UNIVERSITY OF PADUA

DEPARTMENT OF COMPARATIVE BIOMEDICINE AND FOOD SCIENCE

Master Course in Biotechnologies for Food Science

Evaluation of the nutritional features of wild fish species in Santa Pola port (Es) used for human consumption with a related study of the presence of toxic contaminants

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ACADEMIC YEAR 2013-2014

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ABSTRACT

Currently, few foods are particularly appreciated as the fish in the daily diet of the Spanish people. In general, fish has a low calorie content, it is a good source for protein of high biological value, provides vitamins, many minerals and it is also rich in ω-3 polyunsaturated fatty acids. The excellent nutritional quality of this product, the beneficial effects on health and the large number of fish varieties in the market, make sure it is widely accepted by consumers of all ages and in all circumstances. Moreover, we are aware that fish species, through biomagnification process accumulate heavy metals in the edible portion throughout the entire food chain. This is a critical and important factor concerning the interest for the public as well as the environmental health.

In recent years in the Spanish community the demand for fishery products has grown more and more rather than for all the other products of animal origin, with a higher consumption of fresh fish recorded in 2008. According to what we have stated so far, we have evaluated the chemical composition and the nutritional features of three fresh fish products of usual consumption in all the Spanish community: *M. merluccius, P. blennoides* e *M. barbatus,* getting to know in detail how much the mineral salts content meets the daily nutritional needs. Then we analyzed also the content of heavy metals in order to understand the general levels of toxic elements and, in particular, if the levels of cadmium, mercury and leads are on a safety range or if they are bad for the consumer's health. The whole study is carried out in order to make the costumer acquainted of both the beneficial characteristics of these raw material intended for commercial use, and also acquainted of the possible risks that may arise because of toxic elements in the edible part. We were able to perform all this work through standard laboratory procedures, as Kjeldahl and Soxhlet method to search for the total protein and lipid, the ICP-AES and AFS spectrophotometer to search for the essential mineral elements and toxic ones.

Keywords: *M. merluccius, P. blennoides, M. barbatus*, heavy metals, chemical-nutritional composition, RDA, Regulation (EC) n.629/2009 of the Commission of the 2nd of July 2008.

RIASSUNTO

Attualmente pochi alimenti vengono particolarmente apprezzati come lo è il pesce nell'alimentazione quotidiana degli spagnoli. Il pescato, in generale, presenta un contenuto calorico basso, è una buona fonte di proteine ad alto valore biologico, apporta vitamine, numerosi minerali ed è anche ricco in acidi grassi polinsaturi ω-3. L'eccellente qualità nutrizionale di questo prodotto, gli effetti benefici sulla salute e il gran numero di varietà presenti nel mercato, fanno si che sia ampiamente accettato dai consumatori di tutte le età e circostanze. Si è a conoscenza oltretutto che le specie ittiche, attraverso il processo di biomagnificazione, accumulano metalli pesanti nella porzione commestibile lungo tutta la catena alimentare. Questo fattore è di seria importanza per quanto riguarda l'interesse della salute pubblica nonché di quella ambientale.

Negli ultimi anni, nella comunità spagnola, è cresciuta sempre più la domanda di prodotti della pesca rispetto a tutti gli altri prodotti di origine animale, con un maggior consumo associato al pesce fresco osservato nel 2008. Con ciò che è stato esposto fino ad ora si è valutata la composizione chimica e le caratteristiche nutrizionali di 3 prodotti ittici freschi di consumo abituale in tutta la comunità spagnola, *M. merluccius, P. blennoides* e *M. barbatus,* capendo in dettaglio quanto il contenuto di sali minerali soddisfa le esigenze giornaliere nutritive. È stato analizzato poi anche il contenuto di metalli pesanti per comprendere i livelli generali di elementi tossici presenti e, in particolare, se la presenza di cadmio, mercurio e piombo si trova in un range di sicurezza oppure nuoce alla salute del consumatore. Il tutto viene fatto al fine di mettere a conoscenza il consumatore sia sulle caratteristiche benefiche di queste materie prime destinate al commercio sia sugli eventuali rischi che possono intercorrere data la presenza di elementi tossici nella porzione edule. Si è potuto svolgere tutto questo lavoro grazie a metodiche di laboratorio standard, come il metodo Kjeldahl e Soxhlet per la ricerca della proteina e del grasso totali, lo spettrofotometro ICP-AES e AFS per la ricerca di elementi minerali essenziali e tossici.

Parole chiave: *M. merluccius, P. blennoides, M. barbatus*, metalli pesanti, composizione chimico-nutrizionale, RDA, Regolamento (CE) n.629/2009 della Commissione del 2 Luglio del 2008.

Chapter I: INTRODUCTION

This thesis has the objective to explore and deal with two important issues that go along with the concept of health and safety of the people with regard to the consumption of sea fish products of interest.

The first part deals in particular with the research and analysis of nutritional features, highlighting the moisture percentage, total ash content, the nutritional intake of protein, lipids, and some of the most important essential mineral elements (especially Ca, Cu, Fe, K, Mg, Mn, Mo, Na, P, Zn).

The second part focuses on the accumulation of contaminants in the edible portion of the fish, facing the issue of heavy metals and aiming to consider the extent of the damage their concentration can cause to the consumer.

The study was focussed particularly on three fish species of wide commercial interest and usually sold and consumed in Spain (Fernández *et al.* 2005) and these are: *Merluccius merluccius*, *Phycis blennoides* and *Mullus barbatus*, caught in Santa Pola port (Valencian Community) (García-Rodríguez *et al.* 2005; García-Rodríguez *et al.* 2006a; Benedicto *et al.* 2008).

The fish is one of the fundamental pillars of the Spanish diet, providing high quality and variety, and it has been rated as one of the main components of the Mediterranean diet. It is therefore clear that its consumption is highly recommended (Santaella 2011). The excellent nutritional quality of the base product makes it welcomed by consumers of all ages. This acknowledgement is due mostly to the huge variety of products available in the market and to the great improvement in food technology that have enabled the development of new products (Martìnez Alvarez 2005).

The observations relating to human health state that the success of this product comes from some elements: a) the consumers appraise it as a digestive, nutritious and healthy food, both for a healthy person and for someone who suffers from frequent pathologies b) The fish is characterized as an important source of nutrients, mainly proteins of high biological value and lipids rich in polyunsaturated fatty acids omega-3 type, it also contains minerals and vitamins, for this reason it is regarded as a food with high nutritional quality (Iglesias & Goméz 2005).

According to the data obtained from the Ministry of Agriculture, Food and Environment during the period 2007-2012 (Magrama 2012), Spanish families yearly consumed a large amount of fishery products even though there was a decrease in consumption of 1.3kg per capita. There was a peak in 2008 which recorded 1,230.2 mln kg of fish consumed (26.8kg per capita). The most remarkable use regards fresh fish (almost 12kg per person per year), which in percentage represents the 44% of the total consumption of fishery products. Then follows the consumption of crustaceans and molluscs, which covers the 15.7% of the total consumption (4.2kg per person per year), canned fish consumption is 15.7% (4.2kg per person), frozen fish 12.3% and finally the consumption of shellfish, frozen and cooked crustaceans which covers a share of 11.7% of the total.

Considering the above data, these lead us to focus our research on the analysis of the most consumed products in Spain. We considered important to carry out a study aimed at the detection of the nutritional properties of a number of species of fish of wide consumption.

This part of the work aims to identify the nutritional value in relation with the beneficial properties of the value itself, with regard to wild species consumed without any type of processing (smoked, salted, canned etc.). It is provided the analysis of samples of wild fresh fish from Alcantarilla wholesale fish market from Pescados Beniajan Company, SL (Murcia).

Following, this research has focused on another important pillar closely related to human health concerning food safety: the presence of chemical contaminants, and in particular heavy metals in the edible portion of fish products.

Since in recent years there has been a growing demand for fish products, mainly due to their better nutritional properties with respect to the meat traditionally consumed (Moretti & Busetto 2010), and as in the Spanish community there is a high demand and consumption of fishery products (Magrama 2012), this undoubtedly implies increasing the monitoring from health inspectors and veterinarians, and in case the stop of marketing for species potentially toxic or allergenic (Busato 2010).

For this reason the study included also the search for toxic and dangerous contaminants for human health, since this is an essential factor to understand whether or not the products represent a risk for the consumer.

1.1 Nutritional value of fish

The main chemical components of the fish are: water, proteins, and lipids. These components have the greatest importance in terms of nutritional value (Ros *et al.* 2010).

Nutritional value of the fish depend on many factors, that can be divided into: a) intrinsic factors (species, age, sex and physiological factors), b) dietary factors (quality of diet: wild/farmed...); c) environmental factors (food availability, salinity, temperature) (Grigorakis 2007).

It is obvious that, considered the above b) point, we can state that there is a big difference in the chemical composition between an aquaculture fish and a wild fish. For example, a farmed fish subjected to a regime of intensive growth has a higher percentage of fat and a low percentage of water compared to wild specimens from sea fishing (Mnari *et al.* 2007; Santaella *et al.* 2007). Also with regard to the minerals amount, many studies have shown that the concentration in minerals is greatly affected by different environmental factors and intrinsic such as those mentioned above in a) and c) (Thodensen 2001; Roy *et al*. 2006).

Water is the element that we find in greatest quantity in the composition of fish species and its presence is inverse to the percentage of fat (Wheaterley *et al.* 1983).

In whitefish and in semi-oily fish the percentage of water content is between 76% and l'80%, while in oily fish the water content is less, it can reach a maximum concentration of 75%.

Also age, sex, sexual maturity and reproductive period are causes that can modify these parameters. During the breeding season the female can concentrate fat storage in the visceral apparatus: and for this reason we could find a decrease of fat in the meat with a consequent increase of water content (Asknes *et al.* 1986).

As for the protein content, it is in general between 13 and 20% (Ros *et al.* 2010). The protein compounds in fish are made up of all the essential amino acids, with an abundant amount of lysine and tryptophan (alike milk protein, eggs and meat of mammals), and this confirms a high biological value of the fish meat (Aquerreta 2000).

According to fat content in the edible portion of the fish, the fishes can be classified as follows:

- Non-oily fish: with a fat content of up to 2.5% (hake, greater forkbeard, gilt head bream etc.);
- Semi-oily fish: from 2,5 to 6% (mullet, anchovies, carp, etc.);

- Oily fish: from 6% to 25% (salmon, tuna, eel, etc.);

In non-oily fish the lipid content is more concentrated in the liver and mature gonads, while in oily fish it is localized mainly in the muscles and into the subcutaneous tissue, abdomen muscles and the muscles that allow the movement of the tail and fins (Testi 2006; Mnari 2007).

Also these factors greatly differ according to species, sex, season, and especially the diet composition. If we compare farmed specimens and wild ones, It is clear that a farmed fish will have a higher percentage of fat than a wild one, and this is due to the composition of the diet it is fed but especially it is due to the high density of fishes in a same area that prevents them to swim in a free way (Flos *et al.* 2002; Mnari 2007).

The minerals and trace elements become part of the muscles and skeleton of fish; they set acid-base balance and are also an important component of hormones and enzymes (Lall 1995; Alasalvar *et al.* 2002). The majority of the mineral salts, outside their specific function, take part in small concentrations in vital phenomena, as enzymes activators, transporters or regulators. The fish takes minerals, necessary for its normal conservation of vital functions, through its diet and water that normally circulates through the gills or skin (Watanabe *et al.* 1997; Lall 2002).

The minerals and trace elements settle mainly on the fish skeleton as well as on the edible part and organs (Lall 2002). The main constituent elements are phosphorus and potassium (from 200mg/100g to 400mg/100g). Of course we have to take into account that sea fishes have a high iodine content and a relatively low sodium content (between 20 and 140mg/100g in the edible part), and this make them suitable for good diets. A Mediterranean diet, rich in oily fish and all the kinds of shellfish, can meet the 20% or more of the daily needs of phosphorus, iron, selenium and iodine (Pèrez Llamas *et al.* 2005).

1.1.1 RDA (Recommended Daily Allowed)

To ensure the complete daily nutritional needs of a person, a balanced diet is important and this has to bring the right amount of nutrients and energy for health and wellbeing.

It is important to set nutritional standards that refer to the nutrient amount to be taken by a human being to meet daily nutritional needs.

