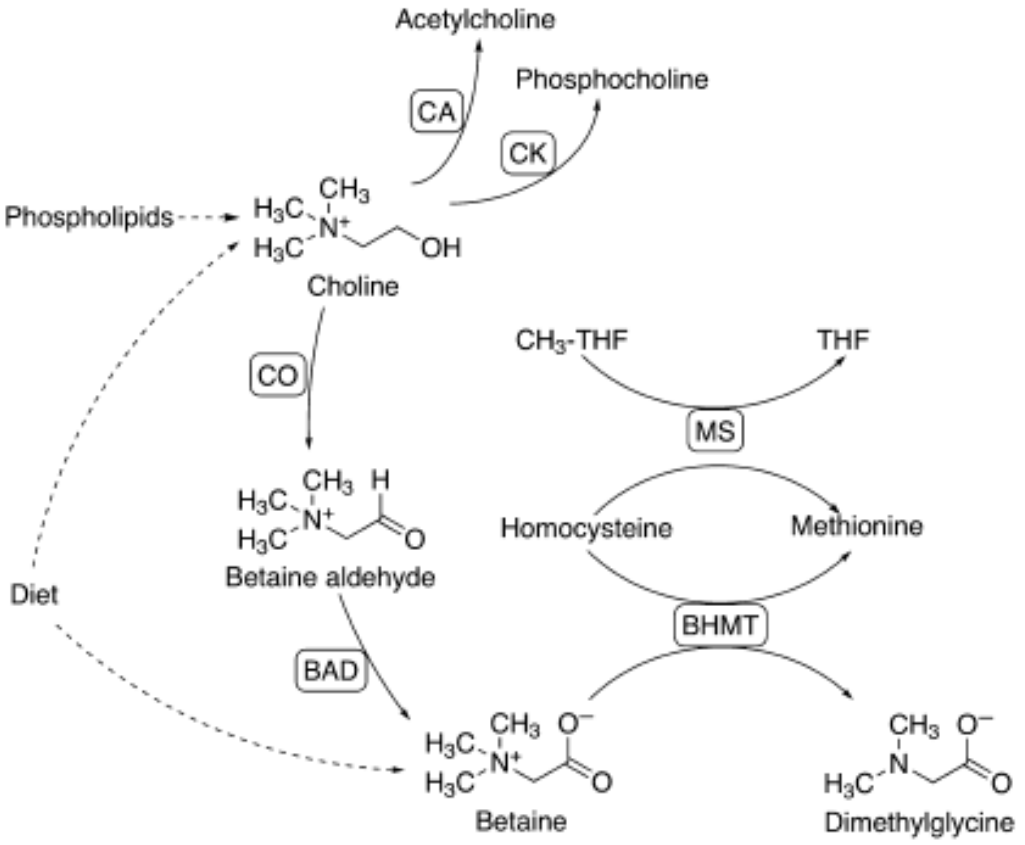


Figure 6: Metabolism of glycine betaine (betaine); in the scheme are pointed out the enzymes involved in the metabolic processes: betaine aldehyde dehydrogenase (BAD), betaine-homocysteine methyltransferase (BHMT), choline acetyltransferase (CA); choline kinase (CK), choline oxidase (CO) and methionine synthase (MS). Moreover the Figure shows the connection with tetrahydrofolate (THF) metabolism (Katayama et al., 2013).

Despite methionine, folic acid, vitamins B_6 and B_{12} are the main nutrients involved in plasma homocysteine regulation (EFSA, 2005b), in case of folate deficiency, BHMT pathway compensates the lower methylation activity (Lever and Slow, 2010); in fact, GB holds up homocysteine methylation also in case of impaired B-vitamin conditions

(Ueland, 2011). This means that GB plays an important role in the maintenance of methionine concentration (Lever and Slow, 2010).

Moreover, BHMT activity regulates cell turgor, maintaining higher GB concentrations in tissue than in plasma (Lever and Slow, 2010), even at orders of magnitudes (Ueland, 2011). In fact the cellular content of GB is regulated by the enzyme BHMT activation in the liver and the kidney (Craig, 2004), where exceptional GB concentrations (generally higher than 100 mM) can be found (Lever and Slow, 2010). Under hypertonic conditions, the activity of renal and hepatic BHMT is downregulated to conserve GB to be used as osmolyte and, under hypotonic conditions, BHMT is upregulated to reduce GB concentration and maintain optimal cell turgor (Craig, 2004).

Definitely BHMT controls the partitioning of GB and regulates its use as a methyl donor or as an osmoprotectant (Ueland, 2011).

BHMT activity is subjected to feedback inhibition by DMG and influenced by hormonal activity (including corticosteroids, insulin, thyroid and sexual hormones), while BHMT gene expression is increased by methyl donor availability (Lever and Slow, 2010).

Several biomarkers related to this metabolic pathway have important roles in human physiology and in the pathogenesis of chronic diseases, such as cancer or cardiovascular diseases (Midttun *et al.*, 2013).

3.3 Glycine betaine in health and disease

3.3.1 Cardiovascular diseases

Elevated plasma homocysteine concentration, a pathologic status called "homocysteinemia", is a consequence of alterations in hepatic transmethylation via methionine-cycle, caused by genetic defects or wrong dietary habits. A severe homocysteinemia, caused by inborn errors of metabolism, is a clinical status called homocysteinuria (Craig, 2004), which can involve disorders in homocysteine degradation or remethylation (EFSA, 2005b). These pathologic conditions may contribute to development of cardiovascular diseases, impairment of hepatic lipid metabolism, renal function and DNA methylation (Bruce *et al.*, 2010). In fact, homocysteinemia have been correlated to vascular illnesses, stroke, birth defects in infants and dementia and Alzheimer's disease in elderly people (Slow *et al.*, 2005); on the other hand, homocysteinuria is associated with skeletal abnormalities, thromboembolism, atherosclerosis, mental retardation, seizure and psychiatric disturbances (Craig, 2004).

Since plasma total homocysteine is a well-attested vascular risk factor (Lever and Slow, 2010), the fact that GB lowers serum homocysteine (Craig, 2004) and increases serum methionine (Hefni *et al.*, 2015) justifies the therapeutic role of GB patients affected by homocystinuria (Holm *et al.*, 2003; Slow *et al.*, 2005) and explains its important role on human health. In fact, in 1966 this compound was recognised by the Food and Drug Administration as an orphan drug, effective for the treatment of genetic homocysteinuria, with doses up to 20 g/day (EFSA, 2005b). GB has been used to treat patients affected by homocysteinemia (Cholewa *et al.,* 2014) and the symptomatology can be considerably reduced with a daily intake of at least 6 g of GB (Craig, 2004; Lever and Slow, 2010; Ueland, 2011). Treatment with such doses of GB were found to be effective also in patients affected by homocysteinuria (Ueland, 2011).

Since GB is highly hydrosoluble and not protein-bound, its bioavailability does not change whether it derives from food or from dietary supplement (Lever and Slow, 2010). Slow *et al.* (2005) asserted that plasma homocysteine levels can be reduced even just with the increased consumption of foods high in GB. In fact Craig, in its in-depth review, reported that "a betaine-rich diet might lower cardiovascular disease risk in healthy humans" (Craig, 2004).

During the years, the homocysteine-lowering effect of GB intake induced many investigations aimed to deep the relationship between GB and risk for cardiovascular diseases (Ueland, 2011). A study on healthy Greek population showed the correlation between high dietary intake of GB with low plasma levels of inflammatory markers, such as C-reactive protein, interleukine-6 and tumour necrosis factor, known to have a key role in atherogenesis. This hints the healthy role of GB against vascular diseases for its anti-inflammatory and anti-atherogenic effects (Lever and Slow, 2010; Ueland, 2011). GB was also found to stimulate the excretion of cholesterol through biliary secretions, which is supposed to be another mechanisms supporting the anti-atherosclerotic action (Craig, 2004).

Despite this, some authors abstained from declaring the effective relationship between GB intake and cardiovascular diseases in healthy people treated with short or medium-term GB supplementation, due to a lack of evidence (Lever and Slow, 2010). For example, scientific studies in humans observed an increment of low density lipoprotein (LDL) cholesterol associated with high doses of GB (Ueland, 2011). In addition, confusion arises also because of GB intestinal transformation in trimethylamine increases the blood concentration of its metabolite (trimethylamine-N-oxide) which is thought to be involved in atherosclerotic plaques formation (Steuer *et al.*, 2016). Even though, the long-term treatment of population affected by GB loss promoting pathologies should be investigated, since it is expected that they benefit from GB supplementation (Lever and Slow, 2010).

In 2011 the Panel on dietetic Products, Nutrition and Allergies (NDA) of European Food Safety Agency (EFSA) published a scientific opinion about the effects of GB on cardiovascular system, concluding that there is a significant relationship between GB

intake and the maintenance of a physiologic homocysteine metabolism, in general population. In fact, a significant and dose-related decrease in homocysteine plasma concentrations was reported, and the NDA Panel considers this effect on homocysteine metabolism beneficial for human health, achievable with the daily intake of at least 1.5 g of GB (EFSA, 2011a).

The aforementioned increment in LDL cholesterol was observed also by the Panel, but it was concluded that, for doses below 4 g per day, this unpleasant effect could be considered insignificant (EFSA, 2011a).

3.3.2 Osmoregulation

When GB is not catabolized by the aforementioned BHMT pathway, it acts as organic osmolyte. Cells reply to external osmotic stress accumulating inorganic ions (sodium, chloride and potassium) and organic osmolytes (Craig, 2004). An insufficient cell volume regulation can cause apoptosis (Lever and Slow, 2010), furthermore the balance of cell hydration state is essential to maintain its physiological functions, such as protein turnover, metabolic pathways, membrane transport, bile excretion, pH control and gene expression (Häussinger, 1996).

GB is the most efficient organic osmoprotectant (Singh *et al.*, 2011) and does not affect cellular functions, being highly compatible with enzyme and hormones activity (Craig, 2004). The accumulation of inorganic ions, can instead destabilize protein's folding; therefore organic osmolytes, replacing inorganic salts, play an important role in preserving the structural integrity of membranes and other cellular components in various animal tissues (Chary *et al.*, 2012), such as kidney, liver, intestine, brain and skin (Ueland, 2011). GB also prevents protein aggregation and corrects mutant protein defects (Singh *et al.*, 2011): it stabilizes and protects proteins from the denaturing effect of urea, for this reason it is particularly active in kidney (Ueland, 2011). In renal medulla, where urinary concentration processes take place, GB protects cells from

osmotic damages produced by high extracellular osmolarity by electrolytes and urea; it also prevents myosin structural changes due to urea activity (Craig, 2004; Ueland, 2011).

In addition, GB helps to maintain albumin and haemoglobin solvation in blood, and modulates ATPase activity on erythrocyte membrane in regulating cellular volume.

In liver GB preserves the immune function of Kupffer cells from osmotic stress due to tumor necrosis factor release and phagocytosis activities, and regulates water balance and nutrients movements across the intestinal epithelium (Craig, 2004).

3.3.3 Liver diseases

GB plays also an important role in preventing or reducing lipid accumulation; it is indeed considered a lipotropic factor. This property is probably related to the presence of electrophilic methyl groups, which contrast the reductive and oxidative damages occurring during pathological states (Craig, 2004).

Liver methylation is important for the physiologic synthesis and secretion of very low density lipoproteins (VLDL) and the minimisation of lipid accumulation in the liver. In addition GB is one of the major liver osmoprotective agent (Lever and Slow, 2010).

In many cases hepatic steatosis is a consequence of obesity, wrong dietary habits, diabetes and alcohol consumption (Craig, 2004). As alcohol inhibits methionine synthase, alcoholic beverage consumption requires an increment of GB intake to maintain methylation activity (Lever and Slow, 2010). GB promotes the synthesis of S-adenosine and protects human liver from ethanol-induced fatty infiltration (EFSA, 2005b), especially in case of hepatic steatosis (Ueland, 2011),

Anyway the most common liver dysfunctionality is the non-alcoholic fatty liver disease, which is typically associated to metabolic syndrome; its incidence seems to be modulated by oestrogen activity, being less frequent in premenopausal women compared to men and postmenopausal women (Ueland, 2011).

Experimental results obtained by studies on animal models promote a possible role of GB supplementation in the treatment of liver steatosis, considering the stimulation of methionine synthesis via BHMT pathway at the base of the beneficial effect (Ueland, 2011). A one-year clinical study on humans demonstrated the efficacy of the treatment of non-alcoholic steatohepatitis with 20 g of GB per day, which reported significant biochemical and histological improvement in patients (Lever and Slow, 2010).

GB supplementation has also been recommended in case of liver disease caused by xenobiotics or bile salts (Lever and Slow, 2010) and it is that improves liver function in people affected by diabetes (Craig, 2004). The supplemental doses recommended for the treatment of obesity and obesity-related diseases are around 2 g per day, which would be cheap and easy to assume (Lever and Slow, 2010).

Other subjects most likely to benefit from GB supplementation are those with increased GB excretion, such as patients with diabetes mellitus or metabolic syndrome, or people suffering from chronical renal failure or lipid disorders (Lever and Slow, 2010). Low plasma GB levels have been found in patients with renal disease probably due to increased urinary excretion (Slow *et al.*, 2005).

3.3.4 Methylation reactions

It is important to consider that metabolic pathways for lipid storage and secretion from the liver consume methyl groups that are no longer available for other methylation reactions (Craig, 2004). Reducing methyl group availability may influence gene transcription and modify genomic stability and imprinting (Ueland, 2011). Disturbances in one-carbon metabolism can lead to incomplete DNA methylation and genetic instability, which can promote senescence and colorectal cancer (Bae *et al.*, 2014). In fact, a diet rich in methylating agents (such as GB) can protect from colorectal cancer (Bae *et al.*, 2014) and possibly reduces risk of breast cancer (Lever and Slow, 2010). This could be related to the provision of methyl groups, important for

epigenetic control of gene function (Lever and Slow, 2010), but also due to the GB ability in reducing hyperosmotic stress that can lead to chronic inflammation, a well-known tumour risk factor (Bae *et al.*, 2014).

This aspect differentiates BHMT from folate methylation pathway, since some authors suggest an increased prostate cancer incidence related to folate (Ueland, 2011) and vitamin B12 supplementation. This trend is not well understood yet, but could be explained by the attitude of these two pathways in increasing the synthesis of the DNA bases (Lever and Slow, 2010).

At the end, interesting conclusions can be drawn on the importance that GB has in human development, from all stages of gestation to early infancy; hence maternal nutrition has a main rule in guarantee the sufficient supply of methyl groups. First of all, low GB intake during pregnancy may induce health complications, which are probably related to high plasma homocysteine (Lever and Slow, 2010). The role of GB during pregnancy gains importance when others methylating agents, such as folate and methionine, are limited (Ueland, 2011). Some studies suggested that, in late pregnancy, BHMT pathway results even more important than folate-dependent methionine metabolism (Lever and Slow, 2010).

In addition, since choline catabolism to GB is irreversible, an adequate dietary intake of GB can ensure choline supply for phospholipid and neurotransmitter synthesis to guarantee an optimal foetal and child neurodevelopment. Furthermore, during embryogenesis the amount of methyl groups is essential for maintaining the methylation of imprinted genes and for a normal embryo development (Lever and Slow, 2010). In fact, high GB intake is correlated to reduced risk in neural tube defects (Ueland, 2011). Thus, the maternal dietary intake of methyl groups during gestation must be optimal, in order to meet the foetal requirements of the developing progeny (Lever and Slow, 2010).

3.3.5 Further applications

3.3.5.1 Human performances and body composition

How GB metabolism affects body fat is already unclear (Lever and Slow, 2010) but its effect in reducing lipogenesis is strongly sustained (Cholewa *et al.,* 2014).Moreover, it seems that an increased methionine availability may enhance protein production and promote lean mass growth (Lever and Slow, 2010). The effect of GB supplementation on humans, in terms of increasing lean mass and reducing fat mass, was demonstrated for the first time by Cholewa *et al.* (2014). The authors justify this mechanism with the expression of an osmoregulated GB transporter in liver and skeletal muscle, which works to maintain GB tissue levels higher than those in plasma.

In the liver, hepatocytes hyper-hydration promotes protein synthesis and hypertrophy, since cellular swelling induces gene transcription and proteolysis inhibition; a similar procedure is supposed to occur in skeletal muscle (Cholewa *et al.*, 2014).

In addition, GB stimulates growth hormone secretion by the hypothalamus, promoting gene transcription of "growth hormone releasing hormone". At the same time, GB can modify body composition also enhancing the hepatocyte secretion of insulin-like growth factor-1 (Cholewa *et al.*, 2014) and improving insulin sensitivity (Pekkinen *et al.*, 2013).

Many authors have also reported that GB supply of methyl groups can be used for the biosynthesis of carnitine and creatine. In fact, elevated muscle carnitine levels were measured after GB supplementation. The synthesis of creatine may be a key factor to explain the positive influence of GB on human athletic performances, together with its capacity in reducing cellular acidosis and enhancing glycolytic metabolism. Furthermore, the osmoprotectant effects of GB, optimizing the cellular state and increasing protein stability, ultimately improve glycolytic flux and muscle oxygen consumption (Cholewa *et al.*, 2014). GB promotes high-intensity fatiguing aerobic performances in general population and improves sense of well-being, reduced fatigue, greater body strength and endurance during recovery in poliomyelitic patients.

In addition, GB enhances cardiac function in people affected by cardiac decompensation and congestive heart failure (Craig, 2004).
Finally, since elevated GB concentration were found in sweat, any people, after a profuse sweating, is expected to need GB supplementation, due to the huge loss. Since studies about GB supplementation conducted on athletes resulted inconsistent, further in-depth analysis have been suggested in this field (Lever and Slow, 2010).

3.3.5.2 Animal breeding

For all the beneficial properties previously described, GB found its primary application in animal breeding, as a feed supplement (Craig, 2004). The mean purpose of world's GB production is in animal feed, with the aim to increase lean muscle and lower lipid content of meat (Lever and Slow, 2010). This compound can alter nutrient portioning enhancing carcass protein deposition and reducing carcass and visceral fat (EFSA, 2005b). Than GB promotes body growth, resulting in improved efficiency of food utilization in pigs and chicks (Craig, 2004).
Furthermore, as hinted by Lever and Slow (2010), the GB osmotic activity in intestinal cells probably affects nutrient digestibility and partitioning. In poultry breeding GB is also used to protect chick intestinal cells from coccidia infection, which affects gut ionic balance. Avoiding the detrimental effects related to this disease, including maldigestion, malabsorption and dehydration, is the way in which GB improves poultry growth performance (Craig, 2004).
Another application of this substance is in aquaculture: GB is administered to farmed fish with the aim to prevent the osmotic stress during changes in water salinity (Craig, 2004).

3.3.5.3 Clinical diagnosis

GB can be efficiently used as dietary biomarker to draw people dietary profile, since the endogenous synthesis is scarce in mammals and a dose-dependent increment in

blood levels can be measured after ingestion (Lenky *et al.*, 2012). Hanhineva *et al.* (2015) declared that GB is an interesting biomarker for the identification of rye intake and its impact on human metabolism.

On the other hand, it is becoming increasingly obvious that this compound plays an important role in human health and is essential for normal body function; consequently, disturbances in its supply and metabolism would lead to pathological consequences. Therefore, measuring GB and its metabolites in humans, by means of clinical laboratory assays, may be useful to draw new strategies for diseases prevention (Lever and Slow, 2010; Ueland, 2011). For example, in people suffering for vascular diseases, the measurement of GB plasma and urine concentrations can predict secondary cardiovascular events (McEntyre, *et al.*, 2014; Ocque *et al.*, 2015; Steuer *et al.*, 2016). Plasma levels of GB in healthy people are rather stable, usually higher in men than in woman, but in general highly individual. They are slightly influenced by osmotic stress, but rather by liver GB concentrations, which are dose-related to dietary intakes (Lever and Slow, 2010).

Low levels of GB in human plasma have been associated to lipid disorders, the metabolic syndrome and diabetes mellitus (Kirsh *et al.*, 2010). In fact, GB concentrations in plasma were found to be negatively correlated to markers of obesity (body mass index, percentage of body fat and waist circumference). Moreover, human scientific investigations observed elevated plasma lipids related to low levels of GB, which suggest a relationship between GB deficiency and lipid abnormalities (such as dyslipidemia). In fact, GB affects body portioning of lipids, in particular it regulates the excretion of triglycerides. This probably explains the inverse relationship found between plasma GB and lipid-related markers (triglycerides, LDL cholesterol and apolipoprotein B) highlighted by several authors, supporting the correlation between low plasma GB and increased vascular risk (Lever and Slow, 2010).

Urinary GB excretion is very poor, and successfully measured in relation to creatine, with 2-35 mmol of GB excreted per mole of creatinine. This ratio is not affected neither by plasma osmolarity or by food intake and it could represent an useful tool to identify patients who are potentially GB deficient or differentiate from those with folate or B_{12}

deficiency. An abnormal GB excretion with urine was found in diabetes and renal patients, together with those suffering from lipid disorders and metabolic syndrome; These population groups are likely to benefit from GB supplementation. An elevated urine excretion can also be promoted by the intake of food containing proline (present in large amounts in orange juice and in legume sprouts); therefore measuring simultaneously GB and proline during diagnostic test is recommendable to monitor this biological effect (Lever and Slow, 2010).

The "methionine load test" is considered a test for betaine sufficiency, and it measures the increment of plasma homocysteine levels after the ingestion of a load of methionine. Tissue stock of GB is the major source of methyl groups, therefore its supply is verified by post-methionine increment in homocysteine levels (Lever and Slow, 2010). Olthof *et al.* reported that 6 g per day of GB during a 6 weeks supplementation in healthy subjects can reduce the increment in homocysteine plasma levels up to 40% compared to no supplemented (control) patients (Olthof *et al.*, 2003).

In recent years, there had been a growing interest in understanding the relevance of GB and its metabolites in clinical field (Zhao *et al.*, 2015). Recently, these compounds have been largely investigated by metabolomic profiling (Schicho *et al.*, 2012; Skappak *et al.*, 2013; Pekkinen *et al.*, 2013; Midttun *et al.*, 2013; Katayama *et al.*, 2013; Xie *et al.*, 2015; Hanhineva *et al.*, 2015; Ji *et al.*, 2015; Lee *et al.*, 2015) to quantitatively measure the alterations of these indicators during pathological changes (Xie *et al.*, 2015).

Since GB deficiency is easily treatable with adequate supplementations, its detection in urine is clinically useful; anyway some authors suggest to extend the investigations to other useful biological matrices, such as sweat.