The nutritional standard that we took into consideration in this study is the RDA (Recommended Daily Allowed) which corresponds to the average daily intake level adequate to meet the needs of nearly all the healthy persons in a particular life stage and gender (see Tab.1). Nutritional standards are intended to protect the entire population from the risk of nutritional deficiencies, to provide the basis for assessing the nutritional adequacy of the average diet of a population, for food education and food labelling. In this study we take into account the daily intake of mineral salts of an average human being (male/female, 30-55 years) in good health and average weight (65-80kg).

ELEMENT	Unit of measure	Men 30 – 55 years	Women 30 – 55 years	Riferimento bibliografico
Ca	mg/die	800 - 1000	800 - 1000	*(AFSSA 2001)*
Cu	mg/die	1 – 2.3	0.9 – 1.8	*(Van Dokkum 1995)*
Fe	mg/die	8 – 10	18 - 20	*(SCF 1993)*
K	mg/die	3100 - 3500	3000 - 3200	*(SCF 1993)*
Mg	mg/die	350	330	*(FNB 2004)*
Mn	mg/die	1 – 10	1 - 10	*(WHO 1991)*
Mo	g/die	75 - 250	75 - 250	*(WHO 1996a)*
Na	mg/die	4000 - 6000	4000 - 6000	*(SCF 1993)*
P	mg/die	800 - 900	800 - 900	*(FNB 2004)*
Zn	mg/die	15	15	*(Van Dokkum 1995)*

Table 1. Recommended Daily Allowance (RDA) of some mineral elements (macro and micro elements) considered in this study

1.2 Heavy metals and biomagnification

Heavy metals are one of the categories most studied and controlled or monitored. It is well known that during the last decades, contaminants amount released into the environment by humans, particularly in the Mediterranean Sea, has increased more and more, reaching levels of alert which required in-depth studies and significant legislative action. Moreover, the Mediterranean Sea being a semi-closed basin and surrounded by countries that are

among the most populated and industrialized in the world since ancient times, brings a level of pollution by toxic compounds which is constant in time and space. An important component of heavy metals in the Mediterranean is due to direct input, in coastal waters of municipal and industrial waste (Moore & Ramamoorthy 1984; UNEP 1996; Riba *et al.* 2005; EEA 2006; Benedicto *et al.* 2008).

Heavy metals have a strong tendency to amass in the soil (Hg, Pb, Cr, Cd, Co, Ni, etc.) and to enter the food chain, causing toxic and harmful effects on living beings even at concentrations not high [2]. Some of these xenobiotics are completely devoid of biological activity (Hg, As, Pb) and if accumulated at a certain dose in edible products can cause harmful effects to the consumer in the short/long-term (Marchetti *et al.* 1998).

The sea food web is the access point for the substances in the environment and they enter into the organisms through different processes moving from one trophic level to another (Hassan *et al.* 2010). The possible ways through which a pollutant enters a sea organism are 3: a) the respiratory system (the gills), b) the ingestion of water and food (gastrointestinal tract) c) skin absorption (McKay & Fraser 2000; Peña *et al.* 2001; Quero Llor 2011). The diet is one of the main way through which the animal organism stores environmental pollutants (Peña *et al.* 2001; Quero Llor 2011). Biomagnification is the process that involves the transfer of pollutants from the food into the organism and this leads to a higher concentration of xenobiotics in the organism than in the food itself (Gray 2002). Following the food pyramid, the accumulation of toxic substances states an increase of accumulation from the bottom upwards (Fig.1). Obviously, a sea organism that is a hunter high in the food pyramid will have a greater concentration of xenobiotic compared to another one which is at a lower level (Canli & Atli 2003).

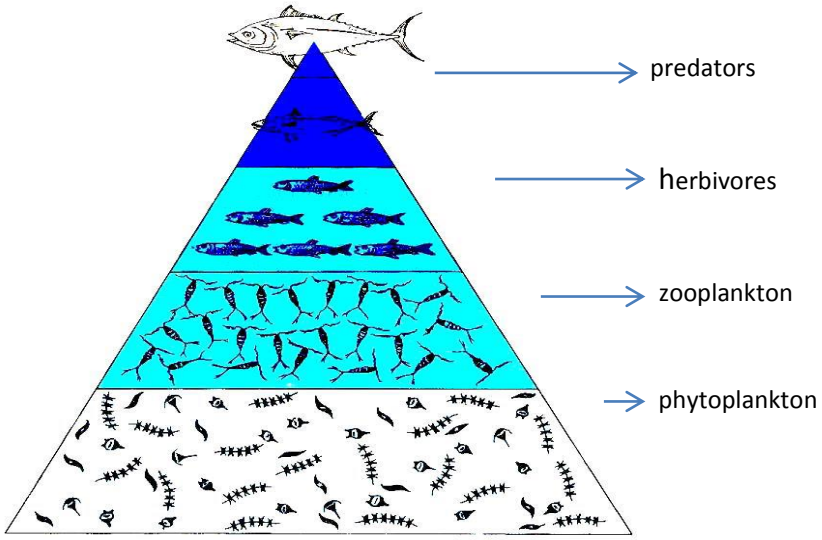

Figure 1. The biomagnification

Biomagnification is the amplification of a pollutant that moves up towards higher levels of a food chain (Rand *et al.* 1995). Heavy metals can be taken through the diet, in this way they move from a lower food level to a higher one reaching the human being. Our body cannot remove them with normal detoxifying processes, without the use of a chelating agent (Dimercaprol, D-penicillamine, Dimercaptosuccinic acid, EDTA), capable of binding to the metal and move it outside the body. So toxic contaminants remain in the tissues, even for decades, and are a serious threat to human health (Vallee & Ulmer 1972; Fortuna 2009).

Every day the human being accumulates more and more heavy metals in the body and these ones stop the activity of several enzymes complex, since the elimination takes place only minimally: through salivation, perspiration, breastfeeding, etc. The metals concentrate particularly into some organs (such as brain, liver and kidneys) and bones, damaging them and they are often a factor that worsens many chronic diseases (Storelli *et al.* 2001). The toxicity of heavy metals, that means their ability to move through biological membranes of the target body, depends on their physical-chemical properties (molecular size of the xenobiotic, degree of solubility in water, ionization, etc.), but the most important is their lipophilic property. In fact the concentration of harmful elements in the target body varies with the lipid content and it is directly proportional to it (Gray 2002). The fat-soluble chemical stressor enters through the diet and crosses the lipid phase of the biological membrane. Once through the cell membrane, following different pathways (transport and

passive diffusion or endocytosis), the xenobiotic interacts mainly with the active sites of enzymes (such as -OH,
-SH, COOH, NH2), causing the loss of their functionality, or replace an essential metal in an enzyme or in a necessary protein. Of course, the sites of action may differ depending on the degree of affinity between the metal and the part of the body. Obviously the target body tries to activate possible elimination pathways to expel harmful element, starting the metabolism of contaminants through detoxification (Klaassen 1990; Cussi 2010).

1.2.1 Information about the present laws

The European Union (EU) set maximum levels for some pollutants, in order to decrease their presence in food to the minimum levels that can reasonably allow good agricultural or manufacturing procedures. The objective is to obtain a high level of public health protection, in particular concerning the sensitive groups: children, allergic people, etc.
The current legislation follows the Regulation (EC) n.629/2009 of the Commission of the 2nd of July 2008 amending Regulation (EC) No. 1881/2006 of the 19th of December 2006 setting maximum levels for some pollutants in foodstuffs: nitrates, mycotoxins (aflatoxins, ochratoxin A, patulin and Fusarium-toxins), heavy metals (see Table 2, 3 and 4: lead, cadmium, mercury), the monochloropropanediol 1.2 (3-MCPD), the dioxins and PCB dioxin-like, polycyclic aromatic hydrocarbons (PAH) and inorganic tin [2]. The food with higher values of pollutants than the ones showed in the following regulation can't be placed in the market. These limits are related to the edible part of the foodstuffs and shall also be applied to mixed or processed, dried or diluted food products, and if necessary, with a concentration or dilution factor, that means taking into account the related proportions of the ingredients in the product mixture.

	Foodstuffs [1]	Maximum levels (mg/kg of fresh weight)
3.1	**Lead**	
3.1.5	Muscle of fish (24) (25)	0.30

Table 2. Regulation (EC) n.629/2009 Commission of the 2nd July 2008 amending Regulation (EC) n. 1881/2006 of the 19 th December 2006

	Foodstuffs [1]	Maximum levels (mg/kg of fresh weight)
	Cadmium	
3.2.5	Muscle of fish (24)(25), excluding species in points 3.2.6, 3.2.7 e 3.2.8	0.050
3.2.6	Muscle of fish of the following species (24)(25): Atlantic bonito (*Sarda sarda*) two-banded seabream (*Diplodus vulgaris*) European eel (*Anguilla anguilla*) Thicklip grey mullet (*Chelon labrosus*) Atlantic horse mackerel (*Trachurus species*) louvar or luvar (*Luvarus imperialis*) scomber (*Scomber species*) Europian pilchard (*Sardina pilchardus*) Sout American pilchard (*Sardinops species*) tuna and little tunny (*Thunnus species, Euthynnus species, Katsuwonus pelamis*) wedge soe (*Dicologlossa cuneata*)	0.10
3.2.7	Muscle of fish of the following species (24)(25): auxis (*Auxis species*)	0.20
3.2.8	Muscle of fish of the following species (24)(25): anchovy (*Engraulis species*) swordfish (*Xiphias gladius*)	0.30

Table 3. Regulation (EC) n. 1881/2006 of the 19 th December 2006

	Foodstuffs [1]	Maximum levels (mg/kg of fresh weight)
3.3	**Mercury**	
3.3.1	Fish products (26) and muscle of fish (24) (25), excluded the species listed at point 3.3.2. The maximum level applies to crustaceans, excluded the dark parts of meet of crab and the head, the thorax of lobster and similar large crustaceans (*Nephropidae* and *Palinuridae*).	0.50
3.3.2	Muscle of fish of the following species (24)(25): fishing-frog (*Lophius species*) seawolf (*Anarhichas lupus*) Atlantic bonito (*Sarda sarda*) eel (*Anguilla species*) roughy (*Hoplostethus species*) rock grenadier (*Coryphaenoides rupestris*) Atlantic halibut (*Hippoglossus hippoglossus*) kingklip (*Genypterus capensis*) marlin (*Makaira species*) four-spot megrim from genus Lepidorhombus (*Lepidorhombus species*) red mullet (*Mullus species*) pink cusk-eel (*Genypterus blacodes*) northern pike (*Esox lucius*) plain bonito (*Orcynopsis unicolor*) poor cod (*Trisopterus minutus*) Portuguese dogfish (*Centroscymnus coelolepis*) ray (*Raja species*) redfish of Sebastes genus (*Sebastes marinus, S. mentella, S. viviparus*) Indo-Pacific sailfish (*Istiophorus platypterus*) silver and black scabbarfish (*Lepidopus caudatus, Aphanopus carbo*) pagellus (*Pagellus species*) sharks (all the species) escolar (*Lepidocybium flavobrunneum, Ruvettus pretiosus, Gempylus serpens*) European sea sturgeon (*Acipenser species*) swordfish (*Xiphias gladius*) tuna and little tunny (*Thunnus species, Euthynnus species, Katsuwonus pelamis*)	1.0

Table 4. Regulation (EC) n.629/2009 Commission of the 2nd July 2008 amending Regulation (EC) n. 1881/2006 of the 19th December 2006

1.3 Description of the fish species

In this study we analyzed three different fish species of sea water, and we chose these as they represent some of the most consumed products in the region of Murcia and in the whole Spain (Fernández *et al.* 2005; García-Rodríguez *et al.* 2005, 2006b). In particular:

Merluccius merluccius (Linnaeus, 1758), *Phycis blennoides* (Brünnich, 1768) and *Mullus barbatus* (Linnaeus, 1758). They represent an important source for the commercial fishing port of Santa Pola for marketing on the Spanish wholesale fish market.

It is important to know the trophic level they belong to and the kind of diet of each analyzed specimen to be able to carry out some observations concerning the accumulation of pollutant elements. The breeding season too is an important factor to talk about concentration of lipid accumulation and consequently xenobiotics accumulation. For this reason hereafter we show the morphological features, in addition to the type of food, the reproductive period and geographical distribution.

1.3.1 Hake (*Merluccius merluccius*, Linnaeus 1758)

The hake[1] (*Merluccius merluccius*) is a white fish belonging to the order of Gadiformes (Tab.5), it is one of the most abundant species and consequently the most studied species in the demersal communities of the whole Mediterranean Sea (De Juana *et al.* 1987; Garcìa Rodriguez *et. al.* 1995; Moranta *et al.* 2008).

The scientific name wrongly mixed up the hake with the codfish, however these are two different fishes, even if they belong to the same species: the hake is nothing else but the young codfish. The two specimens present only weight and age differences and another morphologic difference in the caudal fins.

KINGDOM	Eukaryota
PHYLUM	Chordata
CLASS	Actinopterygii
ORDER	Gadiformes
FAMILY	Merluccidae
GENUS	*Merluccius*
SPECIES	*M. merluccius*

Table 5. Scientific classification of *M. merluccius*

[1]Synonyms: Spanish :Pescada; Pescadilla; Pijota. – French. Merlu; Merluchon; Lausse. – German: Seehecht; Hechtdorst. – Eng: Hake. – It: Merluzzo; Nasello. – Port: Pescada; P. Branca; Marmota. – Cat. Lluç. – Gal. Peixota.

The scientific name *Merluccius* comes from the word used to indicate the hake in the middle age: Maris Lucius, that is “sea pike”, because of its resemblance with the pike (De Juana & De Juana 1987).

The hake is a fish of medium size (Fig.2). It has a cylindrical body shape, elongated, thin and little compressed, a head quite long with the top flattened. His body is covered with small scales, it has a steel-gray colour on the back, silvery along the sides, white on the belly, while all fins are gray. The eyes are round and quite small, considering it is an inhabitant of the dark deep waters (Inada, 1981). In the Mediterranean the average size is 25-30 cm reaching a maximum of 60 cm. It’s a necto-benthic species with a huge range of bathymetric distribution (20-1000m) although usually it is caught at depths of less than 500m, in particular between 100 and 300 m since it prefers muddy and sandy sea bottoms of medium depth. (Oliver & Massuti 1995).

Figure 2: Hake (*Merluccius merluccius*) [3]

It is a voracious predator and it has a lively curiosity: he feeds mainly on small crustaceous of Pleuroncodes and Cervimunida genus and squids, but it doesn’t mind as well small teleosts (Casey & Pereiro 1995). It hunts always in midwater and never on the bottom. It may come up at lower depths to chase prays trying to escape. It has a nocturnal activity, in the daytime it stays in the depths and in the night it comes up to feed (Casey & Pereiro 1995). The reproductive period lasts almost all year round, although there are activity peaks that go from February to June in waters very far away from the shore (Olaso 1990; Martin *et al.* 1999; Nannini *et al.* 2001; Belcari *et al.* 2006).

The hake is an abundant species in the Mediterranean Sea, especially in the north. It is also found in the eastern Atlantic Ocean, from Norway to the parallel 21N of Cabo Blanco in Mauritania (Recasens *et al*. 1998).

The hake is the most marketed species in Spain, a kind of fish whose consumer is loyal.

The genus *Merluccius* includes 13 species and the distribution of twelve of these is shown below in Figure 3:

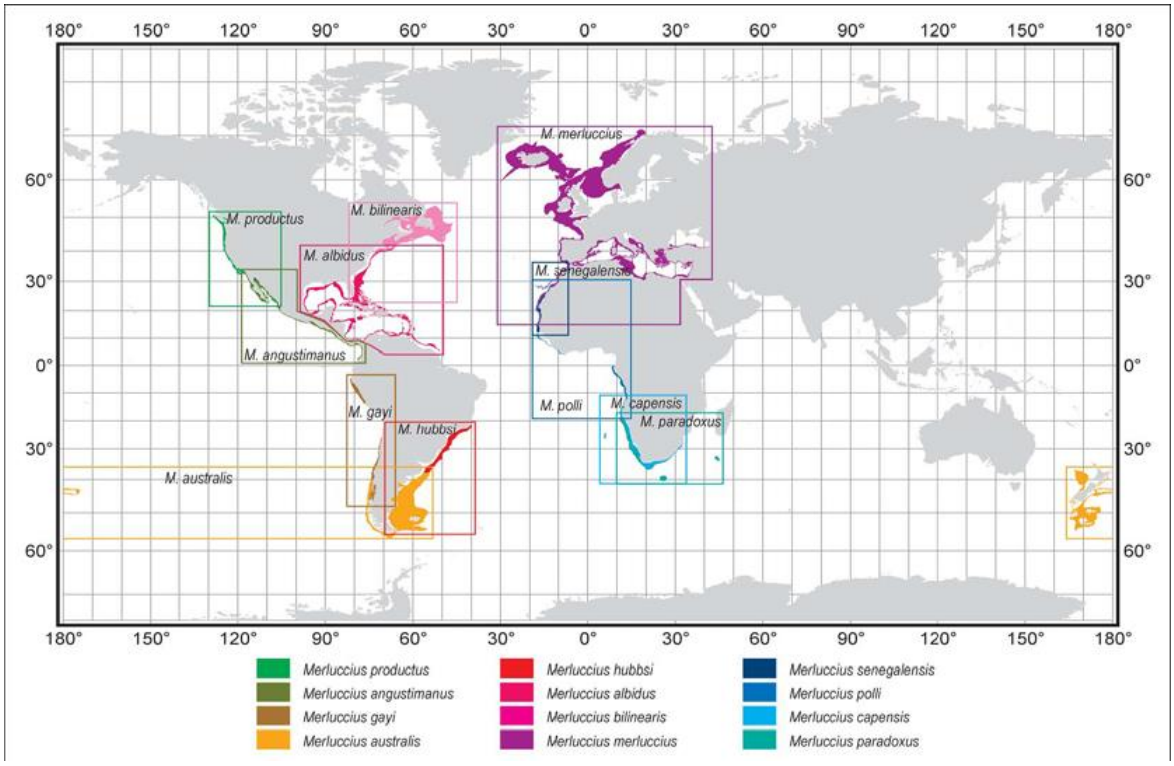

Figure 3. Geographical distribution of *Merluccius* genus

The thirteenth species is *M. patagonicus*. Until 2003, year when it was discovered, this species was mixed up with *M. hubbsi* and *M. australis*.

1.3.2 Greater forkbeard (*Phycis blennoides*, Brünnich, 1768)

This sea water fish is commonly known as Greater forkbeard[2], is a white fish belonging to Gadiformes order (Tab.6). Three species of *P. blennoides* are known: *P. blennoides*, *P. phycis*, *P. chesteri*.

[2]Synonims: Sp: Alfaneca; Escolano; Loncha. – Fr: Moustelle brune. – Ger: Miltermeer; Eng: Forkbeard. – It: Musdea. – Port: Abròtea. – Cat: Mòllerab roquera. – Gal: Barbada; Bertorella; Lorcha.

KINGDOM	Eukaryota
PHYLUM	Chordata
CLASS	Actinopterygii
ORDER	Gadiformes
FAMILY	Gadidae
GENUS	*Phycis*
SPECIES	*P. blennoides*

Table 6. Scientific classification of *P. blennoides*

The greater forkbeard has an elongated body and rather strong (Fig.4), getting more and more compressed towards the tail, its body length is approximately 9 times the size of its height. Its colour considerably varies depending on the place it lives, generally in surface waters it has a dark brown colour, in order to camouflage partly, blending with the colour of the rocks where they usually lurk, while in deeper waters it becomes considerably lighter (Cohen *et al.* 1990; Based Peired 2002).

The head is very small, quite flattened with large eyes and protruding. Two barbels with taste cells sprout up under the jaw.

The greater forkbeard can reach a maximum size of 60cm.

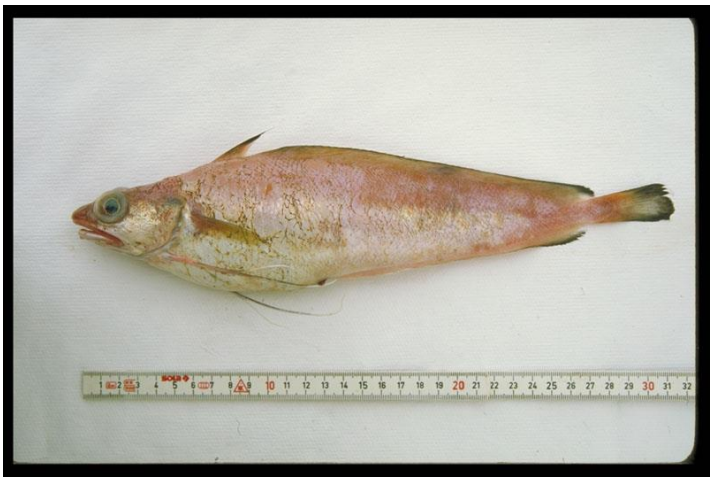

Fig. 4 Greater forkbeard (*Phycis blennoides*) [4]

It is a carnivorous species and it belongs to benthic sea and stays in muddy seabed between 200 and 800m. It feeds exclusively on small preys such as fishes and crustaceans and among them shrimps, prawns, crabs and then small pelagic creatures such as anchovies and

mackerels and moreover demersal and benthic creatures such as squids (Moranta *et al*. 2008).

The reproduction of this species takes place in spring time, preferably during April-May.

The greater forkbeard is found in the Mediterranean Sea and in the Northern Atlantic Ocean between Morocco and Northern Norway, including Iceland and Azores Islands (Fig.5). Usually it can be found in the darkest recesses, at different depths, in general between 100 and 800 m, but smaller specimens can be found inshore. It has typical sedentary habits. It is possible to find a greater forkbeard where the submarine geology has cracks and crevices (Ardizzone *et al.* 1998c; Riede 2004).

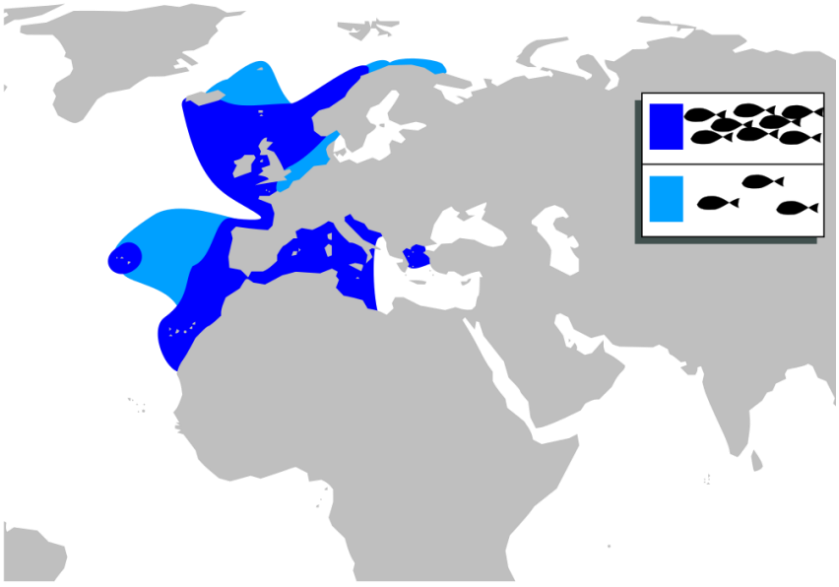

Figure 5 .Geographical distribution of *P. blennoides* [5]

1.3.3 The red mullet (*Mullus barbatus,* Linnaeus 1758)

The red mullet, *Mullus barbatus* (Linnaeus 1758), is a demersal species, it belongs to the Mullidae species (Tab.7). Indeed in the Mediterranean Sea there are two species belonging to genus Mullus: *M. barbatus* and *M. surmuletus.* Worldwide are known five species belonging to this genus: *M. argentinae*, *M. auratus*, *M. barbatus barbatus*, *M. barbatus ponticus*, *M. surmuletus*.