Some authors sustain that the request in GB-related tests is likely to grow in the near future (Lever and Slow, 2010).

3.4 Toxicological studies

In 2005 EFSA, on a request of the European Commission, published a scientific opinion on the use of GB an a novel food for use in beverages and dairy, confectionary and cereal products. In this occasion, the Scientific Panel on Dietetic Products, Nutrition and Allergies evaluated the evident safety of betaine confirmed by animal and human study, but found that certain aspects need to be clarified. The absence of available data on reproduction and developmental toxicity, chronic toxicity and carcinogenicity on animal models prevent the set of a no observed adverse effect level (NOAEL) and, as a consequence, an acceptable daily intake could not be established (EFSA, 2005b).

Subacute and subchronic toxicity studies on animal models demonstrated that liver is the main target organ in rats treated with up to 4.4 g/kg b.w. doses of GB: hepatomegaly, cytological lesions in hepatocyte, followed by increased serum levels of liver enzymes, occurred; symptoms resulted largely reversible. Moreover, no mutagenic effects have been observed after somministration, demonstrating the non-genotoxicity of this substance (EFSA, 2005b). From studies conducted on rats, GB intake was established to be safe at daily somministration between 9 and 15 g (Craig, 2004).

In addition, a human clinical assay reported the efficacy of the treatment of non-alcoholic steatohepatitis with 20 g of GB per day. This one-year study demonstrated the safety of this compound and the significant biochemical and histological improvement in treated patients (Lever and Slow, 2010).

Longer-term animal studies on GB supplementation claimed that prolonged treatments are unlikely to be harmful, but even beneficial against obesity and metabolic syndrome (Lever and Slow, 2010).

The general conclusion is that GB is well tolerated at daily intake up to 30 g, but additional animal studies are needed, in order to establish NOAEL values (EFSA, 2005b).

3.5 Methods of analysis of glycine betaine: state of the art

Glycine betaine (GB) is a zwitterionic compound, composed by an inner salt within the quaternary ammonium group, permanently positively-charged, and the carboxyl group, negatively charged (Wood *et al.*, 2002; Chary *et al.*, 2012). This chemical structure makes GB a very polar molecule with a strong water solubility. At the same time, it is scarcely solubile in most organic solvents so it is not easily extracted from biological samples (Lever and Slow, 2010).

Measuring GB concentrations has always been a challenging issue for the analysts due to its peculiar structure (Lever and Slow, 2010; Li *et al.*, 2010). At first, the analysis of GB and related compounds were performed by qualitative or semi-quantitative colorimetric tests (Dragendorff's reagent) applied on planar chromatography (Li *et al.*, 2010). Thin layer chromatography on silica or alumina plates exploited the partition between an hydrophilic solid phase and a more hydrophobic mobile phase containing organic cations (Storer *et al.*, 2006). However, these methods shown limited sensitivity, selectivity of detection and analytical accuracy, then the absolute concentrations of GB in biological tissues were difficult to calculate. Another option was the pyrolytic dealkylation which allowed its detection by gas chromatography in its dealkylated forms. However, this method tends to overestimate levels of tissue betaines, lacking of sufficient specificity (Li *et al.*, 2010).

Other recent strategies to measure GB concentration exploited the low solubility of quaternary ammonium tri-iodides or the action of betaine-homocysteine methyltransferase in enzymatic essays (Lever and Slow, 2010). The use of radioenzymatic assays and high-performance liquid chromatography (HPLC) associated to electrochemical detection (Zhao *et al.*, 2015) have also been reported. The first was found to be cumbersome and variable in recovery, while the second needed complex derivatization and extraction steps (Holm *et al.*, 2003).

Despite the absorbance spectrum of GB does not show any peak in the visible, or near-UV regions, but only a weak signal close to 200 nm (Lever and Slow, 2010; Chary *et al.*, 2012), HPLC coupled to ultraviolet detector have been largely used. This technique demonstrated a sufficient sensitivity for measuring GB in matrices at high concentrations (tissue, for instance), but not for plasma or serum, where a derivatization step resulted necessary to enhance the sensitivity (Lever and Slow, 2010). As the quaternary amine group is unreactive an UV-absorbing or a fluorescent functional group is usually added by alkylation to the GB carboxyl group. Since this group is often deactivated, it is difficult to derivatise (Lever and Slow, 2010). The most common derivatising agents used for the analysis of betaines were p-bromophenacyl esters (e.g. 2'-bromophenacyl bromide and 2' bromophenacyl triflate, that show a max ultraviolet absorption at 262 nm) or 2-naphthacyl triflate (Lever and Slow, 2010; Chary *et al.*, 2012). The use of fluorescent derivatives compounds and those with a higher UV absorbance, permitted to simplify the extraction procedure and to handle a smaller aliquot of sample (Lever and Slow, 2010). Techniques using chromophoric derivatives had been developed for GB detection in tissue (Mar *et al.*, 1995), plasma, urine, cells (Storer *et al.*, 2006) or food (de Zwart *et al.*, 2003) which permitted to analyse that matrices with sufficient sensitivity. Anyway they turned out not to be suitable for routine clinical applications (Lever and Slow, 2010). Holm *et al*.(2003) reported that HPLC methods for the analysis of derivatised GB in blood were “characterized by low sensitivity and limited throughput”. Moreover the selectivity and accuracy of these methods could be compromised by the abundance of numerous other acidic metabolites in the matrix, which possibly interfere with the reaction and makes laborious extraction procedure necessary for their removal (Li *et al.*, 2010). Similar procedures were developed using capillary electrophoresis coupled to UV detection, but there were met the same complications (Li *et al.*, 2010). Storer *et al.* reported that the sensitivity limitations of this technique was probably due to the derivatization step with 2-naphthacyl triflate (Storer *et al.*, 2006).

The small dimensions, the high polarity and the permanent cationic moieties of GB makes mass spectrometry (MS) an attractive choice for its analysis (Airs and Archer,

2010; Li *et al.*, 2010; Chary *et al.*, 2012). At first, MS analysis of betaines were realized using desorption methods, such as desorption chemical ionization, fast atom bombardment (FAB) and plasma desorption (PD) (Chary *et al.*, 2012). The first shown low in sensitivity and incomplete resolution for the analysis of mixture or betaines, while FAB and PD-MS resulted more performant (Chary *et al.*, 2012). The sensitivity of FAB-MS technique could be improved by derivatization-step but renouncing to the rapidity and efficiency in recovery of the method (Chary *et al.*, 2012).

Another spectrometry technique found suitable for GB determination was nuclear magnetic resonance (NMR), which has the advantage to require fast sample preparation. Its suitability for the analysis of GB in urine and tissue has been tested (Lever and Slow, 2010) and, since the interest about GB dietary intake was increasing, surveys of the its levels in foods have been developed (Hefni *et al.*, 2015). Recently NMR has been largely adopted also in metabolomic studies (Schicho *et al.*, 2012; Ji *et al.*, 2015; Zotti *et al.*, 2016), but its main limitation remains the low sensitivity and sometimes the lack in selectivity. Besides, the high costs of NMR equipment makes routine analysis prohibitive and not likely to be realised for diagnostic purposes (Lever and Slow, 2010).

In early work, the spectrometric analysis of GB was carried out in combination with gas chromatography (GC) after conversion to volatile derivatives (Lever and Slow, 2010). The lack of useful chromophores and their permanently charged groups preclude GC separation in a non-derivatized form (Li *et al.*, 2010). An example of GB detection in serum by GC-MS without derivatisation has been published, but in this case GB had to be enzymatically converted to dimethylglycine to be detected (Holm *et al.*, 2003). The derivatisation-step made GC-MS a tedious and low specific choice (Zhao *et al.*, 2015).

MS coupled with liquid chromatography (HPLC-MS) has been recently tested, without the need for derivatization-steps (Lever and Slow, 2010). The advent of soft ionization techniques (e.g. electrospray ionization) provided a perfectly suited mean for an efficient and selective detection of compound like GB (Airs and Archer, 2010; Li *et al.*, 2010). Liquid chromatography–tandem mass spectrometry (LC-MS/MS) became the first choice for analysis of betaines because, compared to NMR or chromatographic

techniques using UV or fluorescence detection, resulted more performant, in terms of rapidity and selectivity, especially if there is the possibility that isomeric compounds could be present in a complex mixture (Lever and Slow, 2010; Chary *et al.*, 2012). Multiple-reaction monitoring (MRM) made MS/MS detection highly selective and suitable to carry out even large epidemiological investigations (Lever and Slow, 2010). Chary *et al.* characterized 25 betaines by using a MS/MS technique (Chary *et al.*, 2012). Wood *et al.* in 2002 developed a MS/MS method for the analysis of GB in plants and described the advantages of electrospray ionization (ESI)-MS/MS against PD-MS in the discrimination of isomeric ions (Chary *et al.*, 2012). They used an off-line chromatographic clean-up by strong-ion-exchange columns but, as the elution of the analytes was achievable only under strong basic conditions, decomposition artifacts could occur during the measurement (Li *et al.*, 2010).

For what concerns liquid chromatography, over the years several stationary phases have been tested for separations of polar metabolites (Li *et al.*, 2010). Ion-exchange chromatography (IEC), for example, has been largely applied for the analysis of derivatised GB in plasma, urine, cells, tissue (Storer *et al.,* 2006) and food (de Zwart *et al.*, 2003; Hefni *et al.*, 2015), generally associated at UV detection. It resulted to be a simple and generally available technology (Hefni *et al.*, 2015), but time-consuming. IEC assays for GB determination without derivatization steps had been conducted in plants (Gorham, 1984), phytoplankton (Keller *et al.*, 2004) or in fish (Charest and Dunn, 1984; Wongso and Yamanaka, 1996) by HPLC-UV or in spinach leaves (Di Martino *et al.*, 2003) by spectrophotometer. As regards the coupling with MS, IEC was found to be not perfectly compatible with ESI sources due to the presence of salts in the mobile phases which can induce ionic suppression (Li *et al.*, 2010). Recently an HPLC-IEC-MS (Stiboller *et al.*, 2015) and an HPLC-IEC-MS/MS (Wang *et al.*, 2014) methods were developed to determine and quantify GB in biological samples, using buffers with volatile salts compatible with ESI technology (ammonium acetate or ammonium formiate).

It is well known that very hydrophilic analytes, such as GB, are weakly retained by reversed-phase (RP) columns and their separation from polar matrix interferences may

be difficult (Jandera, 2011). To retain these compounds on a RP substrate, derivatization and ion pairing techniques have been tested (Pesek and Matyska, 2007); however, the time-consuming steps due to derivatisation and the difficulties in ionization associated to the presence ion pairing agents in the mobile phase (Bell and Jhones, 2005), penalised the use of HPLC-MS in this field (Pesek and Matyska, 2007). Airs and Archer (2010) measured GB in marine plankton using an octadecyl silica column (RF) coupled to a HPLC-MS and reported that the mechanism of retention was based on the affinity of the cation for residual silanol groups (basically an ion-exchange interaction) in the stationary phase.

For what concerns normal-phase (NP) chromatography, strongly polar compounds, as GB, could be excessively retained by a polar stationary phase, or even not be sufficiently soluble in non-polar organic solvents (Jandera, 2011). Besides, typical NP mobile phases are not suitable for direct interfacing to ESI sources because they do not favour the ionization and the charge separation mechanism (Zhao *et al.*, 2011).

The developments of silica-hydride surfaces simplified the separation of these compounds, with a mechanism of retention that mimics a NP chromatography (Pesek and Matyska, 2007), allowing the use of highly polar solvents, such as water (Jandera, 2011). The mobile phases utilized were aqueous mixture of organic solvents (methanol and acetonitrile), typical of the RP chromatography; modulating the composition of these mobile phases, it was possible to obtain the retention for both hydrophobic and hydrophilic compounds, operating respectively in the RP mode (increasing the water percentage) or the NP mode (increasing the organic percentage) at the same time. This dual mechanism is generally identified with the term of "aqueous-normal phase" chromatography (ANPC) (Pesek and Matyska, 2013). Several application of this technique were reported the study of Koc *et al.* (2002), who published for the first time a LC-ESI$^-$MS method for the quantification of GB in tissues and foods, and Holm *et al*. (2003), who measured GB in human plasma and serum. The latter HPLC-MS/MS method resulted suitable for large-scale epidemiologic studies (Holm *et al.*, 2003). Inspired by Holm *et al.*, Lenky *et al.* in 2012, adopted a hydride silica column to determinate GB in the same matrices (Lenky *et al.*, 2012) and, recently, Hefni *et al.*

used the same stationary phase to assay the presence of GB in foods (Hefni *et al.*, 2015). Meanwhile Li *et al.* (2010) proposed a further LC-MS/MS method for separation of seven betaines from coral tissues using a pentfluorophenylpropyl column (Chary *et al.*, 2012). Fluorinated stationary phases show both RP and NP retention for polar analytes, depending on the percentages of organic modifier in the mobile phase, and exhibit a particular retention effects on basic compounds; this suggest the presence of an ion-exchange interaction between analytes and stationary phases (Bell and Jhones, 2005).

An analogue chromatographic technique, characterised by the use of NP stationary phase in combination with an RP mobile phase, is the "Hydrophilic Interaction Liquid Chromatography" (HILIC) (Jandera, 2011; Zhao *et al.*, 2011). It represents a considerable alternative when there is no need to separate mixture of both hydrophilic and hydrophobic compounds (Zhao *et al.*, 2011) because provides good retention of strongly polar molecules. HILIC technology mainly differs from ANPC for the high content of organic solvents (more than 50%) presents in the mobile phase (Jandera, 2011). These solvents (often acetonitrile) are especially compatible with ESI source, due to its low viscosity, then provides higher performances in terms of sensitivity, selectivity and separation efficiency (Jandera, 2011; Zhao *et al.*, 2011).

Several authors in the last years exploited this technology for the quantification of GB in biological liquid matrices, including plasma (Bruce *et al.*, 2010; Kirsh *et al.*, 2010; Mueller *et al.*, 2015), serum (Steuer *et al.*, 2016) urine (Zhao *et al.,* 2015; Ocque *et al.*, 2015), amniotic fluid and cerebrospinal fluid (Kirsh *et al.*, 2010), seaweed (MacKinnon *et al.*, 2010), cereal products (Bruce *et al.*, 2010; Ross *et al*., 2014), and foods (Zhao *et al.*, 2011; Xiong *et al.*, 2012).

4. Glycine betaine analysis

4.1 Materials and methods

The aim of this part of the work was to develop a method for the detection of glycine betaine (GB) in the edible portion of clam (*Tapes philippinarum*) using ultra performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), following the guidelines provided by the Commission Decision 657/2002/EC.

This technology is of increasing interest as support for quantitative analysis and validation of biomarkers assays (Ackermann et al., 2006), although there is no published consensus approach in the field of validation of methods for the analysis of endogenous compounds (Houghton et al., 2009).

GB an endogenous osmolyte present in very high concentrations in the target matrix; therefore, particular expedients have been evaluated and applied, in order to achieve the validation of this analytical method.

The implementation of the analysis started in the department of food technology of the "Instituto de Investigaciones Marinas (IIM)" of Vigo, Pontevedra (Spain) and completed in the Laboratory of Analytical Bio-Agroalimentary Chemistry (CABA-Lab) of the Department of Veterinary Medical Sciences of the University of Bologna (Italy).

4.1.1 Glycine betaine analysis in IIM

4.1.1.1 Standards

To assess the feasibility of the method and set the instrument, commercial standards of glycine betaine and choline deuterated analogue were employed. All the standards were purchased by Sigma-Aldrich (Missouri, USA) in powder form:

- Betaine BioUltra ≥ 99.0%
- Choline chloride-(trimethyl-D_9) 98.0% used as internal standard

For each compound a "stock solution" was prepared in methanol (HPLC grade) at a concentration of 100 µg/g, dissolving 10 mg of standard powder in 100 mL of solvent. All solutions were stored in refrigerator at 4°C.

4.1.1.2 Reagents and chemicals

Solvents employed for LC analysis:

- Acetonitrile, LC-MS grade (Fisher Scientific);
- Methanol, LC-MS grade (Fisher Scientific);
- Ammonium acetate, LC-MS grade (Carlo Erba);
- Acetic acid, LC-MS grade (VWR);
- Ultrapure water (Fisher Scientific);

All solvents and buffer prepared were ultrasonicated before the injection in the in HPLC-MS/MS system.

4.1.1.3 Equipment

HPLC-MS^n

The equipment employed for glycine betaine analysis consisted of a Agilent Infinity 1260 quaternary HPLC system coupled with a mass spectrometer Thermo Scientific LTQ Velos Dual-Pressure Linear Ion Trap quadrupole.

Chromatographic separation was achieved using a Waters Spherisorb® SCX (5µm 4.6 x 250 mm) column.

A Thermo Scientific Xcalibur processing and instrument control software was used to acquire data.

The ultra-high-purity helium gas needed for the analysis in HPLC-MS/MS was supplied by a tank of pressurised helium (Alphagaz).

Other equipment

For the HPLC-MS^n analysis, the following equipment was employed:

- Ultrasonic bath (JP SELECTA S.A., Abrera, Barcelona, Spain)
- Analytical balance (Denver Instrument, Bohemia NY, USA)

4.1.1.4 Instrumental conditions

LC conditions

Chromatographic analysis were carried out under programmed conditions, at flow rate of 0.4 mL/min.

The mobile phase consisted in:

- Phase A: methanol
- Phase B: acetonitrile

The program started with 15 min of 5% A in isocratic conditions to equilibrate the column, then the percentage of methanol was increased to 95% A in 1 min and maintained for 13 min. Then, in 11 min the initial conditions were set (5% A) and hold for further 15 min, to restore the column for following injections.
The table below (Table 4) resumes the chromatographic program:

Time (min)	Phase A (%)	Phase B (%)
0	5	95
15	5	95
16	95	5
29	95	5
30	5	95
45	5	95

Table 4: Mobile phase gradient program of HPLC-MS system

During the analysis, samples were maintained at 25°C in the autosampler and the injection volume was 20 µL, in "full loop" mode.

MS^n conditions

The linear ion trap mass spectrometer operated in positive electrospray ionization (ESI+) mode.
The instrument settings were:

- Sheath gas flow rate: 55
- Auxiliary gas flow rate: 15
- Ion spray voltage: 5.5 kV
- Capillary temperature: 250 °C
- RF lens: 64.6%

Analysis were performed in SRM (selected reaction monitoring) mode, following two transitions for betaine and the internal standard (D_9-Choline).

Helium was used as collision gas.

In Table 5 there are reported the precursor-to-product transitions for betaine an the internal standard, with the correspondent collision energy (CE).

Analyte	Transitions (m/z)	CE (eV)
Betaine	118.08 > 59	27
	118.08 > 58	27
D_9-Choline	113 > 69	26
	113 > 66	26

Table 5: Monitored transitions and their relative specific parameters.

4.1.2 Glycine betaine analysis in CABA-Lab

4.1.2.1 Samples

Samples of *Tapes philippinarum* employed for method development and validation were purchased by a research group of the Department of Veterinary Medical Sciences of the University of Bologna, in the headquarter of Cesenatico.

They had been suppressed by congelation and stored at -18 °C in a freezer until the extraction procedures.

4.1.2.2 Materials

Standards

To verify the method performance and for samples quantification, commercial standards of glycine betaine and its relative deuterated analogue were used. All the standards employed were purchased by Sigma-Aldrich (Missouri, USA) in powder form:

- Betaine BioUltra ≥ 99.0%
- Betaine-(*trimethyl*-D_9) hydrochloride ≥ 98.0% used as *internal standard.*

For each compound a "stock solution" was arranged following the procedure described below:

- Glycine betaine stock solution (BET) was realised at a concentration of 5000 µg/g, dissolving 250 mg of betaine powder in 50 mL of an acetonitrile:water 1:1 solution.
- Betaine-(*trimethyl*-D_9) stock solution (D_9-BET) was prepared dissolving 10 mg of betaine-(*trimethyl*-D_9) powder in 100 mL of the same solution, obtaining a concentration of 100 µg/g.

All solutions were stored in freezer at -18°C.

Reagents and chemicals

Solvents employed for sample preparation and LC analysis:

- Distilled water (produced directly in the laboratory);
- Dichloromethane, laboratory grade (Merck);
- Acetonitrile, LC-MS grade (Sigma Aldrich);
- Methanol, LC-MS grade (Sigma Aldrich);
- Ammonium acetate, LC-MS grade (Sigma Aldrich);
- Formic acid, LC-MS grade (Sigma Aldrich)
- Ultrapure water (produced directly in the laboratory)

4.1.2.3 Equipment

UHPLC-MS/MS system

The equipment employed for betaine analysis consisted of a Waters Acquity UPLC® binary pump (provided with degasser, thermostated autosampler and column compartment), coupled with a Waters Quattro Premier XE™ triple quadrupole mass spectrometer equipped with an ESCi™ Multi-Mode Ionization Source (Waters Corporation, Milford MA, USA).

Chromatographic separation was obtained using a Waters Acquity UPLC® BEH HILIC (1.7 µm 2.1 x 50 mm), fitted with a Waters VanGuard™ guard column with the same packing (5 x 2.1 mm, 1.7 µm) (Waters Corporation, Milford MA, USA).

A Waters MassLynx™ 4.1 software (Waters Corporation, Milford MA, USA) was used to acquire and process data.

The nitrogen supply required for the mass spectrometer's interface operation was insured by a DBS N2-Mistral-4 generator (DBS Strumenti Scientifici, Padova, Italy).

Other equipment

For the clam sample preparation, the following equipment was employed:

- Centrifuge (Hettich, Kirchlengern, Germany)
- Water purification system (Rephile Bioscience, Shanghai, China)
- pHmeter (Agilent technologies, Milan, Italy)
- Ultraturrax (IKA, Staufen im Breisgau, Germany)
- Analytical balance (Gibertini Elettronica s.r.l., Milan, Italy)
- Automatic pipettes (Gilson, Middleton WI, USA)
- Vortex mixer (Velp Scientifica, Monza e Brianza, Italy)

4.1.2.4 Instrumental conditions

LC conditions

Chromatographic analysis were carried out under programmed conditions, at constant flow rate of 0.6 mL/min.

The mobile phase consisted in:

- Phase A: ammonium acetate solution 10 mM acidified with 0.05% formic acid (pH 3.65)
- Phase B: acetonitrile

The program started with 1 min of 5% A in isocratic conditions, then the percentage of water gradually increased to 60% A in 1 min and hold for 0.5 min. After that, the initial conditions were rapidly restored to 5% A in 0.5 min and hold for further 1 min, to equilibrate the column.