KINGDOM	Eukaryota
PHYLUM	Chordata
CLASS	Actinopterygii
ORDER	Perciformes
FAMILY	Mullidae
GENUS	*Mullus*
SPECIES	*M. barbatus*

Table 7. Scientific classification di *M. barbatus*

The red mullet[3] has a slender body and not very compressed at the sides (Fig.6). The profile of the head is convex, the head is large with changing colour: when irritated it becomes pearly at the sides with carmine-coloured marks. It has always a longitudinal red stripe from the eye until the peduncle of the caudal fin. The mouth is small and it can extend two tactile barbels or "whiskers" very mobile that are used to search for food in the seabed where it lives; these barbels are generally so long to reach the opercula (Bianchi & Morri 2000).

Figure 6. Red mullet (*Mullus barbatus*) [6]

The eyes are very large, very close to the profile of the head and are very fragile. Its colour is typically carmine on the back and gradually becomes lighter on the sides and belly, up to take on a yellowish colour. Four longitudinal yellow stripes run through the sides (Based Peired 2002).

[3]Synonims: Sp: S. De mala casta; Mijarco; Iguelo. – Fr: Rouget de vase; R. Barbet. . Ger: Roter Meerbarbe. – Eng: red mullet. – It: Triglia di fango. – Cat: Moll fanguer. – Gal: Salmonete; Barbo.

The red mullet can reach a size of 40 cm when it has completed its development. Basically it is lazy, agile in the movements and it has gregarious habits (Tursi *et al.* 1994).

The red mullet feeds on small crustaceans and molluscs which hunts out with the barbels, as well as fry, polychaetes and especially worms.

The reproductive period lasts from April till August. During the breeding season, according to areas and climatic trend, the red mullet spawns very small pelagic eggs (De Ranieri 1979).

Red mullet as its similar species the striped red mullet (*M. surmuletus*) needs warm waters. It is a common species in the Mediterranean waters: the search for it goes from Scandinavia (but seldom) up to the coasts of Senegal (Labropoulou & Eleftheriou 1997; Tserpes *et al.* 2002) (Fig.7). It is a demersal species that lives in close contact with the seabed, mostly because of trophic reasons (Demestre *et al.* 1998; Tserpes *et al.* 2002; Moranta *et al.* 2008).

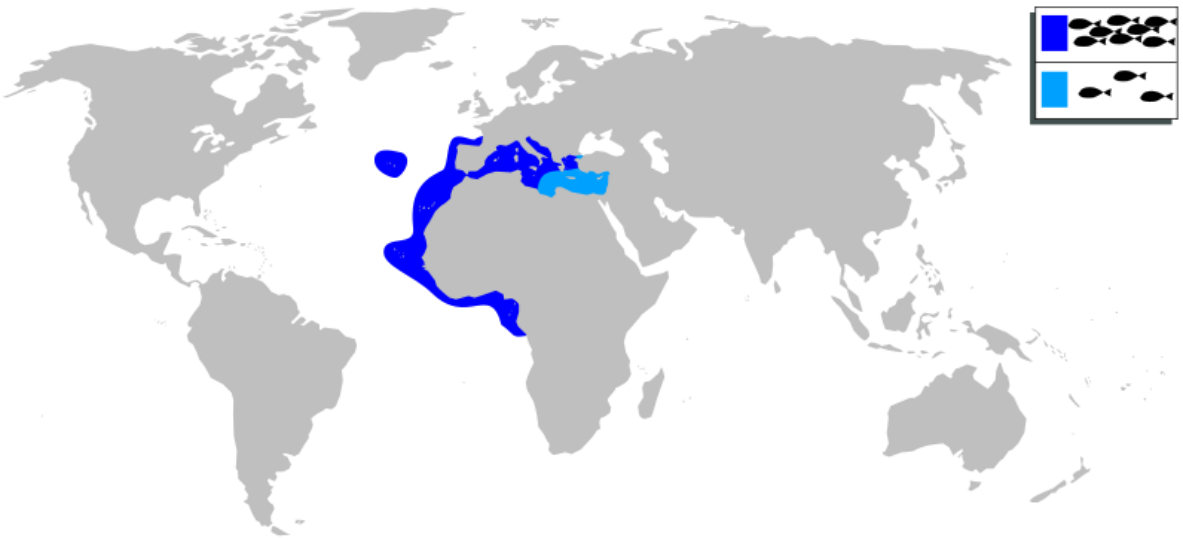

Figure 7. Geographical distribution of the *M. barbatus* species from [7]

1.4 Area of study: Santa Pola port

According to FAO the Mediterranean Sea is located in the so called zone 37 (Fig.8). Looking in particular at the sub-area 37.1 we find the division 1.1 which is the collection zone of the fresh samples of our study that is Santa Pola port (in the red circle).

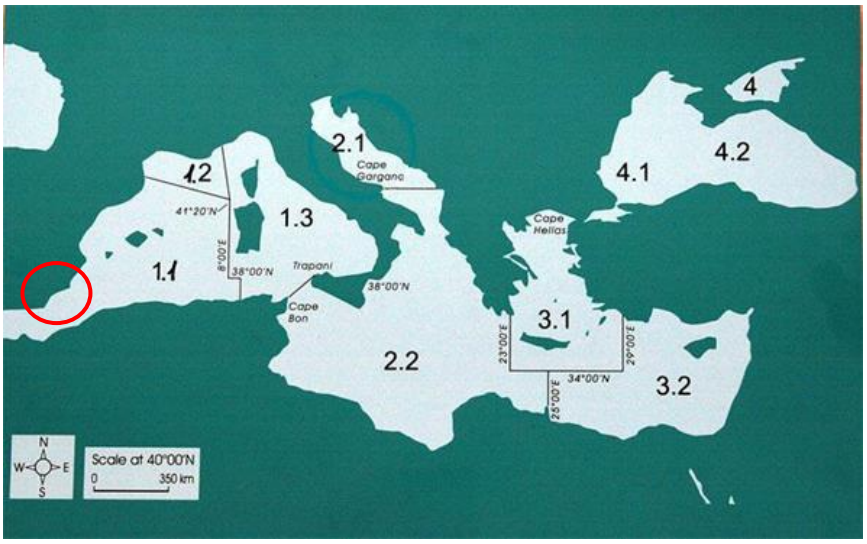

Figure 8. FAO zone 37 [8]

Costa Blanca is a coastline zone of Spain by the Mediterranean Sea, more precisely between Cape de la Nao and Gata Cape. It's a coastal stretch of southern and south-eastern Spain. Traditionally it is the coastline of Alicante province; in a more extensive sense, it includes the coastline of the province of Murcia and part of the province of Valencia and Almeria.

The south east zone of the Iberian Peninsula, between Cabo de Palos (Murcia region) and Javea (Alicante region) includes twelve fishing ports (among them Santa Pola port) and it has always been an important and rich fishing area, of these twelve ones the majority involves small-scale fishing and trawling fishing. Santa Pola port is located in this same city that is Santa Pola, on Costa Blanca (White Coast), in the region of Alicante. Its activities are mainly fishing on sandy and muddy sea floor of the continental shelf between 50 and 800 m depth. Catches are multi-species and they vary in composition according to the depth: between 50 and 150 m are fished mostly red mullets (*Mullus barbatus* Linnaeus, 1758), common octopus (*Octopus vulgaris* Linnaeus, 1758) and cuttlefish (*Sepia officinalis* Linnaeus 1758). Following, fishing between 150 and 350 m we find the target species that is hake (*Merluccius merluccius* Linneaus 1758), Norway lobster (*Nephrops norvegicus* Linnaeus 1758), white shrimp (*Parapenaeus longirostris* Lucas 1864), blue whiting (*Micromesistius poutassou* Risso, 1826), and angler (*Lophius* spp. Artedi 1758). In deeper waters is caught red shrimp (*Aristeus antennatus* Risso, 1816), greater forkbeard (*Phycis blennoides* Linnaeus 1758) and the adult codfish (Fernández *et al*. 2005; García-Rodríguez *et al.* 2005, 2006b).

Chapter II – AIM OF THE THESIS

The work for this thesis was carried out mostly in the laboratory of Food Technology, Nutrition and Food Chemistry (Bromatology) of the Faculty of Veterinary Medicine of the University of Murcia, in cooperation with the Department of Chemistry and CEBAS Research Institute of the University of Murcia (Es).

The purpose of this thesis was the investigation of the chemical composition and the concentration of mineral elements and heavy metals in fish species of wide consumption in the Region of Murcia, and in general, of wide consumption in the whole Spanish community. The data that we obtained are useful to carry out some assessments, in the strict sense, on the interspecies variability of all the components we sought. In the first part of our study we focused on the search for the chemical-nutritional components and this in order only to have a general and temporal idea of moisture variation, ash, protein content and total lipids of *M. merluccius, P. blennoides* e *M. barbatus.*

Then we shifted our attention on the search for the mineral salts content (macro and micro elements) with the purpose to consider their concentration in order to carry out some calculation concerning the meet of daily percentage need (RDA) that this fish products offer when they are sold fresh. In this stage of the study we worked in partnership with CEBAS Research Institute (Murcia) which cooperated with us providing the results of the spectrophotometer ICP-AES analysis.

During the last part of our work we concentrated on the search for the concentration of heavy metals, thanks to the cooperation once again of CEBAS Research Institute and the Department of Chemistry (University of Murcia).

The search for heavy metals is important to judge if selling these products could represent a risk for the consumer health (concerning Cd, Hg e Pb), and if the products are below the maximum limits laid down by EC Regulation n.629/2009 of the Commission of the 2nd of July 2008, amending EC Regulation n.1881/2006 of the 19th of December 2006. Is also possible to make a series of assessments of the levels of pollution in Santa Pola port and whether they represent a threat to flora and fauna.

Chapter III: MATERIALS AND METHODS

All the fishes subjected to the experimental analysis are picked up weekly from Alcantarilla wholesales fish market (Fig.9) by Pescados Beniajan Company, S.L. (Murcia). The three species of our interest are here represented by wild specimens fished in Santa Pola harbour (Autonomous Valencian Community). After their collection they are subjected to all the chemical nutritional (traditional methods) and toxicological analysis for this study.

Figure 9. Alcantarilla fish market

3.1 Samples preparation

When collecting the specimens at the fish market, the samples are placed in plastic bags and stored in a container filled with ice. In general the next day the following steps are performed in the following order: dissection, shredding and either further storage or direct use.

3.1.1 Material

The following list includes the material used for the samples preparation:

- A knife or a scalpel;
- A scissor;
- A chopping board;

- plastic food conteiners;
- A spoon;
- A black permanent marker;
- A balance (Bifinett, made in Germany, 2006);
- A refrigerator (ZANUSSI Model ZRCC37R, Made in Sweden 2007);
- A freezer -85°C Thermo ELECTRONIC CORPORATION VXE 380 Jovan made in Rep. Ceca);
- An electrical grinder (Moulinex 1000W, made in France)

3.1.2 Dissection

Nutritional chemical analysis of the fish species have been carried out on a variable number of samples for each species for a total weigh of approximately 1 kg for each group. The procedures include a series of chemical analysis relating to the nutritional chemical composition of the fish: moisture content analysis, total protein percentage and total fat quantity.

Have been carried out weekly analysis on approximately: 1 kg of *Merluccius merluccius*, 1 kg of *Phycis blennoides* e 1 kg of *Mullus barbatus*.

All the procedures for in this study are carried out weekly on fresh sample. The samples to be analyzed are represented by a weekly average of:

- 12 - 15 samples of *Mullus barbatus* (mullet);
- 6 - 8 samples of *Phycis blennoides* (greater forkbeard);
- 4 - 6 samples of di *Merluccius merluccius* (hake)

The useful part for the nutritional analysis is the entire fish fillet. Therefore it is carried out a dissection taking care of eliminating the fishbone and the skin, all the internal organs, the head and the tale.

We start the dissection with all the specimens of the same species, and once we have finished we take care of washing very carefully all the tools before proceeding with the second group, in order to avoid mixing different samples.

The first thing to do, before starting the dissection, is to weigh every fish by itself and write down the weight of each one.

The dissection is carried out using a knife or a scalpel and scissor; the technique doesn't require particular care regarding the cut and the sample pick up as the useful part to be analyzed is the entire musculature and all the rest has to be removed.

We start making a crosscut with the knife from the gills removing the head from the body and eliminating the useless part, that is the head. Then starting from the front part, we make a second cut following the mid line using the scissor, going through the anal part and reaching the caudal pole (Fig.10). We go on cutting the tail and we remove it. Then we remove all the organs (the visceral track, heart, lungs, swim bladder etc.) and we continue making a cut close to the fishbone and with our hands we pull out the fishbone going on cutting with the scissor and making our best to avoid leaving some spines in the meat. To complete the total removing of the meat from the rest of the body we take more quantity as possible from the sample using the knife, avoiding spines and skin (Fig.10). The meat taken from a single sample is put into a single food container and as we finish the dissection of all the samples of the same species, we go carefully back over the meat taking away some parts if not needed (Fig.10).

We take a fresh sample of approximately 100g, we weigh it on the Bifinett balance and we put it in a plastic bag providing it with: sample title, total weigh and date. We will use the quantity of the sample that we took and put in the bag for all the next analysis with the exception of mercury toxicological analysis, for which we need to use a frozen sample not a fresh one. For this reason the remaining part of the sample will be frozen at a temperature of -85°C.

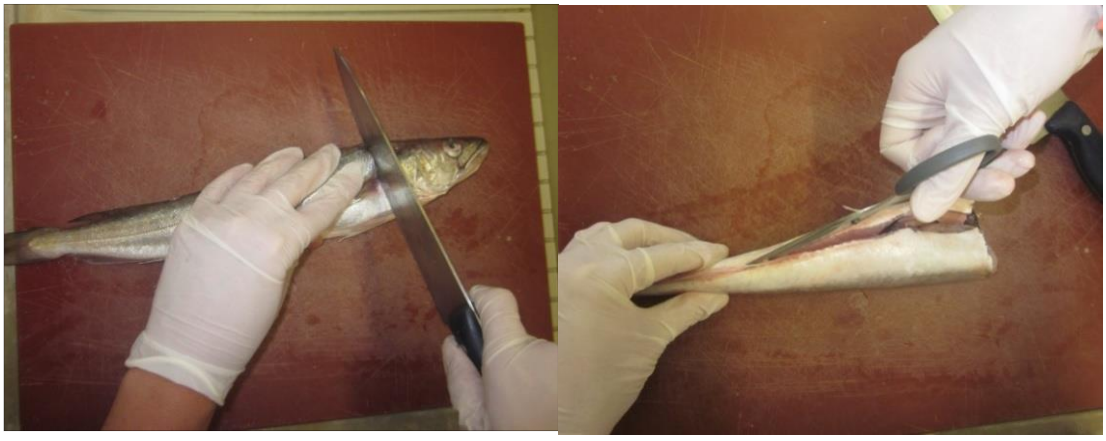

Figure 10. Fish dissection procedure

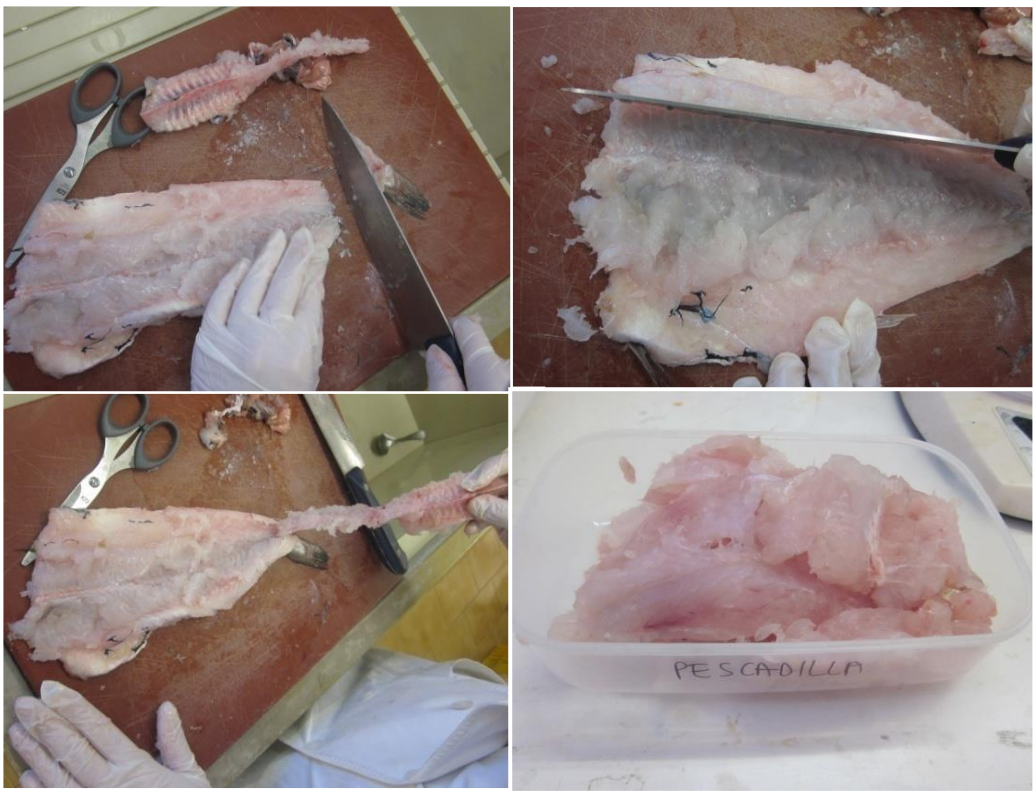

Figure 10. Fish dissection procedure

3.1.3 Shredding

As we finished the dissection of all the specimens of each group we should continue with the shredding to get a better homogeneous sample.

In this stage it is important to wash and wipe carefully the grinder at the end of each procedure, before starting the next one and this is necessary to avoid mixing the different species.

Now we store the individual samples in different initialed plastic bags, some of these will be used right away, while other ones suitably stored in the freezer.

3.2 Moisture analysis

All the food contains water in variable proportion, either it was subjected to processing or it is a raw material. The percentage of water in the natural foods ranges from 65% to 90%. In animal tissues and vegetable tissues we find water in two different conditions: water in free structure and water in imbibitions, which is bound or absorbed. The last one, which

represents only the 4% of the total, is electro statically bound to the polar groups of the proteins and it is mechanically held by the protein structures. On the other hand the free water, which represents the prevalent part, is not bound to particular bonds with the soluble components of the food; therefore it is used in the microbial metabolism. In fact this free water is involved in foods changes as the microorganisms necessarily need water to increase and moreover they need other suitable conditions like temperature, acidity and availability of nourishment. (Hart 1991). These two structures are easily broken down by heat. The quantity of water in the fresh meat is a very important parameter to define the good quality of the food and this especially when it is compared with protein content. It is easy to calculate the water: it is calculated through the drying of the sample in a drying oven at 80°C. The resulting difference between the weight of the plate with the dried sample and the empty plate gives us the moisture content of the food. (Fennema 1993)

3.2.1 Materials and equipment

The material used for the moisture analysis is the following:

- Analytical balance Explorer model (Ohaus, 26adrid, España) sensitivity of 0.0001g;
- Drying oven 201 (P- Selecta, Barcelona, España);
- 9 glass plates Petri (Pobel, Madria, España);
- Two spoons;
- Kartell desiccator, made in Italy

3.2.2 Moisture analysis procedure

The procedure to rate the moisture percentage in the fish uses the 934.01 method of the A.O.A.C. International (1999).

For each species we work on a triple sample. The day before the analysis we put the Petri plates in the drying oven at 100°C. The next day we take them out of the oven and we let them cool down in the Kartell desiccator. Later when they have completely cooled down, we weight them without the lid using the precision balance Explorer model (Ohaus, Madrid, España) and we write down the weight of each plate. We have to properly initial all the Petri plates.

We go on taking all the fresh samples from the plastic bags stored in the fridge and we weight them using the analytical balance Explorer model (Fig.12). The pickup corresponds approximately to 25g of the sample for each Petri plate. The fresh sample has to be evenly distributed on the entire plate surface and to do so we use a spoon. All the plates bearing the sample are now put in the drying oven for 24h at a temperature between 80 and 100°C (Fig.12). The following day the plates with the samples are taking out of the oven, put in the desiccator and let cool down and then we weight them (remembering to remove the lid) and we write down the weight of the edible dry part that we obtained.

Figure 11. On the left an example of the weighted sample and on the right samples put in the drying oven

We use the following equation to calculate the percentage of the moisture:

$$Water\_grammes\%(G_A) = (M_0 + M_1) - M_2$$

$$Moisture(\%) = \frac{(G_A`*100\ )}{M_1}$$

That is:

M_0= weight (g) of the empty plate (without the lid);

M_1= weight of the fresh sample;

M_2= weight of the plate + dried sample;

G_A = weight (g) of the water in the fresh sample.

3.3 Search for the protein

The total protein content, which is calculated to make general observations about the nutritional composition of the samples, is not affected by extrinsic factors (such as the external environment or diet), but by intrinsic factors such as sex, size, weight, species (Shearer 1994).

To search for the protein in the meat of the fish we follow a traditional procedure called Kjeldahl method and this gives results for the organic nitrogen. The method is based on combustion through heating with concentrated sulfuric acid in the presence of a catalyst in order to reduce the organic nitrogen to ammonia that in this way is turned into ammonium sulfate with heat release (exothermic reaction). The ammonia solution obtained is alkalinized by sodium hydroxide and is distilled. The distillate part is collected in a solution of boric acid and follows the titration with a known volume of HCl. The obtained value is converted through a mathematical equation into crude proteins content.

3.3.1 Materials and equipment

The materials and equipment used for the protein analysis are listed here below:

- Analytical balance Explorer model (Ohaus, Madrid, España 2006) sensitivity of 0.0001g;
- A plastic container;
- Plastic wrap;
- Spoons;
- Catalyst Kjeldahl (Cu-Se) powder RE Panreac Quimica SA (Barcelona, España). Composition: Potassium sulfate 96,5%, Copper sulfate (II) pentahydrate 1,5%, Selenium 2% ($Se/CuSO_4.5H_2O/K_2SO_4$);
- Glass balls, Paninsulab, Gestion Integral de Laboratorios, S.L. C/La Mata, CARTAGENA (Murcia);
- Kjeldahl digestion glass pipes Kjeldahl;
- Digestor FOSS 200-240V, 50-60Hz (Tecator^{TM}Digestor, Made in Sweden);
- Scrubber FOSS (Tecator^{TM} Digestor, Made in Sweden) H_2O e NaOH; 100-240V, 50W, 50-60Hz (Made in Sweden)
- Fume Hood (for chemical use) SIEMENS model VG 150, 1990 Revised in 2011;
- Mineralization appliance;
- Sulfuric acid 96% PA-ISO H_2SO_4 (PANREAC Quimica S.L.U.);

- Pipette 5-30ml div. In 0.50 ml Ex 20gradic (FINNPIPETTE Thermo SCIENTIFIC);
- Erlenmeyer flasks of 250 ml (Pobel, Madrid, España);
- Graduated cylinders of 100 ml (Pobel, Madrid, España);
- Distilled water;
- Boric acid (H_3BO_3) 4% (131015 Panreac, Barcelona, España);
- Mixed indicator solution (4%): prepared by dissolving 2 g of methyl red and 1 g of methylene blue 1 L of ethanol 96%;
- Plastic pipettes
- Kjeltic™ distiller 2100, 200-240V, 1900W, 50-60Hz (Tecator™ Technology FOSS) Made in Sweden;
- Magnetic stirrer model IKA-RTC Basic (IKA, Staufen, Germany);
- Hydrocloric acid (HCl) 0,1 N (181023 Panreac, Barcelona, España);
- Burette with stand;
- magnets;
- pH meter CRISON 2V, 50-60Hz, 3.3W (E-08328 ALELLA-Barcelona, España)

3.3.2 Procedure general information

To calculate the protein we follow the validated Kjeldahl procedure, described in the method 955.04 of the International A.O.A.C. (1999).

The product obtained following this procedure is the total content of nitrogen in the organic material. We talk about crude proteins as in addition to the nitrogen mineralization of the amino groups of the proteins also nitrogenous substances are mineralized that are non-protein and these ones basically are a very small part compared to the total proteins, for this reason the error can't be avoided but it's acceptable. To obtain the total value of the proteins we have to multiply the nitrogen value by a coefficient that considers the amino acid composition of the sample.

The procedure is divided into four main phases: the sample preparation, the digestion, the distillation of the digested sample and in the end the titration.