The total run time was 4 minutes. The following table (Table 6) resumes the chromatographic program:

Time (min)	Phase A (%)	Phase B (%)
0	5	95
1	5	95
2	60	40
2.5	60	40
3	5	95
4	5	95

Table 6: Mobile phase gradient program for UHPLC-MS/MS system

During the day of analysis, samples were maintained at 20°C in the autosampler; the injection volume was 1 µL, in "partial loop with needle overfill" mode and the column temperature was set to 40 °C to avoid excessive backpressure.

MS/MS conditions

Mass spectrometer operated in positive electrospray ionization (ESI+) mode.
The instrument settings were the following:

- Capillary voltage: 4.00 kV
- Extractor voltage: 4.00 V
- Source temperature: 140 °C
- Desolvation temperature: 325 °C
- Cone gas flow: 150 L/h
- Desolvation gas flow: 550 L/h

Analysis were performed in MRM (multiple reaction monitoring) mode, following two transitions for betaine and for the deuterated internal standard. Argon was used as collision gas at a flow of 0.35 mL/min.

In Table 7 there are reported the precursor-to-product transitions for betaine an the internal standard, with the correspondent cone voltage (CV) and collision energy (CE).

Analyte	Transitions (m/z)	CV (kV)	CE (eV)
Betaine	118.2 > 59.3	38	16
	118.2 > 58.3	38	23
D_9-Betaine	127.2 > 68.4	38	18
	127.2 > 66.3	38	27

Table 7: Monitored transitions and their relative specific parameters.

4.1.2.5 Extraction procedure

Before starting with the extraction, a pool of samples was prepared. The flesh of 5 frozen clams was minced with a scalpel and homogenised in a glass tube with Ultraturrax. All this procedure has been carried out as quickly as possible to keep the matrix frozen and guarantee an optimal homogenisation.

50 mg of the pool are weighed on an alluminium sheet and put in a 15 mL falcon tube flushing with 10 mL of distilled water. Then 40 µL of D_9-BET (100 µg/g) is added and all the sample is mixed with Vortex for about 1 minute and rests for 5 minutes. The sample is then centrifuged for 5 minutes at 8000 rpm at 25°C and 5 mL of supernatant are transferred in a new falcon tube.

The extract is purified adding 3 mL of dichloromethane and resting for 10 minutes; every 2 minutes the sample is vortexed for 30 seconds. The complete separation in two phases is obtained by centrifugation, at the same conditions described above.

1 mL of the upper aqueous phase is transferred in a tube containing 4 mL of acetonitrile LC-MS/MS, with the aim to obtain the precipitation of proteins and salts contained in the sample. The solution is manually mixed for few seconds and further centrifuged at the same conditions, to promote the deposit of unwanted substances.

Finally 1 mL of supernatant is transferred in a glass vial, ready for the injection in the UHPLC-MS/MS.

In the next page is presented a scheme (Figure 7) resuming the whole extraction procedure.

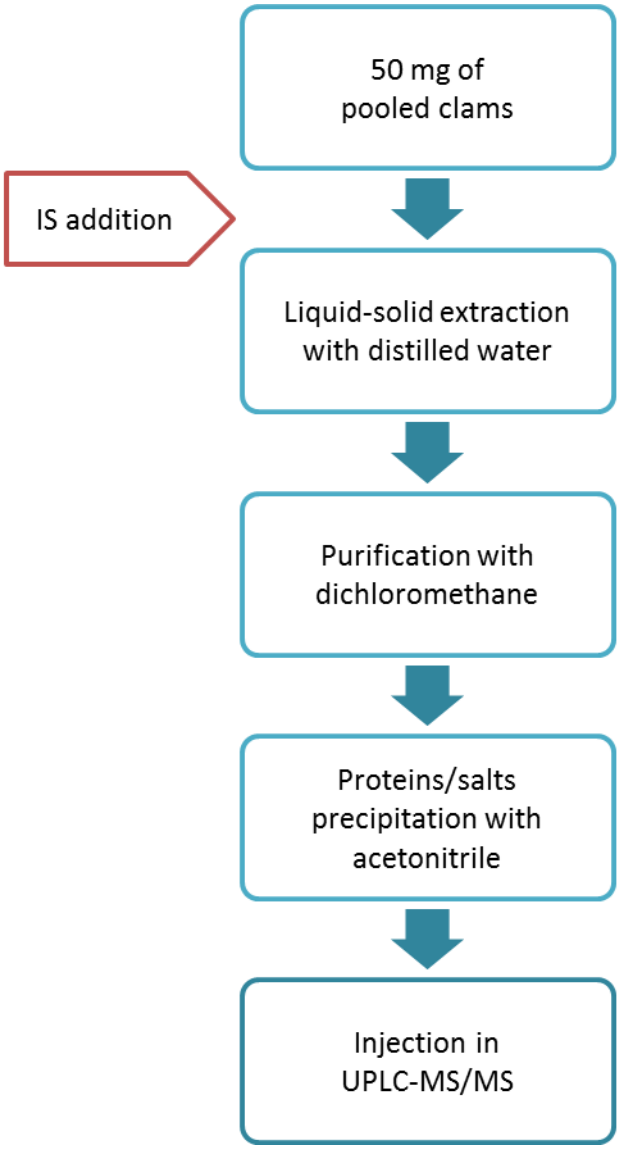


Figure 7: Extraction procedure for GB extraction

4.1.2.6 Method validation

The described LC-MS/MS method was validated taking inspiration by to current European regulations (Commission Decision 2002/657/EC), with the application of appropriate adaptations.

Since no clam tissue was reported to be devoid of GB, the determination of a limit of detection for this method resulted not feasible, as reported in a similar investigation carried out by Li *et al.* (2010).

A deuterated internal standards was employed (D_9-Betaine) in order to ensure the quality of data. Glycine betaine (GB) was then quantified calculating the ratio between its area and that of the internal standard.

Linearity and range

Linearity was evaluated building matrix-matched calibration curves in clam tissue. Curves were prepared during three different days, spiking 50 mg of clam tissue at 4 levels of concentration (plus blank, which contains the endogenous amount of analyte), following the scheme reported in Table 8.

A linear regression model was applied, associating the concentrations to the relative response of target compound.

Name	Concentration (mg/g)	µL D_9-BET (100 µg/g)	µL BET (5000 µg/g)
P 0	endogenous	40	-
P 2.5	2.5 mg/g	40	25
P 5	5 mg/g	40	50
P 7.5	7.5 mg/g	40	75
P 10	10 mg/g	40	100

Table 8: Calibration curve preparation for the analysis of GB

The interval for which suitable precision and accuracy of the measurement stay in the range from 2.5 mg/g to 10 mg/g level of concentration.

The limit of quantification (LOQ) of the method was identified as the lowest point of the calibration curve (P0) since it was no need to validate the linearity of the method below the endogenous concentration of the samples.

To establish the endogenous content of GB in clams, an appropriate number of representative blank samples (a total of twenty) were processed and analysed, as suggested by the Commission Decision 2002/657/EC. The percent standard deviation of the data obtained by the analysis of 20 samples (fortified with the internal standard) resulted below 15%, then the mean value of the responses was used to be matched with the zero value of the calibration curve.

Trueness and precision

Trueness and precision of the method were evaluated by preparing quality control (QC) samples spiked at three different concentration levels (QC low; QC medium; QC high) and realized in triplicate. Sea bream samples were fortified at three different levels of concentration: quality control low (QCL) at 4 mg/kg, quality control medium (QCM) at 6 mg/kg and quality control high (QCH) 8 mg/kg.

Trueness, referring to the closeness of the mean of a set of measurements to the true value, was expressed as bias, that indicates how much the mean of measured values differs from the reference value (QC).

Precision, concerning the closeness of agreements between a set of repeated measurements under unchanged conditions, was expressed as relative standard deviations to the mean (CV%). Values obtained by the measurements should be lower than those calculated by the Horwitz equation:

$$CV\% = \leq 2^{(1-0.5\log C)}$$

C is the mass fraction expressed as a power of 10, in this case -3. Then, should be considered acceptable value of CV% below 6.

Recovery and Matrix effect

The use of the internal standard significantly increases the reliability of the results, then the recovery was calculated in terms of trueness of the method and the matrix effect was considered irrelevant.

Stability

The analyte stability in matrix was assayed under different storage conditions. For this purpose three sample fortified at QCM level were prepared; one was maintained for 24 h in autosampler (bench-top stability) at 20°C and two were subject to freeze-thaw cycles of 1, 2 ,4 and 20 weeks, as suggested by the Commission Decision 2002/657/EC. The acceptance criteria for all stability tests were an accuracy of within ±15%.
Before the analysis of the frozen samples, the vials containing the extracts were left at room temperature for two hours to obtain a complete thaw of the sample.

Carry-over

The presence of a carry-over of the target molecule in the system was assessed following the "Guideline on bioanalytical method validation" published by European Medicine Agency (EMA, 2011).
The presence of chromatographic signals at the specific retention time of the analyte was verified, by means six consecutive injections of a solution free of the target compounds, consisting in water:acetonitrile 50:50 (blank solution). Then, it was assessed that the intensity of eventual signals was not greater than the 20% of those obtained by the injection of the lower point of the calibration curve (P0); likewise, for what about the internal standard, the signal should not overweight the 5% of P0.

4.2 Results and discussion

A chromatographic method coupled to mass spectrometry detector for the quantification of glycine betaine (GB) in *Tapes philippinarum* was developed and validated, taking inspiration by the European regulations (Commission Decision 2002/657/EC).

The development of this method started in the department of food technology of the "Instituto de Investigaciones Marinas (IIM)" of Vigo, Pontevedra (Spain) where the characterisation of glycine betaine (GB) with a linear ion trap quadrupole mass spectrometer was carried out and several chromatographic techniques were tested. The analysis proceeded in the Laboratory of Analytical Bio-Agroalimentary Chemistry (CABA-Lab), equipped with an UHPLC-MS/MS system, and the method has been successfully validated.

4.2.1 Chromatographic separation

4.2.1.1 SCX chromatography

As reverse phase chromatography were considerate not well suited for the analysis of highly polar compounds (Steuer *et al.*, 2016), alternative techniques were tested, starting from the ion exchange chromatography, exploiting the permanent positive charge present in GB.

A Waters Spherisorb® SCX (5 µm 4.6 x 250 mm) was connected to the HPLC-MS^n system, hosted in the IIM, and several chromatographic conditions were tested, taking inspirations by Bruce *et al*. (2010), Zhao *et al*. (2011) and Wang *et al*. (2014).

Water acidified with the 0.5% of acetic acid and acetonitrile were used, both in isocratic and programmed conditions, but it resulted difficult to obtain repeatability of the analysis in terms of retention times.

It was assumed that GB had high affinity with the solid phase and its retention increased at low pH, when the carboxylic group was protonated and the molecule showed only the positive charge. Besides, many bibliographic sources (Williams and Frasca, 1999) highlighted that the optimal elution conditions were included in a pH range between the GB pK_a and the pK_a of the functional group of the column (acid propylsulphonic in this case). This would have meant working at a pH between 1 and 1.83 (pK_a betaine), which were lower than the working range of the column (pH 2.5-7.5).

Other references (Croes *et al.*, 1995) suggested the increment of the ionic strength (for example adding salts) to improve the elution power of the mobile phases. Wang *et al*. (2014), for example, achieved the elution of GB in isocratic conditions with 50% water 10 mM ammonium acetate and 50% of acetonitrile. Therefore, the addition of 5 mM of ammonium acetate to the aqueous phase was tested, without obtaining any improvements in terms of repeatability of the analysis.

The substitution of the aqueous phase with methanol, a solvent in which GB is highly soluble allowed the achievement of repeatable analysis, together with the optimal spray condition for ESI operation.

However, difficulties found in obtaining linearity of responses prompted a change in the strategy for the chromatographic separation, starting from the stationary phase.

4.2.1.1 HILIC chromatography

As mentioned in Section 3.5, Hydrophilic Interaction Liquid Chromatography (HILIC) has been recently recognised as the method of choice for the retention of strongly polar molecules (Jandera, 2011; Steuer *et al.*, 2016).

A Waters Acquity UPLC® BEH HILIC (1.7 µm 2.1 x 50 mm) has been chosen for its high chemical stability, versatility and ruggedness at wide pH operating ranges.

The interactions between the target analyte and the HILIC stationary phase allowed a successfully retention of GB and its internal standard, and the use of suitable mobile phases allowed to obtain good peak shapes.

The mobile phases generally used for GB and related compounds detection are acetonitrile and aqueous solution with 5-20 mM of ammonium formiate or acetate, acidified with formic or acetic acid, to reach a pH between 3 and 3.5.

In order to optimize the chromatographic conditions for GB, 10 mM of ammonium acetate and 10 mM ammonium formiate in aqueous solution were tested, but no difference in terms of sensitivity between the two buffers have been noted.

For what about the pH, better performances have been reached at values of 3.65, obtained adding 0.05% of formic acid, in line with the concentrations used in other studies (Bruce et al., 2010; Kirsh *et al.*, 2010; Ocque *et al.*, 2015).

The programmed conditions, employing increasing concentration of water to elute the analytes, allowed satisfactorily peak shapes for both GB and the internal standard, in a very short chromatographic run (4 min, including column re-equilibration).

The high percentages of acetonitrile (never below 40%) in the ramp also provided high performances in terms of sensitivity due to its high compatibility with ESI source.

4.2.2 MS/MS detection

The most employed technique for the detection of GB is electrospray ionization (ESI), which provided efficient and selective analysis of small and polar molecules. It consists on the application of a high potential difference (at kV levels) on the solvent flow coming from the chromatography system throw a capillary, at controlled atmospheric pressure. This mechanism produces charged molecules dissolved in the mobile phase, which is converted into spray droplets mixing the solution with a nebulization gas (nitrogen).

Depending on the polarity of the employed electric field, positive or negative ions can be produced. The droplets are attracted in the entrance cone of the detector where, hit by the desolvation gas (again, nitrogen), undergo a progressive evaporation of the solvent until the release of the highly charged molecules. Finally the ions are electrically attracted in the detector.

For GB analysis, positive ionization (ESI^+) is applied, exploiting its permanent positive charge. Once in the instrument, molecules are selected by a linear series of 3 quadrupoles where, in the first one (Q1) ions are filtered according to their mass-to-charge ratio (*m/z*), in the second one (Q2) the selected molecules are broken in fragments by the application of a collision gas (an inert gas, such as argon or nitrogen), and in the last quadrupole (Q3) specific fragments for the target analyte are filtered. The procedure described above is illustrated in Figure 8.

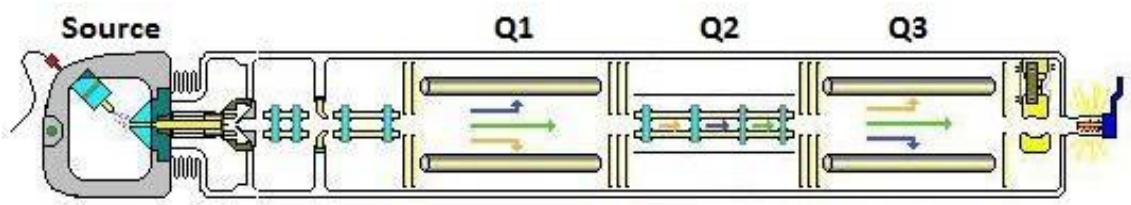


Figure 8: Graphic representation of a triple quadrupole mass spectrometer

As the fragmentation pathways characterises each molecule, MS/MS spectrometry provides selective identification of target compounds, producing signals for precursor-to-product ion transitions. This method is termed “reaction monitoring”, and could operate only for one transition (selected ion monitoring, SIM) or for different precursor–product ion pairs (multiple reaction monitoring, MRM).

In the proposed method, two transitions were monitored both for GB and its internal standard; the most abundant for quantification and the other for confirmatory purpose, in order to obtain an unambiguous determination of the target substances. The fragmentation pattern of GB and the deuterated internal standard is reported in the next page in Figure 9.

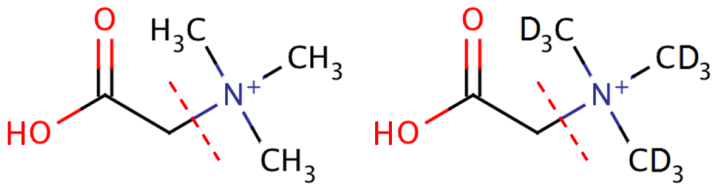


Figure 9: Graphic representation the most frequent break site of GB (on the left) and its deuterated analogue (on the right), which produces the quantification ions, corresponding for each compound to the aminic group.

The optimization of the MRM conditions has been achieved choosing, for each transition, the correct energy applied to the analytes in the source (cone voltage) and in the collision cell (collision energy), on which the generation of product ions depends. The chromatograms below (Figure 10) represent the signals resulting from the analysis of a matrix sample (fortified with D_9-BET), corresponding to the two peaks on the top, and from the injection of a solvent solution containing BET and D_9-BET, on the bottom.

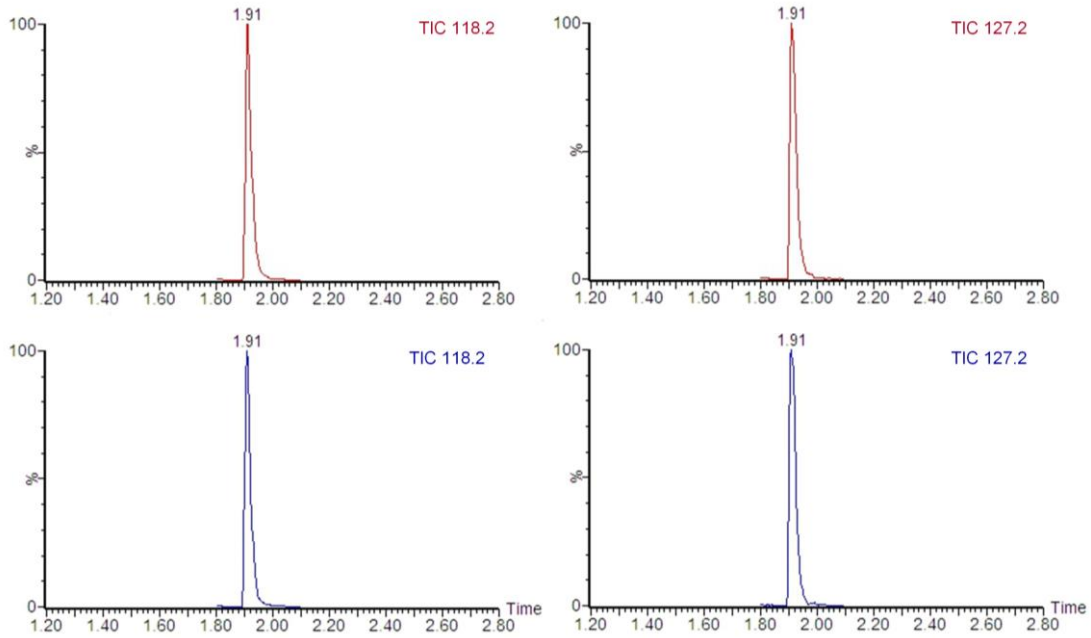


Figure 10: Chromatograms obtained by the total ion count (TIC) of the specific transition for GB (on the left) and for the deuterated internal standard (on the right)

4.2.3 Extraction procedure

The developed extraction procedure consisted in a simple and rapid liquid-liquid extraction (LLE) method using water as solvent, being this molecule highly water-soluble (Lever and Slow, 2010).

Although the complexity of the matrix analysed, there was no need to use organic solvents to promote protein precipitation because GB is not protein-bounded (Lever and Slow, 2010). Only a purification step with dichloromethane resulted necessary to remove non-polar analytes which could interfere during GB ionization (e.g. phospholipids). Then, a SPE (solid-phase extraction) purification step could be avoided, making the method described simple, rapid and cheap.

This extraction procedure is the result of the optimization of two published methods, developed by de Zwart *et al.* (2003) and Hefni *et al.* (2015), which determined GB in food.

These techniques were compared with those of Koc *et al.*, (2002), Zhao *et al.*, (2011) and Xiong *et al.*, (2012), which extracted GB from foodstuff applying modified versions of the Bligh and Dyer (1959) method. This involves the use of mixtures of methanol and chloroform in a specific ratio, to separate the lipid from the aqueous fraction of biological materials. Methanol, as extraction solvent, has been largely used for the analysis of GB in food (Zeisel *et al.*, 2003; Bruce *et al.*, 2010; Ross *et al.*, 2014; Ji *et al.*, 2015; Stiboller *et al.*, 2015) or other biological matrices (Li *et al.*, 2010; MacKinnon *et al.*, 2010; Pekkinen *et al.*, 2013; Wang *et al.*, 2014); however, for the purposes of this work, it resulted not suitable, since it prevents an optimal two-phases separation between water and the non-polar solvent used for the purification step.

Several tests had been carried out to establish the amount of sample to process, 50 mg of clam pulp were selected as the optimum compromise to obtain precise measurements without generating relevant matrix effect.

The extraction efficiency of different solvent volumes had also been tested: the addition of 10 mL (suggested by Hefni *et al.*, 2015) of water resulted the best choice to

extract GB from *Tapes philippinarum*, as the great analyte content in the target matrix allowed high dilution rates.

Extending the extraction time to 30 minutes or adding ultrasonication steps did not lead to any notable improvements in recovery rates of the extraction procedure.

Prior to inject, a dilution 4:1 with acetonitrile was performed with the aim to promote the precipitation of salts and residual proteins, and to further reduce potential residual interfering compounds from the matrix; this step also increased the percentage of organic phase of the injected solution, which promotes the optimal operation of HILIC system.

Most of the publications available in literature reported methods for the detection and the quantification of GB in biological liquid matrices (generally plasma, serum and urine); only few methods has been developed in food, cereal products or other biological matrices.

To the best of our knowledge, only two studies published method for the analysis of GB in clams*:* Li *et al*. (2010) tested in clam the applicability of a method developed in coral tissue; Ji *et al*. (2015) used *Tapes philippinarum* as bioindicator for a metabolomic study using nuclear magnetic resonance spectroscopy.

No other method has been yet developed and validated for the detection and quantification of GB in *Tapes philippinarum* using HILIC technique coupled to MS/MS.

4.2.4 Method validation

Validation of the described method for the identification and quantification of glycine betaine (GB) in in *Tapes philippinarum* was performed as described in Section 4.1.2.6, according to Commission Decision 2002/657/EC concerning the performances of analytical methods and establishing the required parameters.