3.3.2.1 Sample preparation

We use fresh fish samples taken from the plastic bags stored in the fridge (see paragraph 3.1.3). We prepare the Kjeldahl pipes (initialed) and in each one of them we add 0.6 – 0.7 g of sample: we weigh the sample using the precision balance Explorer model and then the

sample is put in the pipe, wrapped inside the plastic film. After we have put all the samples we add 7 g of catalyst Kjeldahl and 10-12 glass balls in each pipe. We go on working under the fume hood and paying particular attention, we add 20ml of sulfuric acid. At this point the samples are ready for the digestion.

3.3.2.2 Digestion phase

The digestion is the main phase of the entire Kjeldahl method. In this phase is expected an important reaction: the oxidation of organic compounds by adding sulfuric acid. As the organic material oxides, the carbon contained in it is converted in concentrated carbon dioxide and the hydrogen produces water. The nitrogen of the amino groups that are in the peptide bonds of the polypeptide chains is digested initially converted in ammonia and then becomes ammonia sulfate.

The exact aim of the digestion is to break the bonds that unite the polypeptide and convert them into more simple substances like water, carbon dioxide and of course ammonia. These reactions could be considerably accelerated by a catalyst (Cu – Se) that increases the boiling point of the digestion acid and therefore the temperature of the reaction system and allows a smooth conversion to ammonia sulfate.

We put the pipes in the digestor (Fig.12) (that is under the chemical hood) and the cycle is as follows:

- a starting phase that varies according to the water content of the sample, normally the duration is 30 minutes at 240°C;
- a second phase increasing the temperature to 340°C for 30 min. in order to reduce the formation of white fumes;
- a final phase of the duration of 2 h at 440°C to continue and end the digestion.

At the end of the digestion is important to have a visual inspection: the result is a clear transparent liquid with a colour that changes to light blue, depending on the catalyst used. We don’t have to find remains of black colour adhered to the tube wall. After we put the tubes, before switching on the digestor it is important to be sure that the system is connected to the scrubber and that we put the lid on the pipes. The function of this tool, which is connected directly to the digestor, is to extract the acid vapor and neutralize it

avoiding the possible inhalation in the lab (Fig.12). The scrubber is composed of two parts: a container with water whose function is to dilute the vapors coming from the Kjeldahl pipes and a container with caustic soda (NaOH) whose purpose is to neutralize the acid vapor. We add to the container with NaOH an indicator that brings up the purple mixture. During its use the mixture changes its colour to red-orange and once we reached this colouring we ought to replace the reagent as at this point it won't be able any longer to neutralize the vapor.

We switch on the digestor and we set in the monitor time and temperature requested, we switch on the scrubber and we start the digestion. The switch from one phase to the following one is not carried out automatically so the operator has to do it manually. At the end of the cycle we turn off the digestor and we leave the scrubber working for about one hour more, making sure of the complete removal of the gases. After that period of time we can switch it off and we let the pipes cool down till they reach room temperature keeping them closed.

Figure 12. On the left a Kjeldhal digestor working; on the right a scrubber

The chemical reactions that take place in the digestor during the digestion cycle are the following:

$$Phase\,1 \qquad MO + H_2SO_4 \longrightarrow CO_2 + 2H_20 + NH_3$$

$$Phase2 \qquad 2NH_3 + H_2SO_4 \longrightarrow SO_4(NH_4)_2$$

That is:

MO = organic material (sample)

3.3.2.3 Distillation of the digested sample

The following day the distillation is carried out using the Kjeltic™ distiller (Fig.13).

Figure 13. Kjeltic™ distiller

The purpose of this phase is to separate the ammonia (nitrogen) from the digestion mixture, increasing the pH through the addition of NaOH. The addition of sodium hydroxide (automatically added by the distiller) transforms ammonium ions NH_4^+ in ammonia NH_3 that is the dissolved gas in the liquid. We distill the ammonia separating it from the digestion mixture through increasing the temperature till the boiling point and in this way it is transformed in a volatile gas. The vapours are conveyed in a capture solution made by a cooling coil and they are dragged into the flask with boric acid.

At the beginning we prepare the samples adding 50ml of distilled water to the digestion product in order to minimize the mixtures containing high acid/salts percentage. We prepare

the flasks, we initial them and we add 25ml of boric acid to each one. We can continue by either adding 5 drops of a redox indicator (a mixture of methylene blue and phenol red) that is useful for the titration or simply we don't add anything more and we use a pH meter during the following phase.

By adding the indicator to the boric acid the mixture appears of a dark violet colour, this reveals a typically basic environment with a pH generally around 7.4 – 7.6. During the distillation we can notice a change of colour caused by the ammonia moving from the pipe to the flask containing the boric acid and the indicator. This movement causes a pH decrease which consequently causes a change of the mixture colour to green (pH 3.5 – 3.55). In more recent distillations the indicator is replaced by a pH meter, which is a more accurate tool in the titration phase.

We start turning the distiller on and making sure that it contains water and sodium hydroxide, we open the cold water knob in order that the water flows and goes to refrigerate the system and the coil where the digested solution will pass through. The first cycle is carried out using a Kjeldahl pipe with distilled water only and an empty flask. This first cycle is carried out just to warm up the distiller to a desired temperature and to assure a suitable distillation of all the samples and also to clean up the equipment from possible remaining. The samples are distilled one by one. After we have started the distiller we press the heating button and we make the sample boil in the pipe solution (Fig.14): it is important to pay attention to the bottom of the pipe because during this heating phase we have to notice the presence/existence of a gel that, as the temperature increases, comes off the bottom and it blends in the solution. We turn off the heat and we go on neutralizing the sample by adding from time to time 10 ml of NaOH at 38% (automatically dosed by the device). We stop the adding phase after we poured approximately 60-70ml of sodium hydroxide and we turn on again the heat button. If the colour solution appears brown-black we go on boiling in order that the mixture is dragged/drawn (in vapour phase) from the Kjeldahl pipe to the flask and it drops in a 4% boric acid solution, otherwise we keep on adding the reagent. The mixture in the pipe is in a liquid state, it turns into vapour because of the heat supplied, in vapour form it comes up and goes through a cooling coil that brings it back to liquid state, in this way it moves to the flask and if there is the indicator, we can notice the colour changing from violet to green (Fig.14).

Figure 14. On the left a sample at the beginning of the distillation. On the right the solution has already moved to the flask and we can notice the change of the colour mixture caused by the mixture pH change

The reactions that take place during the distillation phase are the following:

$$Kjeldahl\_pipe: SO_4(NH_4)_2 + 2NaOH \longrightarrow SO_4Na_2 + 2H_2O$$

$$Flask: HBO_3 + +2NH_3 \longrightarrow (NH_4)_3BO_3$$

3.3.2.4 Potentiometric titration by using a known acid

At the time that the sample melts into the basic capture solution we notice the colour change to green.

Now we can take the flasks and go through the titration with hydrochloric acid at known title 0.1N to determine the ammonia quantity absorbed by the boric acid. We take the flask from the distiller, we add a magnet and we put it on a magnetic stirrer. We turn on the stirrer and we put it under a burette. In the burette we add a standard solution with a known concentration of HCl and slowly we add it to the basic solution through the indicator (Fig.15). If we are working with the chemical indicator we ought to pay the maximum attention at the moment when the solution turns to green. Just at the moment of the change of colour we have to stop adding the hydrochloric acid. This type of titration is not precise for that the use

of the pH meter is preferable. The only visual perception of the human eye has a margin of error that ranges between +/-0.5 and +/-1 of pH unit and it is unlikely that all the samples will show the same final pH using only the visual perception of the colouring change.

The pH meter is more accurate in this case because it records the initial pH and when we reach the requested pH (that is the starting pH of the boric acid of 3.55) we can stop the pouring of the acid. When the basic acid reaction is finished we write down the known acid volume that we had to add to achieve the final result.

Figure 15. Sample subjected to basic acid titration

3.3.2.5 Formula and calculation for the % of the crude protein

We use the following formula to calculate the protein percentage in the sample:

$$Crude\_protein(\%) = \frac{(V_2 - V_1) \times N}{M} \times 1.4 \times 6.25$$

That is:

V_1 = is the volume of the HCl (ml) solution requested for the proof/test of white (cost = 0);

V_2 = is the volume of the HCl (ml) solution requested to test the sample;

N = is the norm of the HCl (cost = 0.1 M) solution;

M = is the weigh (g) of the fresh sample

6.25 [4]= conventional value that varies depending on the matrix used. As the proteins contain approximately 16% of N so we have to multiply the PG (crude protein) by a stoichiometric coefficient of 1/16;

1.4 = conversion factor to shift from nitrogen to protein.

3.4 Search for the lipids

In the list of the chemical nutritional analysis it is considered also the extraction and calculation of the total lipids percentage in the edible part of the fish. In general nutritional analysis is indeed a useful tool to rate a food product from a macro and microelements, vitamins and dietary fibers content point of view. The search for the lipids quantity is important not only for this reason but also for the possible consideration that we can point out regarding heavy metals accumulation as fat soluble substances.

If sample collection lasts for a certain period of time, building a concentration/time chart, we can view the possible variations due to intrinsic and extrinsic factors (for exemple: breeding season.). To figure out the fat percentage in the fish we follow the Soxhlet method 920.39C of A.C.O.A. (1999).

3.4.1 Materials and equipment

The material used for search for the lipids in the different species is the following:

- Analytical balance Explorer model (Ohaus, Madrid, España 2006) with sensitivity di 0.0001g;
- Drying oven 201 (P- Selecta, Barcelona, España);
- Mortar
- Spoon
- Cellulose extraction thimbles 33x80 mm ALBET labScience (made by Dassel, Germany);
- Supports (iron rings for the cellulose cartridges)

[4] Note (1): This factor has originally been 6.25 based on the assumption that all proteins contained 16% nitrogen. However, it has been known for some time that plant proteins (and gelatin) contain more nitrogen, and thus require a lower factor (Sosulsky & Imafidon, 1990). Different factors originally determined by Jones et al. (1942), are currently used to calculate proximate protein amounts based on nitrogen content in different foods. These factors range from 6.37 for human milk to as low as 5.55 for gelatin and 5.18 for almond. The nitrogen factor for meat and fish is 6.25 (Pirjo & Pekka 1996).

- Cotton
- Metal pipes (FossTecator AB, Höganäs, Sweden);
- Extractor Soxhlet 2055 Avanti model (FossTecator AB, Höganäs, made in Sweden);
- Desiccator Kartell, made in Italy;
- Diethyl ether ($C_4H_{10}O$) p.a. (8033 J.T. Baker, Deventer, Holanda)

3.4.2 Procedure of the search for the total lipids

In this procedure we use the dry sample therefore we collect it from the plates subjected to moisture analysis. We try to collect the entire sample scraping it off the Petri plate and then we put it in the mortar and crumble it carefully almost in powder. We weight it (Fig.16) and put it in the extraction cellulose thimble covering it with a little bit of cotton. For each extraction thimble there have to be an empty and sterile metallic pipe as it is necessary to collect the fat during extraction.

Each analysis is carried out on a triple sample.

The device has to be switched on at least 15 minutes before we use it to allow the pipes cooling down and avoid system overheating during extraction. We insert the extraction Soxhlet thimbles, we put the metallic pipes initialed under each corresponding sample and at the end we add 50ml of diethyl ether for each sample (Fig.16).

We switch on the Soxhlet device, starting the heating plate under the metallic pipes and when we reach a temperature around 72-74°C we can start the cycle that will be the following:

- 10min in which the cellulose thimble is immersed in ether;
- 2h in which the thimbles are taken out from the pipe and they don't come into direct contact with ether;
- 30min in which we let ether flow completely into the underneath pipe.
- 10min end of cycle to allow the gases discharge

When the cycle is finished we turn off the device we take out the metallic pipes and we put them in the drying oven for 24h in order that ether vaporizes and exclusively the fat will be left (Fig.16).

The following day we take out the metallic pipes from the oven, we let them cool down in the desiccator and we weight them.

Figure 16. Overview of the phases for the extraction of total lipids content

3.4.2.1 The 4 phases in details

After we have inserted the metallic pipes, the samples and added the ethylic ether, we can start the extraction cycle. There is a pre-phase 1 in order to warm up the heating plate at a temperature between 72-74°C. It takes about 2 minute.

We have to let the refrigerant work during the entire cycle in order to maintain the system at the requested temperature. Through this the soil around each pipe where the ether flows will maintain such a temperature to allow the recirculation of ether, as from volatile it turns into liquid form.

After this warming pre-phase there is the first phase, it lasts 10 minutes: in this phase the cellulose thimble is in the metallic pipe soaked by the ether previously poured (Fig.17). During this phase the plate heat allows ether volatilization, this one in a vapour form comes up, comes into contact with the pipe cooled by the soil and comes down turning into a liquid form. This continuous liquid-vapour form changing allows the fat separation that thanks to the cellulose thimble and the ether can be separated going through the thimble and in this way drops in the bottom of the metallic pipe.

Then phase 2 follows, it lasts 2 hours. In this phase the metallic pipe remains always in contact with the heating plate, while the cellulose thimble is taken away from the direct contact with diethyl ether (Fig.17). The ether that remained in circulation volatizes, it goes up and turns liquid, comes down and carries with it the remained fat left in the cellulose thimble. At the end of the phase the entire fat in the sample is settled in the bottom of the metallic pipe.

In phase 3 that lasts 30 minutes, we have the cellulose thimble still lifted, the plate still remains at a temperature of 72-76°C (Fig.17), the device collects the entire diethyl ether that remained in circulation and moves it in a gathering storage of the ether which is incorporated in the Soxhlet. In the metallic pipe the amount of ether won't be equal to the initial amount as through volatilization part of the liquid is lost or gathered by the extractor and placed in the storage.

The last phase lasts 10 minutes (Fig.17) and in this one the plate is not heated and the pipe contains only lipid, as the ether has vaporized entirely (or almost entirely).

To vaporize completely the diethyl ether that could have remained, it is necessary to remove the metallic pipes at the end of the cycle and put them in a heater at 80°C for at least 15 min.

Figure 17. Some details of Soxhlet extractor: on the left the first two phases, on the right phases 3 and 4

3.4.2.2 Calculation of lipid percentage

We use the following equation to calculate the lipid percentage:

$$Total\_lipid\,(\%) = \frac{(M_1 - M_2)}{M} \times 100$$

That is:

M_1 = weight of the metallic pipe with the extracted material

M_2 = weight of the empty metallic pipe;

M = weight of the dried sample

This method allows separating the lipid from all the other components that are in the food. The lipid is solubilised by the diethyl ether and with a support as the cellulose thimble it is possible that the soluble lipid passes through the partitions and spill out in the metallic pipe.

3.5 Determination of ash and samples preparation for ICP-AES analysis

The procedure to calculate the ash in a biological sample is required in order to proceed with the analysis to calculate the minerals and heavy metals content. This stage of samples preparation is carried out in the laboratory of Food Technology, Nutrition and Food Chemistry (Bromatology), and then all samples are sent to the laboratory of CEBAS Research Institute for the analysis of mineral elements and heavy metals in the ICP-AES spectrophotometer. Obviously all the results received from the laboratory of Research are important in order to carry out some considerations about the content of essential mineral elements, in order to calculate what percentage meet the daily nutritional requirements. The results of the concentration of heavy metals allow to consider the possible risk that these specimens may represent for the consumer health, these results also provide basic information on the situation of environmental pollution in Santa Pola port. In analytical chemistry the ash is simply the solid residue of combustion, that portion of sample that doesn't burn and that therefore doesn't turn into a volatile substance. It shows a typical light gray colour and it's composed of inorganic elements, mineral salts and it's devoid of water.
The ash determination follows the procedure 945.46 of the O.A.C. International (1999). The following samples preparation for the analysis of minerals and heavy metals involves acid hydrolysis of the ash obtained in a solution of HCl and HNO_3.

3.5.1 Materials and equipment

The material used for the ash preparation that is to be submitted to ICP-AES analysis to determine minerals and heavy metals is the following:

- Porcelain crucibles C-4 (KPM, Berlín, Alemania);
- Analytical balance Explorer model (Ohaus, Madrid, España 2006) sensitivity of 0.0001g;
- Spoon;
- Graduated flasks 50 ml (Pobel, Madrid, España);
- Nitric acid (HNO_3) 65%, p.a. (UN 2031, J.T. Baker, Deventer, Holanda);
- Hydrochloric acid (HCl) 37%, p.a. (UN 1789, J.T. Baker, Deventer, Holanda);

- Deionized water (milliQ);
- Deioniser Agua milli-Q Millipore^{TM} 230v, 50Hz, reviewed in 2012 (made in France);
- Electric muffle furnace 800 degrees Program Controller S27 (Naberthem, Bremen, Germany);
- Drying oven 201 (P-Selecta, Barcelona, España) max 200 degrees;
- Heating plate (Jata 2);
- Plastic pipette;
- Becker (Fisherbrand) cap. 100ml;
- Falcon 50 ml;
- Graduated test tubes with plastic screw cap 15ml;
- A scissor;
- Parafilm

3.5.2 General information on the procedure

All the glassware and the porcelain used to calculate the ash and to prepare the samples are washed overnight in HNO_3 in order to remove any impurities adherent to the sides and then rinsed with deionised water.

The crucibles are taken out from the nitric acid bath and are put in the drying oven for 15 minutes, then are left to cool down, initialled and weighted.

Afterwards follows the combustion of the fresh sample in the oven, the ash calculation and the acid hydrolysis of the ash, in order to continue with the spectrometer analysis.

3.5.2.1 Incineration and ash calculation

The procedure involves the incineration of 1g portions of sample in crucibles (Fig.18) placed in a muffle furnace at 660±10°C until complete combustion of the organic substance and the achievement of a constant mass.

We weight 1 g portions of sample, place them in their correspondent initialled crucibles and we take note of the weight. We place the crucibles in the muffle furnace for 24h at 660±10°C (Fig.18) until we obtain an ash completely white, without any remaining of organic substance. The following day we remove the crucibles from the furnace paying great attention to the huge temperature change: in order to avoid breaking the crucibles we turn

off the furnace, we let stand for about one hour and then we open the furnace, we leave it another half an hour to gradually decrease the temperature avoiding breakage.

Figure 18. On the left: weighing the fresh sample; on the right: samples placed into the Program Controller S27 muffle furnace

After we have weighted all the crucibles we can calculate the ash quantity:

$$Ash(g) = A_1 - A_0$$

$$Ash\ (\%) = \frac{A_1 - A_0}{Ash(g)_1} \times 100$$

That is:

A_0= weight of empy crucible:

A_1 = weight of crucible with ash

$Ash(g)_1$= crucible weight with ash.

3.5.2.2 Acid hydrolysis of the ash

The ash obtained is submitted to acid hydrolysis on a heating pad and it is melted in HCl and HNO_3 (Fig.19). This phase involves acid hydrolysis of the sample, a crucial step to continue with the spectrometer analysis ICP-AES. It's necessary to work under a fume hood avoiding inhalation of possible vapour produced by the chemical reaction.

We place the crucibles on the heating plate and we add 3ml of HCl and 2ml of HNO_3 in each one. We turn on the plate and we wait for the mixture to boil (approximately half an hour) (Fig.19). When we reach the boiling point we let evaporate most of the mixture and after that we take away the crucibles from the plate, we add deionised water and we let cool it down. The solution inside each crucible has to be transferred in a 10ml test pipe for the spectrometer analysis and for that we should continue pouring the samples into the initialled flasks and taking care of mixing well. While transferring the solution into the flask we should work changing the pipette every time in order to avoid possible contamination or transfer of substance from a flask to another (Fig.20). After we have poured all the solution containing the dissolved ash we add deionised water until we obtain a 50mL volume. Then we seal the flask with parafilm and we turn upside down 3–4 times the flask to mix well.

The solution of the flask is transferred into a 10mL falcon and the remaining one in a 50mL falcon and we store it in a fridge.

At this stage the samples are ready for ICP-AES spectrometer analysis.

Figure 19. On the left reagents necessary for acid hydrolysis; on the right acid hydrolysis on heating plate

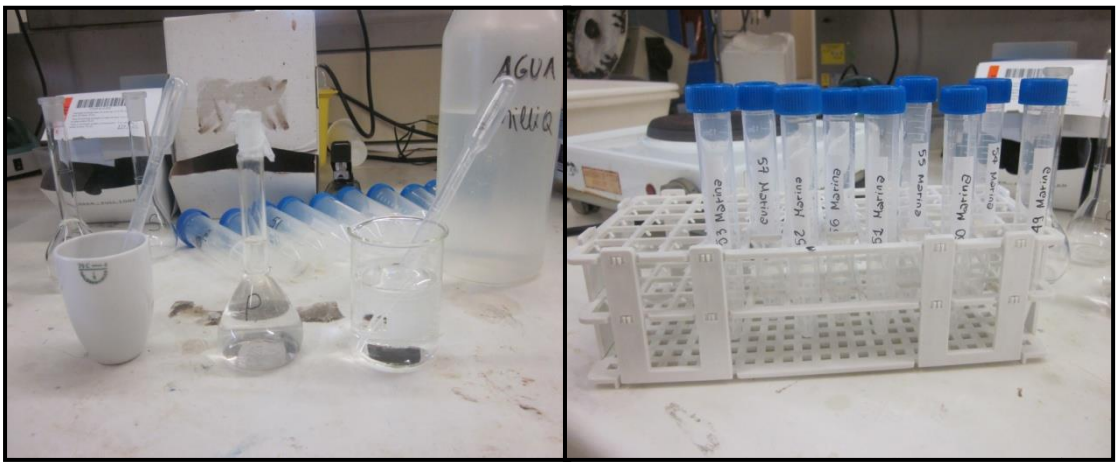

Figure 20. On the left material used to transfer a sample in a flask; on the right samples on the 10 mL falcons and ready for spectrometer analysis

3.6 ICP-AES analysis

The ICP-AES spectrometer analysis is carried out by the Ionomic Service, a support analysis service to the scientific research since 2006, a public laboratory for scientific research of Murcia (CEBAS-CSIC) which sends all the results of the weekly analyses. The analysis of the essential mineral elements and heavy metals for the three species of our study aims to detect the concentration of each element considered in all the sampling, so it is possible to make some considerations about the nutritional features and concentrations of harmful elements for the consumer health.

3.6.1 General information

The method of analysis procedure used to detect minerals and heavy metals amount in the fish (excluding mercury that follows a different method) involves multi-elemental analysis through atomic emission spectrometry employing inductively coupled argon plasma with optical detector (ICP-AES Inductively Coupled Plasma-Atomic Emission Spectrometry) (Fig.21). The basic principle of this technique involves the atomization of the sample to be analyzed and quantified, that is the transformation of the solid or liquid matter into atomic vapour (Payling *et al.* 1996).

Atomization occurs by supplying heat at a temperature between 6000 and 8000K: clearly we are talking about a destructive technique since the sample is completely consumed (Broekaert *et al*. 2002).

ICP-AES is one of the most powerful and popular tools used in analytical chemistry to determine the inorganic elements in traces in different matrices (Boss & Freeden 1997). This is used to identify, characterize and determine qualitatively and quantitatively the components of a certain sample as the contaminants (ex. aluminium, arsenic…), the macro and microelements (as calcium, magnesium, zinc, etc.).

The use of the inductively coupled plasma source allows dissociating the atoms or ions that form the sample, these ones being free excite through the presence of the source and plasma, turning to a higher energy state from which then they decay turning again to a ground state with a spontaneous photons emission. The measure of the radiations wavelength emitted by the photons which is characteristic of each element allows detecting the atoms that emitted this kind of radiations and thus we carry out a qualitative analysis, while measuring the emission intensity we can go back to the element quantity (Fassl 1986; Cozzi *et al*. 1997; Polesello 2002).

The amount of each element in the "atomic gas" is measured by the radiation emission in the visible range from atoms in the vapour state.

The liquid and gaseous samples can be injected directly into the device, while the solid ones required a prior extraction or acid digestion in order to place the analytes in a solution.

The elements that are sought in this study are: Ca, Mg, Na, K, P (macroelements); Cu, Fe, Mn, Mo, Zn (microelements); Al, As, Cd, Pd, Ni (contaminants). Among the contaminants there is mercury too, but this involves a different method for its detection.