The complexity in the validation of this method lied in the unavoidable presence and the high levels of glycine betaine in *Tapes philippinarum*, due to the vital importance that this compound has in mollusc organism. As a consequence, it was impossible to find a blank matrix. Anyway the method developed did not involve the use of any surrogate matrix for the validation procedures, but dilution steps of the extract allowed the reduction of the matrix effect (due to the abundant presence of phospholipids in clam tissue) without compromise method performances.

Two studies conducted on the same matrix employed different analytical techniques with other purposes, such as high performance liquid chromatography (HPLC) coupled with UV detector for a large-scale monitoring on GB content in food (de Zwart *et al.*, 2003), or nuclear magnetic resonance for a metabolomics assays on toxicological status of clams (Ji *et al.*, 2015). Li *et al*. (2010) developed and validated a method using HPLC coupled with high resolution mass spectrometry for the analysis of GB in coral tissue; they successfully applied the methods on clams, but any validation procedure on this matrix was lately published.

Linearity and range

Coefficient of determination (R^2), from the injection of calibration curves prepared for each day of validation, were used to evaluate the linearity of the method. Very satisfying results, with R^2 values >0.99 for each curve, demonstrated a good linearity of the method.

Trueness and precision

The correct homogenisation of the pooled matrix and the use of deuterated internal standard, with very similar chemical structure an behaviour, allowed a significant stability of the response and conferred high performances, in terms of trueness and precision, to the method.

In fact, the analysis of quality control samples, fortified as described in section 4.1.2.6, reported very satisfying results: bias were lower than the set limit of ±5.0% and ranged from -2,6% to 3,7%, and coefficients of variation remained below or equal to 6%.

Results are reported in the table below:

Concentration level	TRUENESS (%)	PRECISION (%)
QCL (4 mg/kg)	3.7	5.5
QCM (6 mg/Kg)	1	6
QCH (8 mg/kg)	1	5.5

Table 9: Trueness and accuracy value at three concentration levels (QCL, QCM and QCH)

Stability

The stability of the analyte in the matrix was assessed following the European guidelines (Commission Decision 2002/657/EC).

From what in our knowledge no data on GB stability in extracted clams have been published yet, anyway bench-top and freeze-thaw assays reported in this work show different results from those obtained by the analysis of other biological matrices.

In fact, freeze-thaw stability at -18 °C was maintained only for two cycles (two weeks), according to the limits mentioned in section 4.1.2.6 (±15%). On the contrary, other authors reported a stability of this molecule at -70 °C in plasma and urine lasting even for several months (Kirsch *et al.*, 2010) and for at least 29 years at −25 °C (Midttun *et al.*, 2013). Xiong *et al*. (2012) tested the stability of GB in food extracts and obtained results similar to those published by these authors.

The bench-top assay at 20° showed a loss of GB concentration of about 25% after 24 hours, while other authors reported a stability of the target molecule in plasma for at least 72 hours at 25°C (Holm *et al.*, 2003).

Carry over

After the multiple injection of the blank solvent, as described in section 4.1.2.6, a systematic presence of signals at the specific retention time for GB was observed; however, their intensities resulted far below the threshold (20% of LOQ), therefore the carry over for this compound was considered irrelevant.

For what about the internal standard, no signal were detected at its retention time, demonstrating the absence of carry over for this compound.

Applicability

The applicability of the method was tested on another matrix of interest for the thesis purposes, that is common octopus (*Octopus vulgaris*).

The extraction procedure resulted suitable in terms of handling of the samples and signals obtained by their analysis could be included in the calibration range established for clams.

The sample obtained by a pool of *Octopus vulgaris,* purchased by a research group of the Department of Veterinary Medical Sciences (University of Bologna), was then analysed. GB content calculated was in accordance with data present in literature (Konosu and Hayashi, 1975).

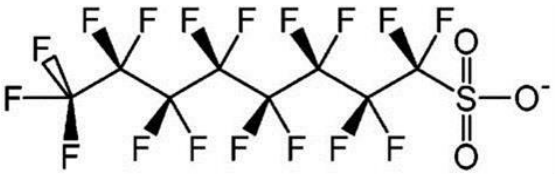
5. Perfluorinated compounds in seafood

Perfluorinated compounds (PFAS) are a group of anthropogenic substances largely used for more than 50 years for industrial and domestic applications (EFSA, 2008).

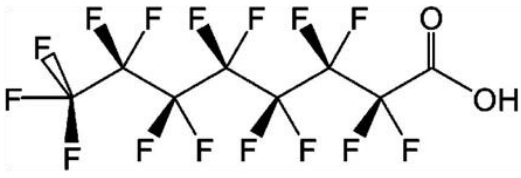
Their collective name derives from the chemical amphiphilic structure, which presents a fully fluorinated carbon chain, with hydrophobic properties, and a little hydrophilic part. The first can be linear or branched and have variable length, between 4 and 16 carbon atoms; the latter can give the molecule negative, positive or neutral charge, for the presence of anionic, cationic or neutral end groups (Buck *et al.*, 2011).

The most important and widespread PFAS are two anionic compounds, both constituted by an eight carbons alkyl chain, but different anionic terminal group: the perfluorooctane sulfonate (PFOS), contains a sulfonate group ($-SO_3^-$), and perfluorooctanoic acid (PFOA) with a carboxylate group ($-COO^-$). These compounds are considered the most representative, and therefore the most studied, among PFAS because of their widespread use and their consequent broad diffusion in the environment. They can also derive from degradation of various florinated precursors (Recommendation 161/2010/UE).

The chemical structures of PFOS and PFOA are reported in the figure below.



PFOS, perfluorooctane sulfonate, $C_8F_{17}SO_3$



PFOA, perfluorooctanoic acid, $C_8HF_{15}O_2$

Fig: Chemical structure of the two main important PFAS

The complete fluorination of the alkyl chain confers these compounds specific chemical properties: PFAS show low boiling points, a weak surface tension and low solubility both in water and in lipids (EFSA, 2008). The high chemical stability it is due to the high energy of the covalent carbon-fluorine bonds, which makes them resistant to heat, hydrolysis, photolysis, extreme pH and biological degradation (Buck *et al.*, 2011). For these properties, starting from the forties, PFAS have been increasingly employed for many industrial purposes, such as stain- and oil-resistant coatings for fabrics and food contact products, fire-fighting foams, surfactant formulations for floor polishes and insecticide (EFSA, 2008).

As synthetic compounds, their presence in the environment, including people and living organisms, is strongly linked to their wide human production and utilisation (Butt *et al.*, 2010; Schiavone *et al.*, 2009). Preliminary investigations revealed increasing levels of these pollutants in the environment and in the general population (EFSA, 2008). Due to their thermal, chemical and biological stability, once introduced in the body they are quite not metabolised and their accumulation does not occur in the fat tissue, but they bind with high affinity to proteins (EFSA, 2008). For these reasons they easily accumulate throughout the food chain and they are often found in human plasma (EFSA, 2011b) with a uniform diffusion in terms of sex, age, etc., among the general population (EFSA, 2008). Diet is considered the main source for human contamination, especially through seafood consumption (EFSA, 2011b).

The effects of these emerging pollutants on human health are not completely clear yet, but the increasing interest of the Scientific community, lead in the last ten years to important discoveries about their toxic potential. Several studies demonstrated their hepatotoxicity, developmental and reproductive toxicity, neurotoxicity and immunotoxicity on living organisms (EFSA, 2008).

The concern about the effects of PFAS on the environment and human health pushed several Authorities worldwide to finance research programs oriented to the study of their presence in the environment and in food; among all PFAS, PFOS was the most investigated compound because the most frequently detected in food products, and at the highest concentrations (EFSA, 2011b). In 2002 the Organisation for Economic Co-

operation and Development (OECD) and, an year later, the United States Environmental Protection Agency (US EPA) published two risk assessments of PFOS toxicity on human health (EFSA, 2008).

With the aim to collect the most data as possible to realize a reliable estimation about human exposure to PFOS and PFOA, in 2008 the European Food Safety Authority (EFSA) requested a group of experts (the CONTAM Panel) to develop a Scientific Opinion on the most important routes of PFAS diffusion in the environment and on the main sources of human exposure, comprehending the relative contributions of different foods and food contact materials. Moreover, EFSA requested the CONTAM Panel to make recommendations about how to manage the risks assessment related to PFAS (Recommendation 161/2010/UE).

In 2009, due to its toxicity, the wide diffusion and persistence both in the environment and in living organisms, PFOS was included in Annex B of Stockholm Convention as Persistent Organic Pollutants (POPs) (Recommendation 161/2010/UE).

5.1 Chemical identity

PFOS is generally used in salt form (potassium, sodium, ammonium), but in water solution (at pH values form 3 to 8) it completely dissociates (EFSA, 2008). PFOA molecules in water solution at pH 4 mainly dissociate and partition between the air/water interface: the perfluoroalkyl chain remains in the surface and the carboxylate group is dissolved in the water (US EPA, 2005).

PFOS is extremely stable and can be incorporated in bigger polymers (EFSA, 2008): in the environment it resists to hydrolysis (estimated half-life >41 years), to photolysis (for more than 3.7 years) and to biodegradation (for several weeks). The only known degradation mechanism for PFOS is incineration at high temperature (3M, 2003).

In the following table (Table 10) values for chemical and physical properties of PFOS and PFOA are reported.

Property	PFOS	PFOA
Appearance at normal P and T°	White powder	White powder
Molecular weight	538.22 g/mol	414.07 g/mol
Vapour pressure	3.31×10^{-4} Pa	0.1 kPa (20 °C)
Water solubility (at 20 °C)	519 mg/L	3.4 g/L
Melting point	>400 °C	45-50 °C
Boiling point	Not measurable	189-192 °C
pK_a	-3.3 (calculated for acid)	2.5

Table 10: Physical and chemical properties of PFOS potassium salt (EFSA, 2008)

Besides their industrial production, PFOS and PFOA presence in the environment can derive from various precursors, by environmental microorganisms degradation or by the metabolism of higher organisms. The number of substances belonging to PFAS family is not defined yet, but it is demonstrated that lots of molecules have the potential to break down to PFOS (EFSA, 2008).

5.2 Production and applications

Due to the aforementioned physical and chemical properties, PFAS present both hydrophobic and oleophobic character, and strong stability towards different types of degradation. This allow their wide employment for more than sixty years in industrial applications and consumer products (EFSA, 2008).

Until the beginning of the Century, PFOS was the most utilised among PFAS, employed for surface treatments, paper protection and performance chemicals. Its annual global production amounted to 4500 t and was mainly concentrated in United States, Europe and Japan (Paul et al., 2009). Regarding to PFOA, the global production reached the 260 t in 1999 (Prevedouros *et al.*, 2006).

PFOS main applications have the purpose to provide water, oil and soil resistance to products like personal apparel and home furnishings, together with grease, water and oil repellence to paper and paperboard used in food contact products; for these reasons it has been largely employed in surface and paper protection treatments. In addition, PFOS-derived salts can be commercialized as finished products in the formulation of fire-fighting foams, denture cleaners, shampoos, chemical intermediates and insecticides (OECD, 2002).

PFOA, instead, is mainly used as a chemical intermediate. Due to the high surface activity, PFOA ammonium salt is employed for the production of fluoropolymers like polytetrafluoroethylene (PFTE) (known with the commercial name of Teflon) and ploy vinilydene fluoride (PVDF) (Lehmler, 2005). The extremely low coefficient of friction and high inertia of PFTE make this compound largely employed as non-stick coating, lubricant and many more. PFOA is also used in the manufacturing of electronic components and food packaging, and in reversed-phased liquid chromatography (EFSA, 2008; US EPA, 2002).

Starting from May 2000, the biggest manufacturing plant of these compounds (the 3M, located in Alabama, USA) voluntarily decided to phasing out the production of PFAS, probably due to the alarming increment of PFAS presence both in the environment and

in the working staff. As a consequence of this decision and of the restrictions laid down by the European Union, PFOS application has significantly decreased and in some areas even ceased, being replaced by alternative substances providing the same functions or by other technologies; this led to a strong reduction of PFAS emissions in the environment (Brooke *et al.*, 2004; Van Asselt *et al.*, 2011).

5.3 Occurrence

The large number of PFAS applications justified their massive production during the second half of 20th century, but few information is available on PFOS production wastes. Data collected by 3M were used to make an estimation: between 1970 and 2002 the global wastes were quantified in 26500 t, of which 24500 t solid, 435-575 t released to air and 230-1450 t to water (Paul *et al.*, 2009). For what about PFOA, global direct emissions in 1999 was about 45 t, with prospect of continue reduction during the years.

However, when talking about emissions in the environment, also indirect sources must be considered. In fact, the presence of PFOS and PFOA is not only a consequence of their manufacture, but it is also consequent to impurity release by production processes, as unintended reaction by-products, and indirect emissions associated to losses during use and disposal of consumer products (Prevedouros *et al.*, 2006).

The extensive use of PFAS, and their consequent release in the environment, caused a global diffusion of these substances, both in high populated and industrialised areas, comprehending regions far from anthropogenic activities (e.g. the Arctic Circle). Recent studies proved the presence of PFOS and PFOA, as well as their salts and precursors, in water, air and soil in many different geographical areas (Recommendation 161/2010/EU).

These substances can contaminate waters directed to human consumption in many ways and virtually everywhere, thus their monitoring is important in order to prevent human exposure due to contaminated drinking water.

PFAS presence in the soil can represent a source for contamination of surface waters (Fromme *et al.*, 2009). Surveys proved that the widespread use of organic and inorganic fertilizer in various agricultural areas can be a source of water contamination, which can reflect in high PFAS concentrations in local population plasma (Skutlarek *et al.*, 2006).

The impact of closeness to human activity on waters contamination had been demonstrated by studies on Tennessee river (Hansen *et al.*, 2002), Yodo River (van Asselt *et al.*, 2011) and in the water environment of Singapore (Hu *et al.*, 2011).

The monitoring of soil and surface waters sampled in the same locations is recommendable, in order to better understand the transfer of these contaminants (van Asselt *et al.*, 2011).

Although current information regarding the environmental sources of PFAS results incomplete, levels found in different animal species and habitats suggest the existence of multiple sources. A study conducted on the presence of various PFAS in a wide number of wild animal species (including birds, mammalian and various species of fish) from multiple areas of the globe stated that higher PFOS concentrations are detectable in species belonging to higher steps of the food chain (predators including polar bears, seals and eagles) and to the proximity to human activities (Giesy and Kannan, 2001).

A survey on PFAS levels in animals belonging to the Great Lakes area confirmed PFOS tendency to biomagnificate in liver and blood of the higher trophic-level animals; while for what about PFOA, a significantly lower biomagnification potential was observed (Kannan *et al.*, 2005).

Furthermore, another study carried out in the New York State area confirmed PFOS bioaccumulative potential and the important role of fish in PFOS contamination along the food chain: piscivorous birds showed values around 2.5 times higher than those of the non-piscivorous species (Sinclair *et al.*, 2006).

The same trend has been observed among fish species: several studies demonstrated the feeding habits influence biomagnification of PFAS in aquatic food webs, since elevated PFAS levels have been detected in piscivorous fishes (Giari *et al.*, 2015).

A French monitoring on marine and freshwater fish species reported that the seconds result in mean about 18 times more contaminated than the firsts; in addition they do not share the same contamination profile, being freshwater fish contamination mostly due to PFOS while that of marine fish is shared between PFOA and PFOS. This trend can be supported by the higher dilution-rate of PFAS in seas/oceans water compared

to that in rivers/lakes and by the different bioaccumulation potency present among those habitats (Yamada *et al.*, 2014).

In contrast with results from Italian study conducted in a marine environment (Nania *et al.,* 2009), no differences were found between PFAS levels in demersal or pelagic species (Yamada *et al.*, 2014). Nania *et al.*, instead. observed higher levels of PFOS and PFOA in benthonic species compared to pelagic ones and concluded that, for those species living on the bottom, the contamination can derive from both water and sediments (Nania *et al.*, 2009).

Several investigations recently conducted on PFOS and PFOA presence in biota from freshwater environments confirmed that PFOS was the predominant PFAS compound (Lam *et al.*, 2014; Pan *et al.*, 2014; Ahrens *et al.*, 2015; Campo *et al.*, 2015; Giari *et al.*, 2015; Svihlikova *et al.*, 2015), and its concentrations in fish was found to be strongly related to those in water (Pan *et al.*, 2014).

Ahrens *et al.* measured the body burden of European perch (*Perca fluviatilis*) from Sweden, and observed higher PFAS levels in gonads, followed by liver and muscle, which resulted more contaminated than blood (Ahrens *et al.*, 2015). On the other hand, an Italian monitoring on eels (*Anguilla anguilla*) reported higher levels of PFOS and PFOA in blood than in muscle (Giari *et al.*, 2015), in accordance with a precedent French survey on biota from Orge River (Labadie and Chevreuil, 2011).

Anyway, a positive correlation between PFOS levels in fish blood and liver was found in a study conducted in Korea, suggesting that blood can be used for non-lethal surveys of PFOS in fish (Lam *et al.*, 2014).

Concluding, PFOS proved to be globally present and at higher levels than PFOA; the latter is detected less frequently and at minor concentrations in biota (Giesy and Kannan, 2001), although its levels in waters are reported to be higher than those of PFOS (Sinclair *et al.*, 2006; Skutlarek *et al.*, 2006).

As already mentioned, PFOS does not tend to accumulate in lipids as other persistent halogenated compounds do, but it easily bind to proteins. Although, its behaviour in animal organism is very similar to that of Persistent Organic Pollutant (POPs): levels in blood are strongly related to PFOS dietary exposure and the measured concentration

increase with the age of the analysed subject (Haug *et al.*, 2010b). This conclusion is shared by Pan *et al.*, who observed that PFOS levels in several fish species from South China increased proportionally with subjects length and weight (Pan *et al.*, 2014).

The calculated half-lives of PFOS and PFOA in the environment are respectively 41 and 8 years, therefore the estimation of the presence and the determination of contamination sources will be of public interest in the decades to come (D'Hollander *et al.*, 2010).

For all these reasons, in 2009 PFOS was included in Annex B of the Stockholm Convention and added to the list of POPs, given its widespread presence, toxicity and persistence in the environment and in biota. As a result of these measures, its employment has been limited to few specific applications (Recommendation 161/2010/UE).

5.3.1 Occurrence in humans

The increasing interest towards the global spread of PFAS and the related risks for human health, led the development of several studies on people contamination measuring their levels in blood, plasma or serum.

Result from several studies on PFOA and PFOS levels in human blood highlighted differences related to sex, age, ethnicity and geographical provenience; generally higher concentrations of contaminants were observed in male people compared to women, and seemed to increase with age.

Several investigations observed the predominant presence of PFOS in the samples, both in terms of frequency and, in almost every cases, in terms of concentration (Kubwabo *et al.*, 2004; Haug *et al.*, 2010b; Ingelido *et al.*, 2010) and confirmed the higher contamination of men compared to women (Harada *et al.*, 2004; Midasch *et al.*, 2006; Fromme *et al.*, 2007b). These findings were in line with the results of a Sweden,

a Spanish and a Greek monitoring (Kärrman *et al.*, 2004; Ericson *et al.*, 2007; Vassiliadou *et al.*, 2009).

However not the whole scientific community shares these conclusion: several surveys on PFAS presence in serum of the Unites States population indicated that no sex-related differences were observed (Olsen *et al.*, 2003a; Olsen *et al.*, 2004).

An extended Australian survey, confirmed an higher contamination from PFOS compared to PFOA, and found increased PFOS levels with age in both genders (Kärrman *et al.*, 2006). The aforementioned Greek study observed significantly high PFOS values in samples belonging to people aged over 40, while no age-related trend was noted for PFOA (Vassiliadou *et al*., 2009). An increment of PFOS levels with the age was also reported by Ingelido *et al.*, who analysed serum samples from two Italian cities (Brescia and Rome), but they observed the same trend also for PFOA. In addition, the study reported higher levels of contamination in people aged from 36 to 65 years, with significant values in females belonging to the 51-65 years group (Ingelido *et al.*, 2010). This study is in agreement with results from the survey of Harada *et al.* (2004) which demonstrated the increased contamination with the age by PFOS and PFOA in female Japanese population, but not in male. As a result, levels in elderly people do not show sex-related differences (EFSA 2008).

Another huge Australian monitoring observed the same gender differences found by other authors, but an interesting peculiarity of this work was that the highest PFOS levels were measured in adults over 60 years, while PFOA concentrations, on the contrary, were higher in children below 15 years (Toms *et al.*, 2009).

On the contrary, in a study conducted on United States population, the sex-related trend was confirmed, while no age-related variations were observed (Calafat *et al.*, 2007). Finally it could be concluded that, as reported by EFSA (2008), no clear age trends have been reported in relation to serum levels of PFOS and PFOA.

For what about the geographical distribution of PFOS and PFOA contamination among human population, Kannan *et al.* conducted an extended survey on blood, serum and plasma samples from Italy, Belgium, Poland, United States, Brazil, Colombia, India, Malaysia and Korea. The highest PFOS concentrations have been measured in samples

from the United States and Poland (>30 ng/mL) and the lowest in those from India (<3 ng/mL). In line with others monitoring, PFOA was generally found at levels 2-7 times lower than PFOS (Kannan *et al.*, 2004).

Regarding European population, EFSA (2008) reported a minimum of 4 ng/mL (in Italy) and a maximum of 55 ng/mL (in Poland) of serum PFOS levels, and concentrations between 4 and 20 ng/mL for PFOA.

An American study published the same year compared United States serum levels with those from Peru: the latter showed extremely lower PFAS frequencies and serum concentrations (Calafat *et al.*, 2006b).

Concerning Asian Countries, a preliminary monitoring carried out in whole blood samples from Tokyo area indicated a mean concentration of PFOS reaching 8.3 ng/mL (Taniyasu *et al.*, 2003). Again in Japan, Harada *et al.* observed significant zone-related differences in contamination between sample of serum collected in Miyagi, the city found with the lowest PFOS and PFOA concentrations, and Akita and Kyoto, which showed the highest levels (Harada *et al.*, 2004).

A more recent and wider monitoring conducted in China, levels of PFOS and PFOA showed a significant zone-related variability: PFOS mean serum concentrations varied from 0.3 ng/mL in those sample collected from a rural area, to 18.8 ng/mL in those from a big town; the same trend was observed for PFOA, which ranged between 0.5 and 25.4 ng/mL among the two collecting sites (Jin *et al.*, 2011).

For what about the influence of the employment situation on human exposure to PFAS, the contamination of occupationally exposed workers was measured and resulted extremely higher compared to that of the general population (Olsen *et al.*, 2003b). Other surveys on occupationally exposed subjects reported results in line with these findings, detecting mean concentrations of 941 ng/mL (range 787-1126 ng/mL) for PFOS and 899 ng/mL (722-1220 ng/mL) for PFOA (Olsen *et al.*, 2003c).

From an analogue study, the analysis of numerous samples from exposed workers showed a very high PFOA contamination levels (range 7-92030 ng/mL), measuring a mean concentration of 2210 ng/mL (Olsen and Zobel, 2007).

The global production and use is in decline since around 2002 and hence contamination levels are expected to decrease. The analysis of numerous serum samples from Minneapolis collected in 2000 and in 2005 suggested that PFAS levels in population decreased after five years from the termination of PFAS production by 3M Company, reporting mean concentrations decreased from 33.1 to 15.1 ng/mL for PFOS and from 4.5 to 2.2 ng/mL for PFOA (Olsen *et al.*, 2007a).

More recently, Toms *et al.* analysed the data obtained by three surveys on PFAS presence in serum samples collected in Australia between 2002-2011, in order to evaluate the trend in the population during an 10 years period. The concentrations measured had significantly decreased during that span of time in both adults and children, although age trends resulted variable along the target period. Sex-related trend were in line with the great majority of surveys, as the concentrations were found higher in males than in females people (Toms *et al.*, 2014). A study conducted on Swedish population confirmed those findings: a monitoring developed during the triennium 2008-2010 reported PFOS and PFOA levels lower than those determined in blood samples of Swedish elderly people collected from the late 1990s (Bao *et al.*, 2014).

For what about ethnic differences in PFAS exposure among populations, two survey projects have been conducted on United States residents belonging to three major ethnic groups (non-Hispanic whites, non-Hispanic blacks and Mexican Americans), in order to evaluate potential ethnicity-depending differences in PFAS serum levels. The analysis reported significantly higher mean concentrations of PFOS in non-Hispanic white subjects compared to non-Hispanic black subjects, and for what about Mexican Americans mean levels were even lower (Calafat *et al.*, 2006a).

The differences found in these results could be due to a combination of factors, including genetic variability, lifestyle and diet. All these parameters can influence human exposure assessments to PFAS, in particular diet seems to be an important route in human exposure to these contaminants (Fromme *et al.*, 2009; EFSA, 2008).

For example, a Polish survey on the inhabitants of the Baltic coast matched the contribution of dietary intake with PFOS and PFOA accumulation in human body: high

fish consumers (mainly from Baltic sea) were found to be more contaminated compared to other subpopulations (Falandysz *et al.*, 2006; EFSA, 2008).

A Sweden study on PFOS serum levels in female population indicated a weak relation to increasing consumption of predatory fish species (such as pike, perch and pikeperch), and such no relations were found with fatty fish consumption (salmon and herring); however, a correlation between shellfish intake and PFOS concentrations in women blood has been reported (EFSA, 2008).

5.4 Human exposure from seafood

PFAS have become of public concern during the last decade, due to their ubiquitous presence and persistence both in the environment and in animal organism, which result in multiple sources of human exposure.

Regarding human exposure to PFAS, currently there are no precise data for its determination, despite numerous information on the presence of these contaminants in the environment, in animals and even in humans are available (Tittlemier *et al.*, 2007).

Among European populations, different values of human exposures to PFAS have been observed, by the monitoring of the presence of PFOS in human blood: high concentrations have been found in Poland, followed by Belgium and Sweden, and lower in Italy (Kärrman *et al.*, 2006).

An estimation of human exposure could be obtained from two approaches: a direct one, measuring biomarkers in humans, and an indirect one, detecting the target compound in environmental and food sources (Picò *et al.*, 2011). The advantage of the direct estimation is in the simultaneous evaluation of all sources of exposure, the disadvantage lies in the impossibility to give an estimation of each source contribution to the total contamination, which is instead provided by the indirect method (Picò *et al.*, 2011). On the other hand, the indirect approach requires specific information on toxicokinetics and data about the timing and scale of exposure to different sources.

According to Picò *et al.*, a combination of both approaches, which involves the quantification of PFAS in food, together with the estimation of their dietary intake by measuring plasma levels, represents the most effective tool to calculate human exposure. Therefore it is possible to reliably characterize the health risk related to food consumption (Picò *et al.*, 2011).

Several authors (Ericson *et al.*, 2008; Kärrman *et al.*, 2009; Tittlemier *et al.*, 2007) demonstrated the correlation between dietary PFAS intake, calculated using toxicokinetic models, and relative blood levels in European population. For what about

Japan, instead, calculated intake values only contributes for about 23% on total PFAS levels measured in population blood. This suggests that contamination sources other than diet are particularly relevant in this Country (Picò *et al.*, 2011).

Although routes of contamination for people have not been completely defined yet (EFSA, 2008), some authors claim that that diet seems to be the major route of exposure (Haug *et al.*, 2010b). However the contribution of the different foodstuff to human exposure is still unclear (Haug *et al.*, 2010b) because data collected on PFAS presence in food are insufficient and it is not possible yet to characterize their level in food (EFSA, 2008).

Anyway, from surveys conducted during the last decade on PFAS levels in food items, PFOS resulted the most frequently detected and was generally measured at higher concentrations than others PFAS. In particular, PFOS has been shown to accumulate in fish with a kinetic bioconcentration factor in the range 1000-4000, mainly in liver (EFSA, 2008).

Levels of PFAS in fish products suggested not only their useful application as bio-indicators in various aquatic ecosystems, but also the importance of this food source for human contamination (Fromme *et al.*, 2009).

Haug *et al.* reported that seafood consumption is one of the major causes of PFAS dietary intake (Haug *et al.*, 2010b), in line with results from studies carried out in Spain (Ericson *et al.*, 2008) and in the UK (Mortimer *et al.*, 2009), where the analysis of numerous sort of foods lead to the conclusion that seafood was the most contaminated food item. This means that eating fish can lead to chronic exposure to high concentration of PFAS and may represent a risk factor for human health (Fromme *et al.*, 2009).

The analysis of seven types of seafood from China proved that PFOS was the most detected contaminant in terms of frequency (it was found in all 27 samples) and concentration, with the highest level (13.9 ng/g) measured in mantis shrimps (Gulkowska *et al.*, 2006).

Within a recent ecological monitoring on river waters in a northern German region involved in a massive PFAS release a few years ago, fish samples belonging to 6

different species were analysed: PFOS was detected in all samples, at concentrations up to 63.8 ng/g. PFOA, were found only in few samples and at relatively low levels, often close to the limits of quantification (Ehlers *et al.*, 2011).

PFOS was found the most detected compound (up to 121 ng/g) also in trout samples from the Great Lakes, in the United States, and the measured levels were correlated with the fishes body weight. Based on data obtained, the authors calculated BAFs (Bio-Accumulation Factors) of 4.1 for PFOS and 3.2 for PFOA (Furdui *et al.*, 2007). Even if at significantly lower concentrations, PFAS levels in seafood have been also measured in remote and allegedly less contaminated areas such as Sri Lanka, where the maximum concentrations measured for PFOS, was 0.012 ng/g (Manage *et al.*, 2005) and even the eastern Arctic, where the reported values for PFOS remained below 1.4 ng/g and were consistently lower for PFOA (Tomy *et al.*, 2004).

A monitoring conducted in Mediterranean Sea (Italy) reported PFOA and PFOS levels in fishes and molluscs lower than those observed in analogue surveys. Although this geographic location is a semi-closed basin with scarce water change, no alarming pollution by PFAS was found. Only a few fish showed high levels of contamination maybe due to a ‘‘dot-like” pollutant release. Moreover, in contrast to what was expected, PFOS and PFOA concentrations in big predators resulted undetectable. This trend is not consistent with the expected biomagnification process for these two pollutants. Moreover, benthonic fish resulted, on average, higher more contaminated than pelagic fish, maybe because the former can absorb contaminants both from seawater and from sediments (Nania *et al.*, 2009).

Two recent studies, one conducted in France (Denys *et al.*, 2014) and the other in China (Pan *et al.*, 2014), reported that, for what about high consumers of seafood, freshwater fishes represent significant contributors to PFAS human exposure, in particular for PFOS.

The Panel CONTAM of EFSA, in it 2008 report, published an estimation of the daily dietary exposure to PFOS from fish consumption for European population. It calculated a value around 60 ng/kg b.w. for the average population and 200 ng/kg b.w. for high consumers. Based on these results, the daily exposure would be below the TDI (150

ng/kg b.w.), except for highly exposed subjects that could even exceed this value. These findings are higher than the results of studies present in literature: a total dietary intake of PFOS equal to 250 ng/day (for an average body weight of 60 kg, as considered by EFSA, it correspond to 4,2 ng/kg b.w.) was calculated for Canadian population (Tittlemier *et al.*, 2007) and a value between 0.89 and 1.06 ng/kg b.w. per day for Spanish people (Ericson *et al.*, 2008) was estimated. For what about PFOA, EFSA reported an indicative daily exposure of 2 ng/kg b.w. for general population and a value of 6 ng/kg b.w. for high fish consumers. The Panel finally highlighted that lack of representative data on PFOA could lead to overestimations (EFSA, 2008).

An English study reported a combined estimated dietary intake of PFOS and PFOA for adult English people of 20 ng/kg b.w. per day (Mortimer *et al.*, 2009), while a Dutch one calculated a mean value between 0.3 and 0.2 ng/kg b.w. per day for the relative population (Noorlander *et al.*, 2011). A Norwegian study reported dietary intakes for PFOS and PFOA of respectively 1.5 and 0.6 ng/kg b.w. per day in that Country (Haug *et al.*, 2010b), while Domingo *et al.* estimated a mean dietary intake for people living in Catalonia region (Spain) of about 97 ng/day, largely deriving from sardine and red mullet (31.4 and 27.4 ng/day, respectively) (Domingo *et al.*, 2011).

The significant variability between results obtained by the studies available in literature can be due to various factors, such as the monitored area or the performances of the employed analytical methods. It must also be considered that the frequency of certain foodstuff intake is not constant, but it is strongly related to dietary habits among Countries and regions; as a consequence, food contribution to human exposure to PFAS can vary significantly. For all these reasons it is quite difficult to define representative data on dietary exposure among the different populations; anyway fish and sea-food seem to represent the most important contributors to the total dietary intake of PFAS (EFSA, 2008).

A recent EFSA report (EFSA, 2011b) found that PFOS was most detected compounds in fish, being measured at the highest levels in fish offal and in fish meat (47 µg/kg and 4.9 µg/kg, respectively). Similar concentrations were also observed in crustaceans and molluscs, even if only few samples were included in the study (EFSA, 2011b).

Likewise, a study conducted in the Netherlands reported that the highest PFOS and PFOA levels were measured in crustaceans and lean fish (825 and 481 ng/g, respectively), while fatty fish, on the contrary, resulted less contaminated (20-100 pg/g) (Noorlander *et al.*, 2011).

During a monitoring in an estuarine area in the south of Japan, PFOS and PFOA showed different exposure and bioaccumulation trends: while the former was the most abundant in animals living in shallow waters, the latter resulted mainly detected in tidal flat species (Nakata *et al.*, 2006).

A multi-site monitoring of various farmed species in Europe, South America and Southeast Asia suggested interesting aspects concerning PFAS contamination among different seafood species. Levels of contamination were sensibly higher in finfish than in shrimps, and carnivorous species (salmon and trout) resulted more contaminated than omnivorous species. In addition, concentrations found in farmed salmon and trout were greater than those measured in lean wild marine fish, while levels detected in farmed shrimp, tilapia and pangasius were generally lower than the wild correspondent. Finally, within the target species, salmon was believed to contribute for 97% to human exposure to PFAS, due to the high contamination levels and the high frequency of consumption (van Leeuwen *et al.*, 2009).

A Norwegian study reported that cod, cod liver, canned salmon and mackerel were the most contaminated fish product by PFAS. The high consumption of seafood in this Country highlights once more the importance of fish as source of exposure for certain populations. The total PFAS average dietary intake estimated in this work was 100 ng/day, with higher values for male subjects compared to female ones (Haug *et al.*, 2010a).

A further survey by the same authors investigated the relations between serum concentrations of PFAS and the estimated dietary intake of these contaminants. This study reported that fish and shellfish contributed for the 93% and 38% of total intake of PFOS and PFOA, respectively. Measured levels were found to be correlated to age and place of origin of the subject: concentrations increased with the age of the people in study, and higher levels were measured in those subjects living near the coast,

probably because of the habit to direct fishing in more contaminated waters. Fish caught from the coast, in fact, presented higher levels of PFAS than those from the open sea (Haug *et al.*, 2010b).

From the 2012 report of EFSA, children resulted the most exposed age group to PFAS through the diet, with exposure values for PFOS up to 19% of the TDI, while those of adults barely reached the 6,7%. This trend is justified by the higher amount of food for kg b.w. ingested by children compared to that of adults (EFSA, 2012). These remarks were confirmed by a survey conducted by Cornelis *et al.* on PFAS exposure in Belgian population, reporting a mean daily exposure of children 3 times higher than that calculated for adults (Cornelis *et al.*, 2012).

The experts of EFSA also estimated a decrease in dietary exposure to PFAS through fish consumption between 3 and 30 times, compared to that calculated in the previous 2008 report. This can be explained by the availability of a greater amount of data about fish, obtained by more performant analytical methods, which provided much reliable data on PFAS concentrations (EFSA, 2012)

Concluding, it must be considered that since diet is likely to be the most important factor during the estimation of human exposure to PFAS, their concentrations should be measured along all the food chain. It is important to identify the sources of these substances and to quantify their contribution, in order to understand how they influence the bioaccumulation in human body (van Asselt *et al.*, 2011).

For these reasons it is recommendable to focus also on the contamination of animals for food production, and to monitor levels of PFAS in feed, water and even in the air inside the farm (D'Hollander *et al.*, 2010).

According to what declared by van Asselt *et al.*, the main part of scientific works published until now are focused on the measurement of PFOS levels in fish and in surface water, but scarce data are collected about PFAS presence in soil and crops. This suggest the need to deepen the studies in those fields, in order to clarify the situation of the production chain; condition required nowadays to carry out any investigation in food security field (van Asselt *et al.*, 2011).

Several authors suggest the development of further studies about the consequences of PFAS exposure on human body. Moreover, to obtain more reliable and representative data, it is necessary to realize further monitoring on these substances, extending the interest to other matrices and analytes, including precursors (Domingo, 2012; Vestergren *et al.*, 2008).

Finally, EFSA suggests the implementation of even more sensible analytical methods for the monitoring of PFAS, in order to improve the capability in quantify samples and then to realise a more accurate estimation of human exposition (EFSA, 2008; Haug *et al.*, 2010b; EFSA, 2012).

5.4.1 Transformation, packaging and cooking procedures

As mentioned in Section 5.2, PFAS are used for several applications, one of these is the production of substances that come in contact with food, representing a potential source of contamination for consumers; then, the contribution of the packaging and the cooking processes to food products contamination should be monitored. Polytetrafluoroethylene (PTFE) is one of the most common product for domestic applications, present in non-stick coatings of pans and in food-contact material, to confer repellence to oil and moisture. Moreover, non-stick coatings are often manufactured using PFAS precursors, which can transfer to food by the same and then, through degradation, contribute to increase human body burdens of substances like PFOS (Fromme *et al.*, 2009).

Investigations on potential risks for the population associated to the residual PFOA in food from PTFE-containing items were conducted by Begley *et al.* (2005). They calculated the amount of PFOA released from non-stick pans and in food-contact paper, and identified the latter as the unique potential source of contamination. Anyway the French Food Safety Agency (AFSSA) concluded that this represents a minor route of exposure for the consumers to PFOA (EFSA, 2011b).

Domingo (2012) reported the results of the studies carried out by D'eon e Mabury, which confirmed that PFOS and PFOA can migrate from the packaging to the food and increase blood levels of PFAS.

A Canadian study investigated PFAS concentrations in raw, baked, boiled and fried fish products and found reduced levels of contamination after all cooking methods. In particular, baking resulted to be the most effective method in lower PFAS levels: after 15 min at 163° C (325 °F) no compound was detected in any samples (Del Gobbo *et al.*, 2008).

A more recent Canadian study conducted by Bhavsar *et al*. (2014) investigates the effect of the same cooking methods on PFAS levels in four freshwater fish species, chosen for their high levels of contamination. The study confirmed the significant increment in PFOS concentrations in all fish species except for common carp, which showed no significant modifications. Since PFOS remains bound to proteins, the moisture loss during cooking processes may explain PFOS consequent concentration. Results from a recent Greek survey (Vassiliadou *et al.*, 2015) are in agreement with these findings.

Based on data from a deep study on Canadian diet, Tittlemier *et al.* (2006) noted that the most contaminated products by PFOS were those from fast food. The authors declared that paper packaged foods, treated to give oil and water repellence, represents the main source of PFOS through the diet. These findings were also supported by the results obtained by a further work of Begley *et al.* (2008), who analysed popcorn and spreadable chocolate package and found that components of packaging emulsify in the food oil.

Despite what declared by Bergley *et al.* (2005) about PFAS contamination by non-stick pans, Sinclair *et al.* (2007) deepened the investigation on the presence of PFAS residues from coating process. They observed that these compounds, remaining on the surface, can be released in a volatile form during the cooking treatments and contribute to food contamination.

A study conducted by Ericson-Jogsten *et al.* (2009) compared PFAS levels in food cooked in non-stick cookware with those in non-cooked samples but did not obtain

any clear conclusions whether non-stick cookwares contribute to PFAS human exposure (Vassiliadou *et al.*, 2015).

Finally, EFSA in its 2011 report suggested the importance to monitor the contamination with PFAS during storage, preparation and serving of food. For this reason, Member States were exhorted to collect more information on packaged food and ready-to-eat composite food (EFSA, 2011b).

5.5 Toxicity

During the last twenty years, several investigations have been conducted in order to deepen the knowledge on the toxic effects caused by PFAS.

Most of the available data demonstrating the toxic potential of these substances were obtained by animal model studies, especially rodents, and few information are available on PFAS toxicity on others mammalians. Some epidemiological investigations have been conducted on exposed populations, such as workers from plants producing fluorinated substances, but collected data resulted still fragmented and incomplete.

Most of the studies concerning toxicokinetic of PFOS and PFOA reported a rather long half-life for these two substances in human body and their accumulative behaviour in liver and serum; on the other hand, they are not found to accumulate in red blood cells (EFSA, 2008).

OECD published in 2002 a hazard assessment on PFOS and its salts, highlighting it's persistent and bioaccumulative nature, with toxic potential in mammals. Based on the results of a 2-generation reproductive toxicity study on rats, a NOAEL (No Observed Adverse Effect Level) for PFOS was set at 0.1 mg/kg/day (OECD, 2002).

EFSA's Panel on Contaminants in the Food Chain (CONTAM) in 2008 proposed lower NOAEL values for PFOS and for PFOA (30 and 60 μg/kg b.w. per day, respectively), and then respective Tolerable Daily Intakes (TDIs) were calculated, resulting 150 and 1500 ng/kg b.w. (EFSA, 2008).

The CONTAM Panel published a Scientific Opinion declaring that occurrences of adverse effects in population due to PFOS and PFOA exposure are very unlikely, although more data are needed to verify this statement, especially with regards to developmental effects (EFSA, 2008).

This opinion was shared by other Authorities: based on toxicological studies on liver, kidney, hematological and immune systems the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment recommended a lower

TDI of respectively 0.3 µg/kg b.w. for PFOS and 3 µg/kg b.w. for PFOA (Committee on Toxicity, 2009 and 2010).

Moreover, the Bundensinstitut für Risikobewertung (BfR, the German Federal Institute for Risk Assessment) expert Panel estimated an even lower provisional TDI of 0.1 µg/kg b.w. for both compounds and confirmed that PFAS exposure through diet is very unlikely, since these levels are considered protective also for those groups of people living in highly contaminated areas (BfR, 2010).

This finding are supported by data from a study of Haug *et al.* (2010b) on dietary exposure to PFOS and PFOA, which reported values respectively 100 and 2500 times lower than their relative TDIs indicated by EFSA. However, these values did not take into account non-food sources, therefore the margin between TDI and human exposure could result smaller for high seafood consumers.

Fromme *et al.* (2007a), instead, referred to both food and non-food sources when assessed the global daily intakes. They reported significantly lower values than even the lowest recommended TDI calculated, at least for adults, since children exposure profile resulted not well defined yet.

However, it must be taken in consideration that most of the available epidemiological studies on humans have been conducted by the major manufacturer of fluorinated chemicals; this may thus lead to tendentious results. Further investigations will clarify the potential correlations between PFAS exposure and risks for human health. EFSA, in fact, strongly recommends the collection of further data on the occurrence of PFOS and PFOA in different foods and feed, together with their levels in humans, in order to better assess the relative contribution of these contaminants to human dietary exposure (EFSA, 2008).

5.5.1 Toxicokinetic

Quite scarce information about PFAS toxicocinetic in humans are available, but several studies on animal models have been carried out, in order to evaluate their kinetic after oral intake. Data collected demonstrated that, after 48 hours, PFOS and PFOA adsorption rates reach the 95% and the 93% respectively, and, after a rapid distribution in the plasma, they can bind proteins, especially albumins in high percentage. PFAS mainly accumulate in the liver, due to the huge affinity for proteins, specific for this organ. Less important amount were detected in kidney and lung, maybe due to an unavoidable contamination by blood (OECD, 2002; EFSA, 2008), but even pancreas and testes are supposed to be target organs (Hu *et al.*, 2005).

Several studies on mice and rats suggested the passage, during pregnancy, of these substances through the placental barrier to foetal blood stream, till the achievement of PFOS and PFOA serum levels very similar to those maternal. The placental passage has also been demonstrated in humans, with the consequent foetal accumulation (EFSA, 2008): a recent study on Norwegian women showed the presence of various PFAS in cord blood, at levels corresponding to the 30-79% of concentration measured in maternal blood. PFOA showed a more efficient transfer to cord blood than PFOS (Gützkow *et al.*, 2012).

Based on investigations conducted in rodents, PFOS and PFOA do not undergo any metabolization processes, nevertheless the biotrasformation of their precursors has been demonstrated, possibly resulting in an increased accumulation of these two compounds in the body (3M, 1999; EFSA, 2011b).

Even if the toxic mechanisms of PFAS towards human body are not well known, these compounds seem to cause adverse effects such as peroxisome proliferation and changes in enzymatic activity. Various Authors reported PFOS and PFOA ability in activate peroxisome proliferator-activated receptors α (PPARα), which are ligand dependent transcription factors, active on genes implicated in lipid metabolism, lipid and glucose homeostasis, inflammation, cell proliferation and differentiation (Shipley *et al.*, 2004; De Witt *et al.*, 2009).

In fact, PFAS can affect the metabolism of fatty acids interfering with their β-oxidation, probably due to the very similar chemical structure to endogenous fat acids; moreover they can cause alterations in acyl-CoA oxidases and dehydrogenases, resulting in decreased haematic levels of triglycerides and cholesterol and in oxidative DNA damage (Hu *et al.*, 2005; EFSA, 2008).

The amphiphilic nature of PFOS and PFOA suggests their tendency to be incorporated into cellular membranes, since they generally occupy the interface zones. This behaviour can lead to the inhibition of cellular gap junction and the intercellular communication (Hu *et al.*, 2002).

PFOS e PFOA excretion occurrs mainly through renal filtration, and scarcely by faeces. In animal studies, gender differences have been noted in PFOA elimination: excretion speed in female subjects was higher than in male. This justifies the different half-life times observed in both sexes (Vanden Heuvel *et al.* 1991), which seem to be related to an hormonal-dependent excretion, involving anionic transporters, competitively inhibited by testosterone (Kudo *et al.*, 2002).

Unlike what happens in animals, renal excretion of both compounds in humans is irrelevant. EFSA established half-lives for these substances in the human body, amounting at more than 5 years for PFOS and about 4 for PFOA; however, further studies on their toxicokinetics have to be carried out, in order to better understand any potential interactions during excretion (EFSA, 2008).

5.5.2 Toxic effects

Several researches have been conducted with the aim to identify the biological effects of the exposure to PFAS.

Toxicological studies in animals proved their immunotoxicity and hepatotoxicity, together with negative effects on reproductive, respiratory and nervous systems and a weak genotoxic and carcinogenic potential (EFSA, 2011b). PFOS and PFOA are also

suspected endocrine disruptors, because of their interference on sexual hormones production; they can cause increased haematic levels of oestradiol and decreased levels of testosterone, demonstrated by evident oestrogenic effects on cell cultures (Jensen and Leffers, 2008).

Several laboratory and epidemiologic researches on humans highlighted the involvement of both humoral and cell-mediated immunity in PFAS toxic effects (Corsini *et al.*, 2011; EFSA, 2012). From animal studies, the effects on immune system are: altered inflammatory responses, increased cytokines and other proteins production, reduction in weight of the lymphatic organs and impaired antibody synthesis (De Witt *et al.,* 2009).

With the aim to support these statements, studies on animal models belonging to the apex of the food chain were conducted, precisely predators such as polar bear (*Ursus maritimus*), artic fox (*Vulpes lagopus*) in Norway and the sled dogs (*Canis familiaris*) in Greenland. After the measurement of PFAS levels in various organs and blood, an alteration in hormonal and vitamin levels able to negatively influence reproductive and immune system has been found. The morphological aspect of liver, kidney and thyroid resulted modified in all sample analysed, and the reduction in bone density, together with pathological effects on nervous system, have been observed (Sonne, 2010).

Experimental studies revealed a scarce acute toxicity due to PFAS administration via inhalation; only respiratory symptoms, such as nasal discharge, and irritation of the skin and eyes were reported. Moreover, after the anatomopathological lesions in the liver were observed, sign of a systemic toxicity. Acute nervous symptomatology was also reported in rats after single oral PFOS ingestion (OECD, 2002). The hyperactivity and the behavioural changes observed were the result of damages towards structures involved in the cholinergic system (Johansson *et al.*, 2008).

Studies on subacute toxicity after oral administration proved the detrimental effects on metabolism, such as loss in weight, increased glycaemia, alterations in thyroid hormone levels, decrease in serum triglycerides and cholesterol. Pathological lesions interested especially the liver, with hepatomegaly and signs of hypertrophy, vacuolisations and necrosis of hepatocytes (Seacat *et al.*, 2003; EFSA, 2008). Primates

showed an higher sensibility compared to rodents, reporting higher mortality rates (Butenhoff, 2002; EFSA, 2008).

The effects on reproductive system and on foetus development in animal models have been evaluated; results obtained suggested a pathological symptomatology proportional to the contamination level and pregnant phase: foetal resorption, abort and growth delays in foetus. Other pathological signs observed were cardiac malformations and palatoschisis, together with high mortality rates and reduction in vitality in new-borns in cases of high maternal contamination (Lau *et al.*, 2003).

Due to PFAS wide diffusion, capacity to cross the placenta, long half-life in humans, and their adverse effects on the development in animals, a number of studies have been conducted in order to verify the occurrence of similar alterations in the population. Data of PFOA and PFOS concentrations in human cord blood were also correlated with gestational age and anthropometric parameters of the new-born and confirmed the association between these substances and birth weight and birth size (Apelberg *et al.*, 2007).

A survey on humans developed by the Danish National Birth Cohort (DNBC) was carried out with the aim to verify whether PFOS exposure could influence the time to pregnancy (TTP, a commonly used parameter to estimate fecundity), defining infertility as a reported TTP of at least 12 months. Data obtained suggested that PFOS exposure at base levels can to reduce fertility (Fei *et al.*, 2009).

Data obtained on the chronic toxicity of PFAS in rodents and primates identified liver as main target organ. In addition to non-specific clinical signs, such as weight loss, ataxia and anaemia, liver damage demonstrated the undoubted hepatotoxicity of these molecules together with the strong suspicion of a carcinogenic effect, due to increased incidence of hepatocellular adenomas. In particular, PFOS seems to be also responsible for formation of mammary adenomas, thyroid follicular cell and fibroadenomas, although data obtained are not sufficient to establish this correlation (OECD, 2002).

For what about PFOA, scientific studies verified the increased risk of pancreatic hyperplasia related to human exposure, and its possible evolution in carcinoma.

Histological lesions in gonads can also occur, together with relatively frequent mammary adenomas (US EPA, 2005; EFSA, 2008).

A monitoring on occupationally exposed population, employee in 3M Company in Alabama, was conducted with the aim to assess if there was a connection between relevant exposure to PFOS based substances and the risk of death from bladder cancer. Based on the scarce data obtained and various shortcomings of these investigations, it resulted quite difficult to make firm conclusions (EFSA, 2008).

In conclusion, the proved carcinogenicity of PFAS is likely to be related to indirect mechanisms, since no genotoxicity for these compounds has been reported by neither in vivo nor in vitro studies. Further investigations are needed to ascertain the described PFAS adverse effects on human health, since others unknown factors are likely to contribute (EFSA, 2008).

5.6 Legislation

The Organization for Economic Cooperation and Development (OECD) in 2002 made an assessment on PFOS relying on information available at that time, and concluded that it is a persistent contaminant, presenting bioaccumulative potential and toxic effects on mammals.

These statements were confirmed also by the Scientific Committee on Health and Environmental Risks (SCHER), so that the European Union decided to restrict the use and marketing of PFOS in order to safeguard human health and the environment.

On December 2006, the Directive 2006/122/EC was issued, which is a modification of Council Directive 76/769/EEC concerning restrictions that must be applied to various dangerous substances and preparations. The measurements of this Directive concern all those non-food products to which PFOS is added on purpose, referring exclusively to new products and should have been applied by Member States starting from June 27, 2008. More in details, it's not allowed to sell or use this compound in concentrations greater than 0.005% by mass, and to place on the market semi-finished items, or parts, containing concentrations of PFOS higher than 0.1% by mass.

Moreover, some minor uses of PFOS are not subjected to these limitations since no alternative substances are available, given that it don't seem to represent a risk for human contamination. Lastly, an important aspect of this Directive is that it pointed out the need to focus the attention also on PFOA and its salts, which are believed to have a risk potential similar to PFOS (Directive 2006/122/EC).

What reported above was subsequently included in Commission Regulation (EC) No 552/2009, on the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) (Commission Regulation 552/2009/EC).

There are currently no restrictions set by the European Community on PFAS presence in food and their use in plastic and paper packaging is allowed in Germany and the Netherlands.

The European Food Safety Authority (EFSA) published a scientific opinion on PFOS and PFOA, reporting complete and updated source of information on PFAS. The CONTAM Panel concluded that the risk for humans related to exposure to these contaminants was considered moderate, but the potential negative effects on the development need to be clarified yet. For this reason, the Panel recommended to collect further data on PFAS concentrations in food and in the population (EFSA, 2008).

The following year, PFOS and its salts were included in Annex B of the Stockholm Convention, which lists persistent organic pollutants (POPs) subjected to restrictions in use and production.

In order to allow a reliable estimation of human exposure, in 2010 the European Commission issued a Recommendation (Recommendation 2010/161/EU) and call Member States to monitor, during 2010 and 2011, the presence of PFOS and PFOA, their precursors and homologues in foodstuffs of both animal and plant origin. Member States were also recommended to periodically provide the collected data to EFSA, in order to expand the European database. Consequently In January 2011, EFSA published an intermediate report on the collective monitoring, summarizing the collected data and divulged recommendations to improve current investigations. However, data analysed by EFSA were provided by only seven Member States and, for some food categories the majority of information derived from a single Member State. For these reasons EFSA exhorted the realisation of a larger European monitoring for every food group, in particular for those with high frequency of contamination but the number of samples analysed in the survey was limited (crustaceans and molluscs) and for those that, even reporting low contamination levels, are inevitably consumed in high frequency (drinking water and baby food) (EFSA, 2011b).

Meanwhile in United States, after the termination of PFOS manufacturing by 3M Company, the Environmental Protection Agency (US EPA) published two SNURs (Significant New Use Rules) in 2002, with the aim to limit the production or the importation of 88 PFOS-related chemicals. These regulations permitted the application of the target substances in a limited number of extremely technical fields, resulting in very low production volumes and negligible releases. US EPA also required

manufacturers and importers a early notify in case of need for any other use (a minimum of 90 days before) (US EPA, 2011).

For what about Canada, PFOS and its related substances were initially inserted in the list of toxic compounds of the 1999 Canadian Environmental Protection Act; later, in 2009, a complete ban was proposed and a regulation was issued to add PFOS and its related substances to the Virtual Elimination List (SOR/2009-15).

In Japan PFOS and PFOA were designated as Type II Monitoring Chemicals in the current regulation for chemicals and dangerous substances (Kashinhou), being considered hazardous compounds for human health. Based on this law, manufacturers and importers have to annually report their production or import volume. Moreover, after the insertion of PFOS in Annex B of the Stockholm Convention, it has been classified as Class I Specified Chemical, and its manufacturing and use is prohibited except for specific essential uses, as what happens in Europe and in the United States (Yamazaki, 2009).

To protect marine ecosystem, in 2008 the European Water Framework Directive claimed the monitoring of PFAS contamination in Mediterranean waters, through the identification of sources, distribution and bioaccumulation patterns. Moreover the European Commission proposed a Maximum Allowable Concentration (MAC) for PFOS and its salts of 36000 ng/L in inland surface waters (Campo *et al*., 2015).

5.7 Methods of analysis of PFAS: state of the art

When performing analysis on PFAS, simple but necessary measures have to be adopted, in order to limit the risk of contaminations or losses, and to guarantee the reliability of the results.

At first, during sample processing, glass or Teflon laboratory equipment should be avoided, because the former can absorb PFAS while the latter is a source of cross-contamination (Hansen *et al.*, 2001); therefore polypropylene should be chosen, being a non-interacting material.

At the same time, since contamination can occur during instrumental analysis, the use of polypropylene vials and PEEK (polyether ether ketone) or stainless steel tubing for the liquid chromatography (LC) system is recommended (Tittlemier and Braekevelt, 2011).

Although not many methods have been specifically developed for the determination of these substances in seafood, techniques employed for the analysis of biological samples can be adjusted and adopted for this purpose (Tittlemier *et al.*, 2007). Samples are usually stored in refrigerators and, in some cases, food have been freeze-dried prior to extraction without causing any analyte losses.

Moreover, to reduce the matrix-effect in case of analysis of complex matrices, pre-treatments are often applied, such as protein precipitation obtained by acetonitrile and formic or trifluoroacetic acid addition, followed by centrifugation. Subsequently, PFAS extraction from seafood samples can be achieved using several different techniques: by solid phase extraction (SPE), often through weak anion exchange cartridges, solid-liquid extraction (SLE), pressurized liquid extraction (PLE), ion pairing extraction (IPE), matrix solid-phase dispersion (MSPD) and solid phase microextraction (SPME), but only for gas chromatography analysis.

This phase can be followed by a further purification step, generally involving SPE cartridges (Labadie and Chevreuil, 2011; Campo *et al.*, 2015; Ciccotelli *et al.*, 2016) or SPE dispersive phase, for the elimination of any residual interfering elements.

IPE, proposed by Hansen *et al*. (2001), is one of the most frequent technique adopted since it showed great flexibility in terms of matrices analysed (liver, muscle, adipose tissue, mollusc, fish etc.). The ion-pairing of the target compounds was performed with tetrabutylammonium hydrogen sulphate (TBA) and the subsequent extraction with methyl tert-butyl ether (MTBE). However this technique resulted laborious and time-consuming, and did not provide a sufficient clean-up of the sample from lipids and other disturbing matrix constituents (Valsecchi *et al.*, 2013).

The applicability of this method has been improved by the introduction of alkaline digestion, with potassium hydroxide, for the extraction of these substances from muscle tissue by Taniyasu *et al.* (2005a). This method was successfully coupled to IPE by Vestergren *et al.* (2012) who proposed the pre-treatment of the samples with alkaline solution to release analytes from the sample matrix (Valsecchi *et al.*, 2013). Other authors modified this method employing, for example, sodium hydroxide (Haug *et al.*, 2010b).

Acetonitrile SLE is the most common method for protein precipitation in tissue in analysis of PFAS in biota samples, due to its easy handling and good recovery performances. Sonication treatment is generally added to SLE extraction for the analysis of a variety of biological matrices (fish, crustaceans and molluscs) and a freezer-incubation step can facilitate impurities (lipids and proteins) precipitation (Valsecchi *et al.*, 2013).

A time-saving alternative proposed by Powley *et al.* (2005) for the analysis of PFOA in soil and sediments was based on SPE dispersive phase with activated graphitized carbon (Envi-Carb), and reported a significant reduction of matrix-effect and good recovery values. The same procedure was lately adopted by Hrádková *et al.* (2010) which applied the method for the analysis of PFOS and PFOA on canned fish and seafood, and later has been used to purify SPE extracts from alkaline digestion (Labadie and Chevreuil, 2011) and IPE (Vestergren *et al.*, 2012).

A QuEChERS (quick, easy, cheap, rugged and safe) technique was introduced for PFAS analysis in milk and fish by Lacina *et al.* (2011) with the result of high productivity and elevated sensibility of the method (Valsecchi *et al.*, 2013). This technique consists in

the simultaneous use of Envi-Carb, C18, and $MgSO_4$ in a SPE dispersive procedure: Envi-Carb and C18 sorbent allowed the removal of sterols, triacylglycerols and other lipophilic compounds, while $MgSO_4$ was added as a desiccant (Valsecchi *et al.*, 2013).

Villaverde-de-Sáa *et al.* (2012) published a MSPD method for determination of PFAS in molluscs, where acetonitrile was used as solvent, diatomaceous earth as solid support and silica as clean-up sorbent. The rapidity and the simplicity of the procedure, together with small amounts of solvent and sample required, are some of the advantages of this method (Valsecchi *et al.*, 2013).

Llorca *et al.* (2009) suggested a simple, rapid and "green" technique using PLE as pre-treatment and followed by a weak anion exchange SPE. The authors reported that this tecnique improved method's performances in terms of limits of quantification (LOQs) and rapidity of implementation, allowing the analysis of a large number of samples. Finally, an example of SPME was proposed by Luque *et al.* (2010) for the analysis of PFAS in biota, and allowed simple and fast extraction with reduced solvent consumption.

For what about detection of PFOS and PFOA, it can be obtained by gas chromatography (GC) preceded by a derivatisation step; however, since this procedure resulted time-consuming and poorly reproducible, LC represents a better alternative for the analysis of these substances (EFSA, 2008). The choice of the stationary phase for LC separation is generally oriented on reversed phase C18 columns, and the mobile phase is composed, in most cases, by a mixture of an organic solvent (such as methanol or acetonitrile) and an aqueous buffers, typically ammonium acetate, at concentrations between 1-20 mM (de Voogt and Sáez, 2006).

Before the introduction of mass spectrometry (MS), several analytical techniques have been tested for PFAS analysis, including combustion methods, neutron activation and x-ray fluorescence, but all three provided non-specific results; other more recent techniques were Fourier transform infrared spectroscopy and nuclear magnetic resonance, which resulted quite unreliable for quantification (EFSA, 2008; de Voogt and Sáez, 2006).

On the contrary MS allowed relevant improvement in the analysis of PFAS, in particular since the introduction of triple quadrupole mass spectrometers (MS/MS). In gas chromatography coupled to mass spectrometry (GC-MS), negative chemical ionization is the most widespread configuration, due to its sensitivity, and it is employed in both positive and negative mode, with methane or ammonia as reagent gas.

For what about LC-MS(MS), the most commonly used interface for PFAS detection systems is electrospray ionization (ESI), working in negative mode due to the strong electronegative character of the fluorinated chain (Tittlemier *et al.,* 2007).

Further detectors, other than triple quadrupole mass spectrometers, have been tested with both LC systems (time of flight, ion trap, fluorescence and conductometric detectors) and GC systems (flame ionization and electron capture detectors); in most of cases, triple quadrupole technology resulted the most suitable detector for this purpose.

Ultimately, LC-MS/MS working in MRM (multiple reaction monitoring) mode with negative electrospray ionization finally resulted the analytical technique of choice for the detection of anionic PFAS in seafood (Tittlemier *et al.,* 2007).

Valsecchi *et al.* suggested that on-line SPE–LC–MS–MS method would represent an interesting future trend for PFAS analysis in seafood, since pre-concentration, purification and analysis occurs in a single step, improving productivity and reducing costs. Another promising technique is turbulent flow chromatography, due to its efficiency in removing proteins (higher than SPE) which can interfere during analysis in ESI-MS/MS (Valsecchi *et al.*, 2013).

Nevertheless data collected in the 2008 report of EFSA were obtained by methods which provided high percentage of non-detected samples and relatively high LOQs (EFSA, 2011b).

An international inter-laboratory study collected data from 29 laboratories analysing environmental and food samples and concluded that differences in collected data can be due to the arbitrary use of labeled internal standard during the analysis and to the not constant chromatographic separation of PFOS interferent (taurodeoxycholic acid). Moreover, the need to detect even lower levels of contamination generally leads to

less accurate and precise results, which is reflected in the between-laboratory variance (Weiss *et al.*, 2013).

Therefore, EFSA suggested the improvement of the analytical performances of the methods for the monitoring of PFAS in food; for example, focusing on the selectively discrimination of the presence of interfering substances, such as taurochenodeoxycholic acid (TCDCA). According to EFSA opinion, the achievement of lower LOQs, in particular, would allow the development of more realistic exposure assessments (EFSA, 2011b).

In 2011 the OSPAR (Oslo/Paris Convention) Commission for the protection of the marine environment of the North-East Atlantic, published a review statement in which it exhorted EU to monitor levels of PFOS in marine environment and recommend to pay attention to any possible risks source that can be considered in the evaluation of human exposure (OSPAR, 2011).

6. Perfluorinated substances analysis

6.1 Materials and methods

The aim of this work was to conduct a preliminary monitoring on the presence of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in the most consumed fish species in the area of Bologna (Italy), by means a previously developed and validated UHPLC-MS/MS method.

Since the main contributors to the total dietary intake of these contaminants seems to be fish and seafood (EFSA, 2008), this study was set up in order to analyse some of the most consumed fish species in Italy (*Dicentrarchus labrax, Merluccius merluccius, Mugil cephalus, Pleuronectes platessa, Scomber scombrus* and *Sparus aurata*) and to provide data for a later risk-assessment on human contamination by PFOS and PFOA.

Successively, it was carried out a single-species monitoring on the presence of these two contaminants in European sea bass (*Dicentrarchus labrax*), from both aquaculture production and capture from wild stocks.

Sea bass has been chosen as target species because of its abundant and frequent consumption among European Countries (Haffray *et al.*, 2007).

Levels of PFOS and PFOA in sea bass fillets were measured with the aim to investigate potential differences between farmed and wild caught subjects, and the influence of different rearing systems on contamination, taking into consideration the morphometric characteristics of fish.

6.1.1 Samples

A total of 26 samples of six different fish species, were provided from local supermarkets in the area of Bologna (Italy) in June 2012.

Fish species, their provenience and number of samples are reported in the table below:.

Specie	Scientific name	Provenience	Samples
European sea bass	*D. labrax*	Mediterranean sea	5
European hake	*M. merluccius*	Atlantic ocean	5
Flathead mullet	*M. cephalus*	Mediterranean sea	5
European plaice	*P. platessa*	Atlantic ocean	5
Atlantic mackerel	*S. scombrus*	Atlantic ocean	5
Gilt-head sea bream	*S. aurata*	Mediterranean sea	1

Table 11: Fish species, their corresponding scientific name, provenience an number of samples

In addition, a total of 140 samples of European sea basses (*Dicentrarchus labrax*) were collected by a research group of the Department of Veterinary Medical Sciences of the University of Bologna, in the headquarter of Cesenatico. The sampling took place during autumn 2011 from 14 different sites in the Mediterranean Sea, except for one in North Atlantic Ocean. There have been collected 10 samples from each location, which could be fish breeding sites or fishing grounds (the sampling sites are reported and described in Figure 12 and Table 12). Before the preparation of the samples, morphological data of fish were noted down.

From each sample the edible tissue (muscle) was homogenized, stored in aliquots of 20 g in polypropylene tubes and maintained at -20°±2 C till the day of analysis. Before the preparation, samples were thawed overnight at 4° C.

Figure 12: Geographic map reporting the sampling locations.

Number	Location	Typology
1	Pakoštane, HR	intensive breeding
2	Monfalcone, IT	intensive breeding
3	Bodrum, TR	intensive breeding
4	Koropi, GR	intensive breeding
5	Isola Figarolo, IT	intensive breeding
6	Pachino, IT	intensive breeding
7	Lavagna, IT	intensive breeding
8	Porto Tolle, IT	semi-intensive breeding
9	Follonica, IT	semi-intensive breeding
10	Porto Tolle, IT	estensive breeding
11	North eastern Atlantic Ocean, FR	fish ground
12	North western Adriatic Sea, IT	fish ground
13	Gulf of Lion, FR	fish ground
14	Tyrrhenian Sea, IT	fish ground

Table12: Description of the sampling locations reported in Figure 12

6.1.2 Materials

Standards

Solutions of PFOS, PFOA and relative $^{13}C_4$-labeled M-PFOA e M-PFOS (employed as internal standards) were used in order to verify the method performance and to quantify the samples.

All commercial standards were purchased from Wellington Laboratories (Guelph, Canada) in methanol, with a purity grade >99%:

- PFOS Sodium perfluoro-1-octanesulfonate: 50 µg/mL, 1.2 mL
- PFOA Perfluoro-n-octanoic acid: 50 µg/mL, 1.2 mL
- M-PFOS Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate: 50 µg/mL, 1.2 mL
- M-PFOA Perfluoro-n-[1,2,3,4-$^{13}C_4$]octanoic acid: 50 µg/mL, 1.2 mL

In order to proceed with the analysis, two mix stock solution and four working solutions were realized from the commercial standards, following the procedures described below:

- PFAS stock solution (0.5 µg/mL) was obtained dissolving 20 µL of PFOS (50 µg/mL) and 20 µL of PFOA (50 µg/mL) in 2 mL of methanol.
- M-PFAS stock solution (0.5 µg/mL) was obtained dissolving 20 µL of M-PFOS (50 µg/mL) and 20 µL of M-PFOA (50 µg/mL) in 2 mL of methanol.

Then, diluting the stock solution in methanol, the following working solutions were obtained:

- PFAS working solution (250 ng/mL)
- PFAS working solution (100 ng/mL)
- PFAS working solution (25 ng/mL)
- PFAS working solution (5 ng/mL)
- M-PFC working solution (25 ng/mL)

Moreover an isomer of the taurochenodeoxycholic acid (TCDCA) was employed to monitor the potential interfering in PFOS identification.
The standard with purity >97% was purchased from Sigma Aldrich (St. Louis, MO, USA).

All solutions were stored in refrigerator at 4 °C and away from light.

Reagents and chemicals

All solvents used for mass spectrometry analysis were LC-MS grade:

- Methanol (Sigma Aldrich)
- Ammonium acetate (Fluka)
- Ultrapure water (produced directly in the laboratory)

Solvents employed during sample treatment were all analytical grade:

- Acetonitrile (Fluka)
- Formic acid (Sigma Aldrich)
- Pure water (produced directly in the laboratory)

For analyte extraction from fish fillets, the following reagents have been used:

- Magnesium sulphate ($MgSO_4$) (Sigma Aldrich)
- Sodium chloride (NaCl) (Sigma Aldrich)
- Bondesil C18 solid phase (40 µm) (Agilent)
- Supelclean ENVI-Carb solid phase (120-400 mash) (Sigma Aldrich)

6.1.3 Equipment

UHPLC-MS/MS system

The equipment employed for PFAS analysis consisted of a Waters Acquity UPLC® binary pump (provided with degasser, thermostated autosampler and column compartment), coupled with a Waters Quattro Premier XE™ triple quadrupole mass spectrometer equipped with an ESCi™ Multi-Mode Ionization Source (Waters Corporation, Milford MA, USA).

For the chromatographic separation a Waters Acquity UPLC® HSS T3 (1.8 µm 2.1 x 50 mm), fitted with a Waters VanGuard™ guard column with the same packing (Waters Corporation, Milford MA, USA) was employed.

A Waters MassLynx™ 4.1 software (Waters Corporation, Milford MA, USA) was used to acquire and process data.

The nitrogen supply required for the mass spectrometer's interface operation was insured by a DBS N2-Mistral-4 generator (DBS Strumenti Scientifici, Padova, Italy).

Other equipment

The following equipment were used for the sample preparation:

- Water purification system (Human Corporation, Seul, Korea)
- Vortex mixter (Velp Scientifical, Monza e Brianza, Italia)
- Centrifuge (Hettich, Kirchlengern, Germania)
- Analytical balance (Gibertini Elettronica s.r.l., Milan, Italy)
- Automatic pipettes (Gilson, Middleton WI, USA)
- Nitrogen sample concentrator
- Waterbath (Grant Instruments, Cambridge, GB)
- Syringe filters Puradisc™in PVDF 0,2 µm (Whatman, Kent, Regno Unito)

6.1.4 Instrumental conditions

LC conditions

Chromatographic analysis were carried out under programmed conditions, at constant flow rate of 0.5 mL/min.

The mobile phase consisted in:

- Phase A: ammonium acetate solution 5 mM
- Phase B: methanol

The gradient started with 0% B maintained for 1 minute, then the percentage of methanol increased to 40% B over 1.5 min and to 95% B in another 1.5 min. The initial conditions were restored in 1 min and hold for 3 min before the following injection, in order to equilibrate the column.

The total run time was 8 minutes; the following table (Table 13) resumes the chromatographic program:

Time (min)	Phase A (%)	Phase B (%)
0	100	0
1	100	0
2.5	40	60
4	5	95
5	100	0
8	100	0

Table 13: Mobile phase gradient program for UHPLC-MS/MS system

During the day of analysis, samples were maintained at 6°C in the autosampler; the injection volume was 10 µL, in "full loop" mode and the column temperature was set to 45 °C to avoid excessive backpressure.

MS/MS conditions

Mass spectrometer operated in negative electrospray ionization (ESI-) mode.
The instrument settings were the following:

- Capillary voltage: 0.4 kV
- Extractor voltage: 4.00 V
- Source temperature: 150 °C
- Desolvation temperature: 450 °C
- Cone gas flow: 100 L/h
- Desolvation gas flow: 800 L/h

Analysis were performed in MRM (multiple reaction monitoring) mode, following two transitions for each analyte and each internal standard; one specific transition was also monitored for TCDCA. Argon was used as collision gas at a flow of 0.35 mL/min.
In Table 14 there are reported the precursor-to-product transitions for PFOS, PFOA and the respective internal standards, with cone voltages (CV) and collision energies (CE).

Analyte	Transitions (m/z)	CV (kV)	CE (eV)
PFOS	498.6 > 80.0	62	44
	498.6 > 99.0	62	38
PFOA	412.8 > 369.0	14	10
	412.8 > 169.0	14	18
M-PFOS	502.9 > 80.0	55	41
	502.9 > 99.0	55	38
M-PFOA	416.7 > 372.0	15	10
	416.7 > 169.0	15	18
TCDCA	498.2 > 124.0	95	52

Table 14: Monitored transitions and their relative specific parameters.

6.1.5 Extraction procedure

Samples were extracted following a procedure described by Lacina *et al.*, 2011: 7.5 g of homogenized muscle were weighted into a 50 mL polypropylene tube and 45 µL of "M-PFAS working solution" (25 µg/mL) together with 10 mL of water were added.

All the mixture was shaken vigorously, both manually and with Vortex, for 1 min before performing a solid-liquid extraction (SLE) by the addition 15 mL of acetonitrile and 0.2 mL of formic acid, and shaking again for 1 min.

Then, 6 g of MgSO4 and 1.5 g of NaCl were added and the sample was immediately shaken for another 1 min. A centrifugation run for 5 min at 9000 rpm allowed the separation of the surnatant of which 12 mL were transferred into a new 50 mL polypropylene tube, containing 1.8 g of MgSO4, 0.18 g of C18 sorbent and 0.09 g of ENVI-Carb sorbent. After shaking the tube for another minute and centrifuging at the same conditions for 5 min, 8 mL of the upper layer were transferred to a 15 mL polypropylene tube, evaporated under gentle nitrogen stream in a waterbath at 40° C and reconstituted in 0.5 mL of methanol.

Before the injection in in LC-MS/MS, the sample was filtered through a 0.2 mm PVDF filter and placed into a polypropylene vial for analysis.

The procedure is schematised in the Figure 13, in the following page.

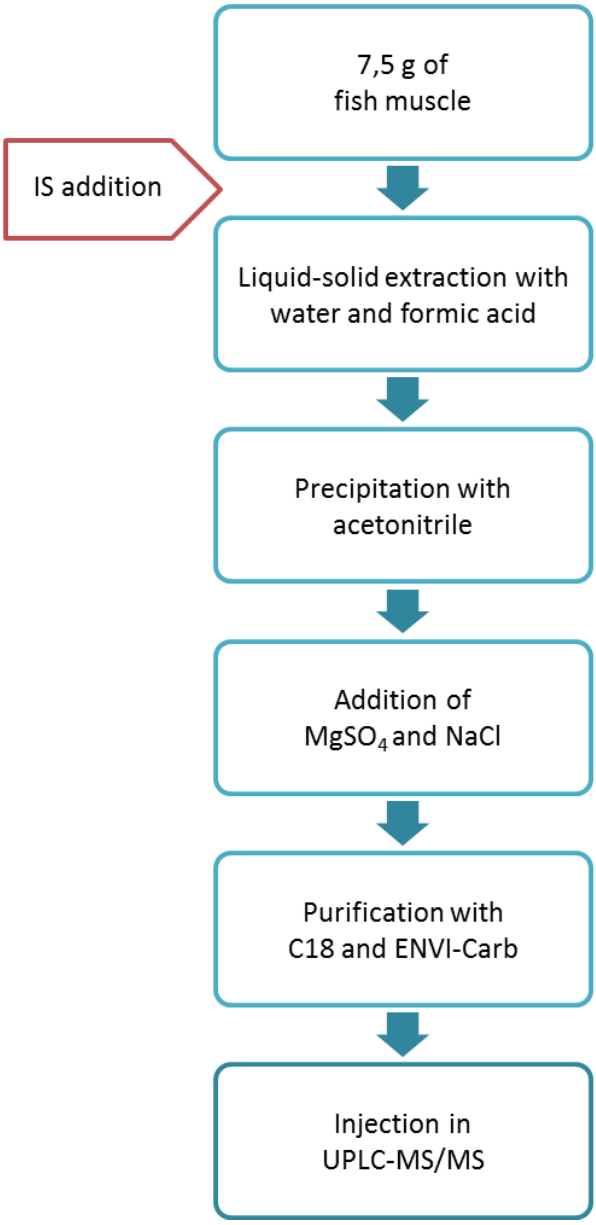


Figure13: Sample extraction procedure

6.1.6 Method validation

The difficulty in finding PFAS free fish samples, made it necessary the use of muscle tissue from sea breams (*Sparus aurata*), purchased at a local store, to build matrix-matched calibration curves for each day of analysis.

Testing the method on this species, the absence of PFOS and PFOA contamination was assessed and the performances of the method were evaluated in terms of specificity, linearity, limit of detection (LOD), limit of quantification (LOQ), trueness and precision, in accordance with current European guidelines (Commission Decision 2002/657/EC).

Specificity

The specificity of the method was assessed analysing non-contaminated sea breams samples and verifying the absence in the chromatograms of potential interferences near the retention times of the target analytes. To keep controlled the specificity of the method, one specific transition of the biliary acids, potentially interfering with PFOS, was constantly monitored during the analysis.

Linearity and range

The linearity of the method was assessed by the injection in LC-MS/MS of a nine-level calibration curve, built spiking the homogenized muscle samples with a fixed amount of internal standards and increasing levels of PFOS and PFOA, following the scheme reported in Table 15, in the next page.

The calibration curve was also used to determine the application range of the method.

Name	Concentration (ng/Kg)	µL M-PFAS (25 µg/L)	µL PFAS (5 µg/L)	µL PFAS (25 µg/L)	µL PFAS (250 µg/L)
P 0	0	45	-	-	-
P 50	50	45	75	-	-
P 100	100	45	150	-	-
P 250	250	45	-	75	-
P 500	500	45	-	150	-
P 1000	1000	45	-	300	-
P 2000	2000	45	-	-	60
P 5000	5000	45	-	-	150
P 10000	10000	45	-	-	300

Table 15: Levels of fortification used to build the calibration curve.

Limit of detection (LOD and limit of quantification (LOQ)

Using the matrix-matched calibration curve, limit of detection (LOD) and limit of quantification (LOQ), have been calculated as the analyte concentration matching the signal/noise ratio (s/n ratio) of respectively 3 and 10.

Trueness and precision

Trueness and precision of the method have been assessed by preparing quality control samples (QC) in four replicates. Sea bream samples were fortified at three different levels of concentration: quality control low (QCL) at 100 ng/kg, quality control medium (QCM) at 500 ng/kg and quality control high (QCH) at 5000 ng/kg.

Trueness was expressed as relative difference between the measured mean value and the spiked concentration, while precision was expressed as relative standard deviation to the mean (CV%).

6.1.7 Sample quantification

To quantify the samples, a matrix-matched calibration curve was prepared for each day of analysis, following the above scheme used for the linearity test (see Section 6.1.6), in accordance with 2002/657/EC criteria. For what about PFOS, the absence of potential interferences near the retention times of the target compounds was verified to avoid the overestimation of the analyte.

6.1.8 Risk assessment

A dietary chemical exposure could be calculated multiplying the concentration of the substance in the food item by the amount of food consumed by a study population, divided by the consumer's body weight.

Data about Italian population food consumption, grouped for by age class, have been provided by the EFSA Comprehensive Food Consumption Database (EFSA 2010). From this database, within the category "Fish and other seafood (including amphibians, reptiles, snails and insects)", the subcategory "fish meat" was selected.

The mean values of PFOS and PFOA levels, measured in the 25 fish samples from this study, were employed as representative of PFOS and PFOA concentrations in all fish species available in the Italian market. This value was multiplied by the amount of fish consumed in Italy (g/kg b.w. per day) reported by EFSA (2010), according to the different age groups.

In particular, for the "average consumers", the exposure was calculated by considering the mean fish consumption, while for the so-called "extreme consumers" by using the 95th percentile. Finally, the percentage of PFOS and PFOA Tolerable Daily Intake (TDI) attributable to fish consumption has been calculated.

6.1.9 Statistical analysis on mono-specie monitoring

Since PFOS and PFOA concentration values obtained by the analysis of sea basses were found not to follow a normal distribution (Shapiro test, p-value <0.001), a nonparametric tests were used in the statistical descriptive and comparative analysis.

The population studied was firstly divided into two groups according to the weight and length of the fish, and the median value calculated was used as cut-off. A Mann-Whitney test was carried out to compare PFOS and PFOA concentration data.

Later, the study population was divided in three groups according to the type of rearing (wild caught, extensively and intensively reared), and a Kruskal-Wallis test was employed to compare the concentration data between subjects grouped. The comparisons between groups were performed giving to all samples with a S/N ratio lower than 10:1, but providing a detectable chromatographic signal, a value equal to the LOQ (9 ng/kg) to in order to be conservative.

Finally, the existence of significant morphometric differences (in weight and length) between fish farmed in intensive rearing, semi-intensive and extensive rearing, and wild-caught was investigated. Then, to assess the relationship between PFOS and PFOA levels, type of farming and morphometric differences of the subjects, two robust multivariate linear regression models were applied.

Potential outliers for both PFOA and PFOS data were investigated employing the Tukey's method, which identifies the values greater than the 75th percentile plus 3 times the interquartile distance, or less than the 25th percentile minus 3 times the interquartile distance, and eventually eliminated them from the analyses.

When concentrations below the LOQ were detected, random values, extracted from a uniform distribution between 3 ng/kg (LOD for both analytes) and 9 ng/kg, were assigned to the samples.

Variables included in the model as predictors were “type of farming” and the morphometric parameters. Considering the collinearity between the two parameters “length” and “weight”, only this latter variable was chosen as predictor. PFOS and

PFOA concentrations were used as dependent variables in the first and in the second model, respectively.

Statistical analysis were performed with STATA 11.2 software (StataCorp, 4905 Lakeway Drive, College 17 Station, Texas, USA), setting significance at $P < 0.05$.

6.2 Results and discussion

A previously developed and fully validated UHPLC-MS/MS method was used for the quantification of the two most important perfluoralkyl substances, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), in seven fish species (*Dicentrarchus labrax, Merluccius merluccius, Mugil cephalus, Pleuronectes platessa, Scomber scombrus and Sparus aurata*).

The good performances of the method adopted allowed the fulfilment of a preliminary monitoring and successively the estimation of a percentage of tolerable daily intake for PFOS and PFOA by seafood from Italy.

Moreover, a mono-specie monitoring on the levels of contamination by these two pollutants was carried out in 140 sea bass samples from different sites in Italy, and different rearing types.

6.2.1 Method optimisation

The extraction procedure was carried out following the method described in the study of Lacina *et al.* (2011).

While analysing PFAS in food matrices, instrumental related contaminations by solvents and laboratory equipment are quite common (Yamashita *et al.*, 2004), therefore some precautions have been applied during samples preparation. The use of glassware was avoided and polypropylene tubes and vials were chosen during sample preparation; moreover, all solvents used for analysis were tested at the beginning of the experiment in order to exclude the presence of chromatographic signals corresponding to target compounds. In this way, background contamination has been successfully controlled.

For what about instrumental analysis, the chromatographic separation was initially obtained with a Water Acquity UPLC® BEH C18, which is used in the majority of publications analysing PFAS in food matrices (Haug *et al.*, 2010a ; Cornelis *et al.*, 2012; Domingo *et al.*, 2012).

As reported by several Authors (Benskin *et al.*, 2007; Kadar *et al.*, 2011), during PFOS analysis in fish, one of four cholic acids may interfere during PFOS analysis in mass spectrometry. It is the taurochenodeoxycholic acid (TCDCA), which naturally occurs in fish and presents the same molecular weight of PFOS and one specific diagnostic transition in common (498,6 > 80). Applying the chromatographic conditions reported in literature, TCDCA shows the same retention time as PFOS, then its quantification should results overestimated.

To avoid this issue, many chromatographic gradients have been tested, in attempt to separate PFOS and the TCDCA in two different peaks, with unsatisfying results. Alternative strategies could be the choice of the PFOS specific transition 499 > 99 for the quantification of this compound or even select a more suitable stationary phase for chromatographic separation. Then, a Waters Acquity UPLC® HSS T3 column, compatible with a 100% aqueous mobile phase, was tested. This configuration made it possible to obtain optimal peak resolution, together with retention times sufficiently different for the two molecules, ensuring the adequate selectivity to quantify PFOS using the transition 498.6 > 80.0. This diagnostic signal resulted the principal in terms of intensity, therefore it was used for the quantification of PFOS; the other one (498.6 > 99.0) was applied for the confirmation and identification, in order to obtain an even more specific method. TCDCA showed a clear signal at its specific retention time, different from that of PFOS; this condition was constantly assessed monitoring TCDCA specific transition 499 > 124.

In Figure 14 it is possible to appreciate the presence of a signal relative to TCDCA in the transition used for PFOS quantification (498.6 > 80.0) and the chromatographic separation of the two compounds obtained by the selected stationary phase; on the left it is shown the chromatogram relative to a blank sample of sea bream (*Sparus*

aurata), used to build the calibration curves, and on the right it is reported one chromatogram obtained by the analysis of a sea bass sample.

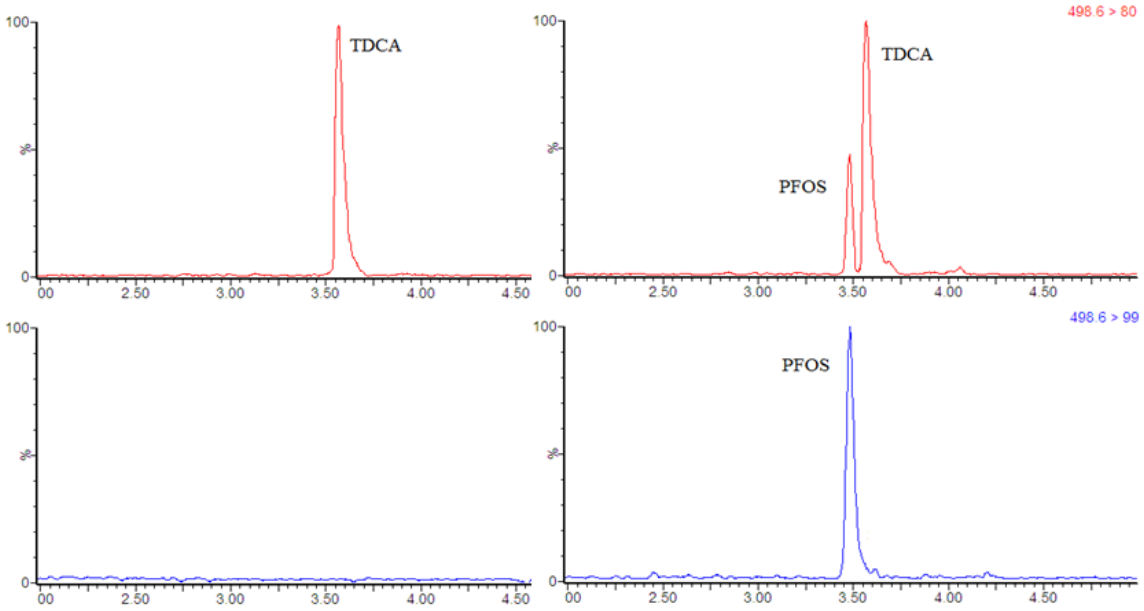


*Figure 14: Chromatograms of the specific transitions of PFOS (*498.6 > 80.0 and 498.6 > 99.0*) resulting from the analysis of a sample of sea bream (on the left) and of sea bass (on the right)*

The UHPLC allowed times of analysis considerably shorter than those obtained by other authors, because most of them used a traditional HPLC. Such a rapid chromatographic run permitted the analysis of a greater number of sample without hinder the accuracy of the measurement, which would be very useful in the perspective of large-scale monitoring of PFAS in fish.

The proposed method demonstrated very satisfying performances and the application of labelled internal standard conferred reliability to the samples quantification. Being chemically identical to the target analytes, the internal standards show the same behaviour as PFOS and PFOA during the different steps of analysis: they establish similar interactions with the matrix and undergo the same matrix effect during ionisation and show similar retention times.

The injection of a calibration curve during every day of analysis proved a good linearity of the method, in the range between 5 and 10,000 ng/kg, because their analysis reported R^2 values always greater than 0.99.

Limit of detection (LOD) and limit of quantification (LOQ), defined as the concentrations providing a chromatographic signal with a signal-to-noise (S/N) ratio respectively of 3 and 10, were calculated as described in Section 6.1.6 and results are reported in the table below (Table 16):

	LOD (ng/kg)	LOQ (ng/kg)
PFOS	2	6
PFOA	3	9

Table 16: Limit of detection (LOD) and limit of quantification (LOQ)of the method

LOQ values resulted particularly lower compared to those reported in literature on the same matrix (Nakata *et al.*, 2006; Berger *et al.*, 2009; Haug *et al.*, 2010b; Ciccotelli *et al.*, 2015). A similar sensitivity is only described by Lacina *et al*. (2011), who employed a more performant instrument (time-of flight mass spectrometer).

Trueness, expressed as relative difference between the measured mean value and the spiked concentration, resulted lower than 13% in every sample, while the precision, expressed in relative standard deviation to the mean (CV%), was always below 11% (values reported in Table 17).

Concentration level	**TRUENESS (%)**		**PRECISION (%)**	
	PFOS	***PFOA***	***PFOS***	***PFOA***
QCL (100 ng/kg)	13,4	12,4	10,4	11,1
QCM (500 ng/kg)	9,2	9,4	6,6	7,5
QCH (5000 ng/kg)	6,8	5,3	4,7	7,1

Table 17: Trueness and accuracy value calculated at three different concentration levels (QCL, QCM and QCH)

Finally, to constantly assess the absence of contaminations throughout all the analysis sessions, a mobile phase sample was injected every five fish samples.
In conclusion, the employed method resulted suitable for the purpose of this research, which required excellent performances in terms of detection capability and specificity in the analysis of a complex biological matrix.

6.2.2 Sample analysis

6.2.2.1 Multi-species monitoring

The present multi-species survey highlighted the constant presence of both PFOS and PFOA in some of the most consumed fish species available on the Italian market, since at least one of the target analytes have been detected in all samples.
As reported in Table 18, PFOS was detected in all target species, at levels between 54 and 1.896 ng/kg, while PFOA ranged from trace levels up to 487 ng/kg.

Species	**PFOS (ng/kg)**		**PFOA (ng/kg)**	
	Range	***Mean ± SD***	***Range***	***Mean ± SD***
European sea bass (*D. labrax*)	703 – 1.243	1.026 ± 205	93 – 487	231 ± 138
European hake (*M. merluccius*)	91 – 1292	716 ± 476	traces – 127	63 ± 52
Flathead mullet (*M. cephalus*)	487 – 1896	926 ± 508	12 – 113	47 ± 35
European plaice (*P. platessa*)	240 – 510	378 ± 99	10 – 36	23 ± 9
Atlantic mackerel (*S. scombrus*)	54 – 120	87 ± 24	traces – 22	9 ± 7

Table 18: Measured PFOS and PFOA levels (range, mean and standard deviation) for each fish species considered (Sparus aurata was used for method validation because found free from contamination). In case of concentration below LOQ, for calculating the mean it was assumed to be equal to LOQ (9 ng/kg) and indicated in the table as "traces"

These results reveal, on the one hand, the frequent seafood contamination by PFOS, and on the other hand the presence of certain variability between fish species. This suggests that the role of this food in human exposure to PFAS can vary significantly.

More in detail, inter-species differences in PFOS and PFOA levels are relevant and some trends can be observed: although the number of samples for each species is rather limited and intra-species standard deviations are not negligible, it could be observed that European sea bass, flathead mullet and European hake resulted the most contaminated species by both compounds, while Atlantic mackerel presented lower levels of PFOS and often only traces of PFOA.

The reasons for such variability can be multiple: an important factor is represented by the different habitat of each species, that has been proved to affect the contamination in fish tissues. Rivers are generally more contaminated than seas, and the coastal environment is usually a more polluted ecosystem compared to the open sea, depending on the proximity of river outflows and the presence of human activities (Sánchez-Avila *et al.,* 2010). According to some authors that found benthic species more contaminated then pelagic ones (Nania *et al.,* 2009; Miniero *et al.*, 2014), the present survey shows higher PFAS levels in European sea bass and flathead mullet, both species living in coastal waters.

Another species-specific factor potentially related to the level of contamination is food habits: various inter-species monitoring suggested that the contamination extent in carnivorous species is generally higher compared to omnivorous species (Van Leeuwen *et al.,* 2009; Shi *et al.,* 2012; Hloušková *et al.,* 2013; Xu *et al.*, 2014; Giari *et al.*, 2015). The relatively high concentrations measured in certain species belonging to this study, such as European sea bass and European hake, might therefore reflect the trophic biomagnification potential of PFOS. Moreover, these latter two, together with flathead mullet, have a lower fat content compared to the others, suggesting that fatty-fish is likely to be a lower contributor to consumers' exposure to PFAS. These findings are in agreement with what reported by Noorlander *et al.* (2011).

Finally, it must be also considered that fish age can play a role in pollutant accumulation. For example, although farmed fish was found to be less contaminated

by PFAS than wild fish (Van Leeuwen *et al.*, 2009; Paiano *et al.*, 2012; Shi *et al.*, 2012), the outcomes of a recent study by Miniero *et al.* (2014) on multiple persistent pollutants suggest that small size wild fish seems to have a similar contamination profile to farmed fish. This means that the level of contamination of a wild fish can significantly vary depending on its age at the moment it is caught.

The data obtained in the present study are comparable with those from similar recent monitoring projects on PFAS presence in fish (Berger *et al.* 2009; Haug *et al.* 2010b; Hrádková *et al,.* 2010; Schuetze *et al.*, 2010; Shi *et al.,* 2010; Domingo *et al.,* 2012; Zhang *et al.*, 2011; Guo *et al.*, 2012; Hloušková *et al.,* 2013), although variability is not so evident in some cases. As it has been discussed, this can be related to multiple factors; however, talking about PFOS, the possibility that some results from other surveys were affected by the presence of the previously mentioned TCDCA-related interference is an important aspect to consider.

6.2.2.2 Exposure estimation

The overall mean concentrations of PFOS and PFOA were respectively 627 ng/kg (SD=489) and 75 ng/kg (SD=106). The mean and the 95th percentile of exposure to PFOS and PFOA, for different age groups, together with the relative percentage of TDI due to fish meat consumption, are shown in the table in Table 19, in the next page.

PFOS				
Age group	***Mean***	***% TDI***	***95th***	***% TDI***
Infants (<1 year)	0.16	0.09	1.32	0.88
Toddlers (1 to <3 years)	1.41	0.94	4.13	2.75
Other children (3 to <10 years)	0.56	0.38	1.90	1.27
Adolescents (10 to < 18 years)	0.32	0.21	1.11	0.74
Adults (18 to <65 years)	0.29	0.19	096	0.64
Elderly (65 to <75 years)	0.30	0.20	1.07	0.71
PFOA				
Age group	***Mean***	***% TDI***	***95th***	***% TDI***
Infants (<1 year)	0.02	0.00	0.16	0.01
Toddlers (1 to <3 years)	0.17	0.01	0.50	0.03
Other children (3 to <10 years)	0.07	0.01	0.23	0.02
Adolescents (10 to < 18 years)	0.04	0.00	0.13	0.01
Adults (18 to <65 years)	0.04	0.00	0.12	0.01
Elderly (65 to <75 years)	0.04	0.00	0.13	0.01

Table: Mean and 95th percentile of exposure to PFOS and PFOA in the different age groups and relative % TDI (PFOS 150 and PFOA 1500 ng/kg b.w. per day) related to fish consumption.

When estimating dietary exposure due to fish consumption, toddlers result the age group with the highest exposure to both PFOS and PFOA, with 1.41 ng/kg b.w. per day (95th percentiles: 4.13 ng/kg b.w. per day) and 0.17 ng/kg b.w. day (95th percentiles: 0.50 ng/kg b.w. per day), respectively.

The lowest mean exposure was found in infants: for PFOS it was calculated 0.14 ng/kg b.w. per day and for PFOA to 0.02 ng/kg b.w. per day, but the 95th percentile increased up to 1.32 ng/kg b.w. per day and 0.16 ng/kg b.w. per day respectively.

In the other age groups, mean values ranged from 0.29 to 0.56 ng/kg b.w. per day for PFOS and from 0.04 to 0.07 ng/kg b.w. per day for PFOA, while the 95th percentile

ranged from 0.96 to 1.90 ng/kg b.w. per day and from 0.12 to 0.23 ng/kg b.w. per day, respectively for PFOS and PFOA.

Finally, the exposure estimated in all age groups and categories of consumers of seafood (average and extreme) resulted far below the TDIs for both PFOS (150 ng/kg b.w. per day) and PFOA (1500 ng/kg b.w. per day).

EFSA declared that the consumption of "Fish and other seafood" is responsible for the 50-80% of PFOS and 7.6-27% of PFOA total dietary exposure, and that "Fish meat" represents more than 80% of the "Fish and other seafood" category (EFSA, 2011b). Consequently, based on the results of this work, the total dietary exposure for the 95th percentile of the most exposed group (toddlers) would reach 9.72 and 8.39 ng/kg b.w. per day for PFOS and PFOA, respectively.

Data obtained by this study on the estimation of the Italian consumers' exposure hint that the risks related to fish consumption are unlikely, even for high consumers. However, the observed inter-species and inter-studies variability suggests that such risk could not be generalized and may depend on multiple factors. As a consequence, further surveys focused on certain species, from different sampling sites, are needed to upgrade the risk assessments on fish meat consumption.

6.2.2.3 Mono-species monitoring

A total of 140 sea basses (70 from intensive rearing, 30 from semi-intensive/extensive rearing and 40 were wild-caught) were processed and data obtained statistically analysed. *Dicentrarchus labrax* was chosen as target specie due to its commercial importance in the Mediterranean area: it is one of the most consumed species and its diffusion has significantly increased with aquaculture improvement.

Since wild sea bass is a top predator in the marine food chain, it could bioaccumulate environmental pollutants; moreover, since its life-cycle is closely associated with coastal environments, sea bass could represent a control species for PFAS contamination.

The analysis of fish morphometric data reported that average weight of the sea basses was 0.570 ± 0.268 kg, the median 0.489 g in a range of 0.301-1.670 kg. The average length was 36.5 ± 4.8 cm with a median of 35.3 cm in a range of 29.9-53.9 cm.

From statistical data analysis it could be possible to state that wild-caught fish were characterized by higher weight and length than fish from semi-intensive/extensive which had in turn higher weight and length compared to those from intensive rearing (Kruskal-Wallis test; $p<0.001$).

For what about contamination, a noticeable presence of PFAS was found in European sea bass fillets included in the study and the results are reported in the table below (Table 20):

Location	Weigh (g)	Length (cm)	PFOS (ng/kg)	PFOA (ng/kg)
1	335,2	31,8	35,7	24,3
2	487,0	35,6	53,8	14,3
3	412,0	33,2	33,9	21,9
4	364,3	32,6	38,3	21,6
5	375,8	33,1	22,6	traces
6	366,1	32,8	21,3	traces
7	421,9	33,7	33,9	22,3
8	530,6	36,2	154,9	36,6
9	670,7	37,9	34,2	18,1
10	658,9	37,8	659,5	30,4
11	713,4	41	1397,1	28,3
12	1288,9	47,4	607,6	117,4
13	655,9	38,6	4353,2	42,5
14	701,0	38,8	3514,9	29,3

Table 20: morphological and contamination data of sea basses analysed, divided among raring typology (groups 1-6 intensive, 7-10 semi-intensive/extensive, 11-14 wild-caught)

PFOS was found in all the subjects with concentrations from 11 to more than 10,000 ng/kg: the highest measured value was 12,405 ng/kg, but since method performances were validated in the 6-10,000 ng/kg range, this measure could not be totally reliable. Median of the data was 49 ng/kg and the mean 783 ± 1878 ng/kg.

PFOA was detected in most of the samples (the results below the LOQ were the 26% of the total) with a maximum concentration of 487 ng/kg. The median was 23 ng/kg and the mean 30 ± 49 ng/kg.

The statistical analysis of the data highlighted some interesting aspects of this monitoring. At first, subjects were grouped according to their morphometric characteristics (weight and length) and for each parameter they were divided in two groups, above and below the median value (≤0.489 kg). Then, subjects were grouped according to the rearing type (intensive, semi-intensive/extensive and wild-caught). Comparison of PFAS concentrations between the two weight-groups showed that subjects below 0.489 kg resulted less contaminated by PFOS and PFOA then those above 0.489 kg (Mann-Whitney test $p<0.001$). Similarly, fishes measuring less than 35.3 cm had significantly lower PFAS concentrations than those longer than 35.3 cm (Mann-Whitney test $p<0.001$).

For what about differences in PFAS contamination among subject belonging to different rearing types, the analysis of data highlighted slight differences between PFOS and PFOA behaviour: the presence of PFOS among the rearing groups was notably variable, resulting wild-caught fish significantly more contaminated by PFOS than farmed fish, and sea basses from semi-intensive/extensive rearing showed higher concentrations of PFOS compared to those from intensive rearing (Kruskal-Wallis test $p<0.001$). As regards PFOA, remarkably lower concentrations were detected in fish from intensive rearing, but between subjects from semi-intensive/extensive rearing and wild-caught levels of PFOA no significant differences have been highlighted by the statistical analysis (Kruskal-Wallis test $p<0.001$).

Results are reported in Table 21, in the next page.

Groups	PFOS (ng/kg)			PFOA (ng/kg)		
	Mean (DS)	***Median***	***Min-max***	***Mean (DS)***	***Median***	***Min-max***
Weight ≤ 489.1 g	168 (530)	33	11-3826	19 (8)	22	9-39
Weight > 489.1 g	1398 (2460)	570*	25-12405	42 (67)	28*	9-487
Length ≤ 35.3 g	213 (596)	33	11-3826	19 (9)	22	9-51
Length > 35.3 g	1326 (2468)	498*	25-12405	42 (67)	27*	9-487
Intensive rearing	34 (16)	32	11-105	18 (8)	21	9-51
S.-int./ext. rearing	283 (291)	130	25-840	29 (11)	29	9-67
Wild-caught	2468 (2897)	1345	112-12405	54 (86)	28	9-487

Table 21: PFOS and PFOA concentrations in sea basses, separated according to weight, length and type of raring in group of 70 subjects (other than semi-intensive/extensive rearing group that was composed of 30 samples and the wild caught subjects that were 40 in total).

** Statistically significant different values among PFOS and PFOA (Mann-Withney test p<0.001)*

Among the analysed samples, some subjects were identified as outliers and excluded, this involves 4 wild caught and 2 intensively farmed subjects for PFOS concentrations, and 4 wild-caught and 2 semi-intensively or extensively farmed subjects for PFOA levels.

After the exclusion of the mentioned outliers, the comparisons of PFOS and PFOA concentrations among the three groups gave the same results obtained when all the subjects were included in the study (Kruskal-Wallis test p<0.001). Indeed, without the outliers, PFOS median (min-max) concentrations in the wild-caught and in the intensively farmed were 1279.4 (112.4-6007.7) ng/kg and 130.1 (24.8-839.6) ng/kg, respectively; similarly, PFOA median (min-max) concentrations in the wild-caught and in the intensively farmed were 27.65 (9.0-101.9) ng/kg and 28.1 (9.0-36.6) ng/kg, respectively.

Moreover, the first robust multivariate linear regression model highlighted that PFOS concentration was significantly correlated with the type of farming, but not with sea

basses' weight. Similarly, in the second model, PFOA concentration resulted significantly correlated exclusively with the type of farming (Table 22).

	PFOS (ng/kg)				PFOA (ng/kg)			
	Coeff.	***[95% Conf. interval]***		***P value***	***Coeff.***	***[95% Conf. interval]***		***P value***
Intensive rearing	*0*	*(reference category)*			*0*	*(reference category)*		
S.-int./ext. rearing	308.3	111.4	649.3	0.006	10.4	3.5	17.2	0.003
Wild-caught	1891.1	1257.6	2524.5	0.000	14.3	6.4	22.2	0.000
Weight	-0.6	-1.7	0.5	0.299	-0.0	-0.1	0.0	0.557
R2	0.45				0.15			

Table 22: Results of the two multivariate linear regression models, setting PFOS and PFOA as dependent variable

Results obtained in the current study are in line with those reported by other recent monitoring on seafood (Berger *et al.*, 2009; Haug *et al.*, 2010b; Hrádková *et al.*, 2010; Schuetze, *et al.*, 2010; Shi *et al.*, 2010; Zhang *et al.*, 2011; Domingo *et al.*, 2012; Guo *et al.*, 2012; Hloušková *et al.*, 2013; Yamada *et al.*, 2014) and confirm the already discussed importance of fish in human exposure to PFAS.

Fish contribution to human contamination should be monitored especially in those Countries where seafood represents a major component of the diet, and particular relevance must be given to those species living in certain habitats and belonging to high trophic levels.

The present results are comparable with those from recent studies investigating PFOS and PFOA presence in fillets samples of *D. labrax*: Paiano *et al.* (2013) analysed sea basses from two fish farms in the Mediterranean Sea and measured maximum concentrations of PFOS and PFOA of respectively 150 and 90 ng/kg; Yamada *et al*. (2014) conducted a survey on French marine and freshwater fish species and detected PFOS and PFOA maximum concentration of respectively 790 and 2600 ng/kg.

In another monitoring project on contaminants in seafood, European sea basses from three different rearing structures in southern Italy coasts were analysed: although the very limited number of subjects included in the study, the same order of magnitude for both PFOS and PFOA levels in fish was observed, reaching the maximum concentration of 59 and 50 ng/kg, respectively (Istituto Superiore di Sanita, 2011).

Different levels of contamination between wild-caught and farmed fish were reported by two recent preliminary studies (Eriksson *et al.*, 2013; Paiano *et al.*, 2012); the main causes of such differences were found in diet and habitat: a higher contamination seems, at least for PFOS, the consequence of biomagnification for predatory habits of this fish, while the feed employed for its farming is supposed to represent a minor source of exposure.

Such dietary differences are also responsible for different muscle/fat ratio: the higher fat content of farmed fish may represent a further reason for lower PFAS levels, since they tend to bind to proteins rather than accumulate in adipose tissue (Nania *et al.*, 2009). This is an important aspect to consider, since it is well known that other halogenated contaminants have significantly higher concentrations in farmed fish compared to wild fish (Antunes and Gil, 2004; Carubelli *et al.*, 2007; Mieiro *et al.,* 2011; Trocino *et al.,* 2009).

At first instance, our results suggested that the concentrations of PFOS and PFOA were not only correlated to the raring type but also to their morphometric characteristics (length and weight). However, after the application of the multivariate linear regression models, it appears evident that the differences observed in the concentration of PFAS are exclusively linked the origin of the fish (wild caught or farmed). This may be due to the different dietary pattern, while weight- or length-related differences are more likely to be a consequence of the fact that farmed fish are also shorter and lighter than wild-caught ones.

As already mentioned in Section 6.2.2.1 , another influencing factor for PFAS presence, especially in caught fish, is sampling location, being the level of pollution of the aquatic environment influenced by multiple factors. In support of this assumption, data from a study conducted on PFOS levels in livers of European sea basses from seven locations

along the Western Scheldt river (The Netherlands) reported a pollution gradient along the river (Van de Vijer, 2005). In particular, contaminations more than 10-fold higher were found in the proximity of the river estuaries and this condition particularly influences the contamination of *D. labrax*, since this species normally uses estuaries as nursery area and juveniles remain in that habitat for about two years more (Martinho *et al.*, 2008).

To our knowledge the present monitoring on the presence of perfluoroalkylated compounds is the first conducted on a large sample of fish of the same species from wild or farmed sources. Notably differences in PFOS and PFOA levels were observed in subjects caught in four sampling locations, three located in the Mediterranean Sea and one in North-east Atlantic Ocean.

In conclusion, the obtained data confirmed the strong influence of the geographical origin on fish contamination. The between-species variability of PFAS levels also confirmed the high species-dependent contamination, which became even more relevant in the multi-species assessment, and is supported by worldwide data available in literature too. For all these reasons, a reliable assessment of the risk of exposure for consumers of this food category is not an easy task.

Finally it is important to perform further adequate investigations, with the aim to identify which seafood products might require precautionary measures, eventually also extending the monitoring to other emerging contaminants, to better estimate the entity of the health risks related to environmental pollutants.

7. Conclusions

A rapid and economic method based on ultra-performance liquid chromatography-mass spectrometry (UHPLC-MS/MS) for the detection of glycine betaine (GB) in *Tapes philippinarum* has been developed. The procedure has been successfully validated in accordance with current European regulation guidelines (Commission Decision 2002/657/EC), modified to make them suitable for the analysis of an endogenous compound in a very complex matrix. The detection of glycine betaine (GB), performed in the Department of Food Technology of the IIM of Vigo, Pontevedra (Spain), was obtained comparing several chromatographic techniques by means of a HPLC coupled with a linear ion trap quadrupole mass spectrometer. The analytical method was completed and successfully validated in CABA-Lab using an UHPLC-MS/MS system.

Although developed on a complex matrix, as clams, the method proposed results rapid and robust, and demonstrate very satisfying performances in terms of linearity, trueness and precision, also thanks to the application of an adequate internal standard.

The applicability of the method developed to another matrix of marine origin was also tested, that is common octopus (*Octopus vulgaris*); data obtained by the quantification of GB in this matrix were found in agreement with those published in literature (Konosu and Hayashi, 1975).

For what in our knowledge this is the first method for the analysis of GB in clam tissue, developed and validated by a UHPLC-MS/MS system.

For what about the analysis of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in seven different fish species, the good performances of the method adopted allowed the fulfilment of a preliminary monitoring on *Dicentrarchus labrax, Merluccius merluccius, Mugil cephalus, Pleuronectes platessa, Scomber scombrus* and

Sparus aurata, and the estimation of the daily contribution of seafood to PFOS and PFOA assumption in Italian population.

Results about the exposure suggested that, both for general population and for high consumers, the risk for human health related to fish consumption is unlikely. However, since an inter-species and inter-studies variability was observed, such risk cannot be generalized and could depend on multiple and still unclear factors. As a consequence, further surveys are necessary to upgrade the risk assessments on fish consumption, focused on certain species and their sampling place.

The subsequent mono-specie monitoring, carried out on 140 sea bass samples from different sites in the Mediterranean area, put on evidence a positive correlation between measured concentrations and fish rearing systems, being wild caught sea basses sensibly more contaminated than farmed sea basses.

Moreover, the study confirmed the strong influence on fish contamination by its geographical origin and the proximity to polluted areas. For what in our knowledge, no other mono-specie monitoring on the presence of PFAS was previously conducted on such a large number of fish of the same species, from wild or farmed sources.

At the same time, the inter-species variability of PFAS contamination found in this study, supported by worldwide data available in literature, suggests that a reliable assessment of the risk of exposure for consumers of this food category is not an easy task. Therefore it is important to perform further adequate investigations, with the aim to identify which seafood products might require precautionary measures, eventually also extending the monitoring to other emerging contaminants, to better estimate the entity of the health risks related to environmental pollutants.

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