Figure 21. Duo ICP-AES Thermo Scientific™ Made in England

It is important, before starting the analytical cycle, to power the ICP-AES device (and then turn it on without plasma) for at least 24h in order to thermostat the optical part. The device requires also a regular maintenance and a careful setup before starting the analytical activity. Every year we suggest a complete maintenance of the device carried out by a specialized technician checking the radio frequency generator.

3.6.2 Analysis of mineral elements and heavy metals

The samples to be analyzed are inside 10 mL test tubes containing the ash dissolved in HCl 37% and HNO_3 65% and diluted with milliQ water. The test tubes are placed in a holder under the fume hood where an auto sampler (provided with a mechanical arm) picks up the solution and passes it to the spectrophotometer.

After switching on the device the first thing to do before starting the analysis is the calibration: we prepare standard samples to compensate any possible fluctuations in the analytical system. The software checks for each calibration that the correlation coefficient is

better than 0.999 with a maximum percentage deviation of the slope of 10%, for each analyte is accepted a maximum error of 15% in the interpolation (es. in Fig.22).

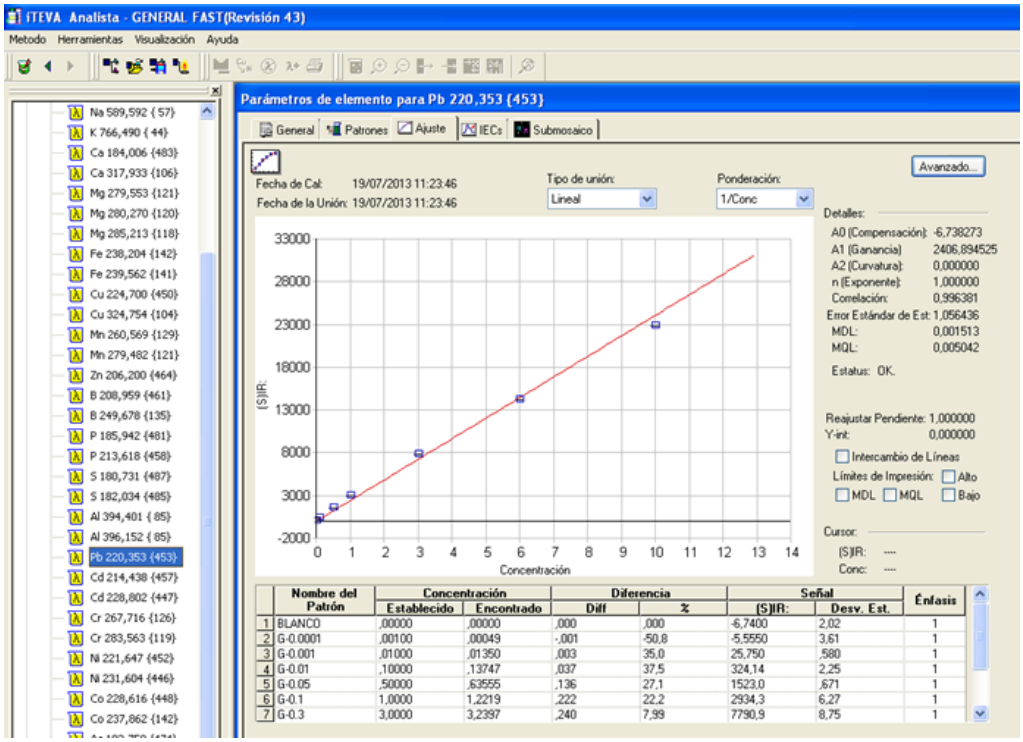

Figure 22. Example of regression in lead analysis, in the range of 0.00049 to 6.4 nm 220.353 mg L^{-1}

Linear calibrations are carried out for each element (white and eight concentration levels shown in Tab.8) The white follows along with the samples throughout the test procedure. The signal of the white is therefore the sum of pollution and noise introduced with the reagents and performed manipulations; this signal is generally subtracted from that of the measurement on the unknown sample. A systematic inspection of the whites is important because if there are sudden changes in values can be related to pollutants in ultra-pure water, in the reagents used for analysis or other deficiency in the procedures of washing the glassware. A careful inspection of the whites is also necessary for the evaluation of the lower limits of detention (LOD) and quantification (LOQ) (Douglas *et al.* 1995; Green 1996).

A regular and systematic storage of all the values of the signal (intensity) of the whites allows to check a good performance of the analytical procedures, and to calculate the best possible detention and quantification limits of the method.

The following table shows all values of the volumes used (mg/L) for each mineral element:

Name of the standard solution	WHITE	G-0.0001	G-0.001	G-0.01	G-0.05	G-0.1	G-0.3	G-0.6	G-1.0
Solution Volume G-1.0 (ml) in 100mL	0	0.01	0.1	1.0	5.0	10.0	30.0	60.0	100.0
Concentration of Cd (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of Cr (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of Cu (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of Mn (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of Ni (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of Pb (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of Zn (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of As (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of Mo (mg/L)	0	0.001	0.01	0.1	0.5	1.0	3.0	6.0	10.0
Concentration of Na (mg/L)	0	0.01	0.1	1.0	5.0	10.0	30.0	60.0	100.0
Concentration of Mg (mg/L)	0	0.01	0.1	1.0	5.0	10.0	30.0	60.0	100.0
Concentration of Al (mg/L)	0	0.01	0.1	1.0	5.0	10.0	30.0	60.0	100.0
Concentration of P (mg/L)	0	0.01	0.1	1.0	5.0	10.0	30.0	60.0	100.0
Concentration of S (mg/L)	0	0.01	0.1	1.0	5.0	10.0	30.0	60.0	100.0
Concentration of Fe (mg/L)	0	0.01	0.1	1.0	5.0	10.0	30.0	60.0	100.0
Concentration of K (mg/L)	0	0.03	0.3	3.0	15.0	30.0	90.0	180.0	300.0
Concentration of Ca (mg/L)	0	0.1	1.0	10.0	50.0	100.0	300.0	600.0	1000.0

Table 8. Concentrations of standard solution for ICP-AES multi-element mg/L

When we have finished the calibration we start with the samples analysis: a part of the sample is sent through the peristaltic pump to the nebulizer which creates an aerosol that is transported to the ICP torch. Here the nebulized sample is placed in the plasma where, at a temperature between 6000K and 8000K involves the excitation of the atoms, taking them to a higher energy level to their ground state. The excited electrons of the atom then return to the ground state, directly or through intermediate energy levels; in these steps takes place the quanta emission of light energy that generates an emission spectrum at different wavelengths (lines). Each line of the spectrum is originated by a particular electronic transition between two different energy levels, and since each atomic species is characterized by a number of possible states or energy levels, the atoms produce their own characteristic emission spectrum. The intensity of a spectral line depends on both the transition probability and also on the number of atoms that are able to execute it. Since the

number of excited atoms is comparable to the total number of atoms, it is possible to establish a good linear relation between the intensity of the emitted electromagnetic radiation and the concentration of the atoms. Because of the phenomena of simultaneous emission by all the elements, with this methodology, there are many spectral interferences with overlapping emission lines from different elements. Therefore, with this analytical technique we should perform a careful control of the interfering elements and determine each analyte with at least two different emission lines, comparing data from the obtained values.

What we obtain from the spectrometer is a peak of emission (Fig.23) from which we can go back to the analyte concentration in 50ml solution. The system itself finds out the quantity of the mineral element and automatically shows the quantity expressed in ppm.

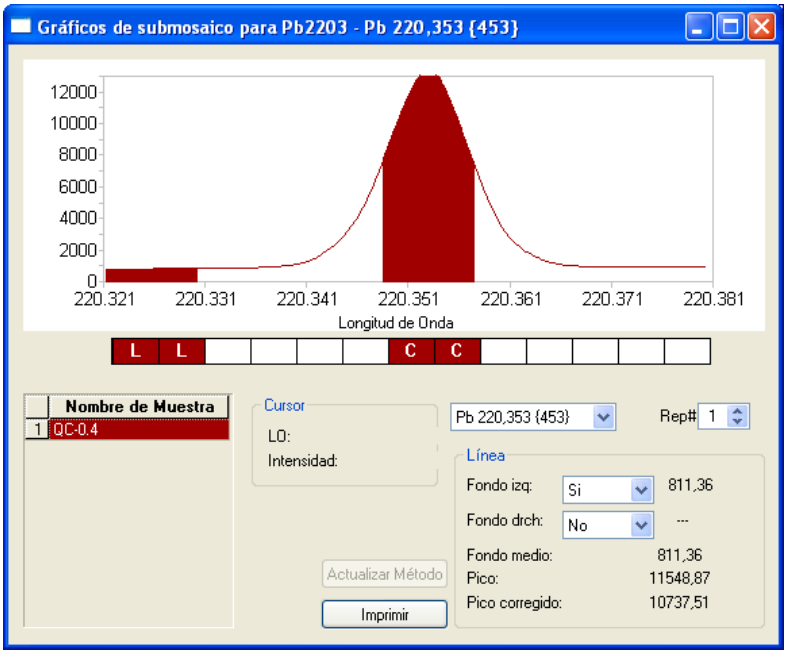

Figure 23. Example of a spectrum of ICP-AES emission obtained in Pb analysis at a wavelength of 220.353nm

3.7 Mercury analysis

For the analysis of mercury traces in the biological samples, a different procedure has been used from the one to analyze other minerals and heavy metals. The procedure involves a previous digestion of the biological material through high pressure mineralization (with micro waves oven), followed by the analysis with atomic fluorescence spectrometer (AFS) in

collaboration with the Department of Chemistry of the University of Murcia. Clearly the choice of the most appropriate method in this case is related to the kind of analyte that we need to search. The work carried out in collaboration with the Department of Chemistry is part of a larger project, where a research team of this Department wants to seek a safer and more precise method to detect mercury. The results obtained from this project are compared with the results of a number of other methods of detection of mercury, to verify the reproducibility of the data and which method offers a greater accuracy in respect to the concentration of mercury.

3.7.1 Materials and equipment

For mercury analysis the material used is the following:

- Analytical balance, Made in Germany;
- Electronic pipette Thermo scientific Etalonnage max 5ml;
- Microwave oven Microwave solvent Extraction lab station, MILESTONE Ethos SEL;
- Plastic sterile containers, 50 ml capacity;
- Fume hood (for chemical use) SIEMENS Model VG 150, 1990;
- H_2O_2 65% pure quality reactive, Panreac;
- HNO_3 30% purity, Panreac;
- Milli-Q PLUS, MILLEPORE Iberica, S.A. Barcelona;
- Tweezers;
- Software easy WAVE 3, program organic (biological samples) up to 0.4g;
- Torque wrench 13.5W power, $HOLEX_R$ brand;

3.7.2 Digestion of food samples

The digestion of the sample in a microwave oven is one of the standard methods to prepare the samples in analytical chemistry. This step is essential for the analysis of mercury in biological samples since the degradation take place through heating all the organic material (at a temperature of 180-200°C) using proper reagents too.

Before starting with the digestion it is necessary to unfreeze all the samples to be analyzed. We pick up a useful amount of each sample (about 30-40g) and we place it in a suitable plastic bag previously labelled (Fig.24). We leave the samples in the refrigerator for about 24 hours. The following day we take from each bag about 0.5-0.6g portion of the sample to be

mineralized. The biological material previously weighed should be placed in a suitable container closed at high pressure (Fig.24).

This mineralization technique at high pressure with microwave oven has some advantages: simplicity, speed, safety, lower risk of contamination and loss of the analyte subjected to investigation. With this technique, through adding 5ml of HNO3 as oxidizing agent, 2ml of H_2O_2 and 3ml of H_2O we have the complete destruction of the organic material of the food matrix (Fig.24). Then we seal hermetically with a torque wrench each container, we place them on a holder and we put it in the microwave (Fig.25). To store the samples after the mineralization it is better to use only disposable containers. The material of which the container is made should minimize the risk of contamination both positive (due to phenomena of release of the inside of it) and negative (that is the loss of analyte by adsorption). We should avoid using glass containers as they are more polluting than the plastic ones (Pomeranz *et al.* 1994; Subramanian 1996).

Figure 24. Series of pictures representing the preparation procedure of the samples to place in the Microwave solvent Extraction lab station

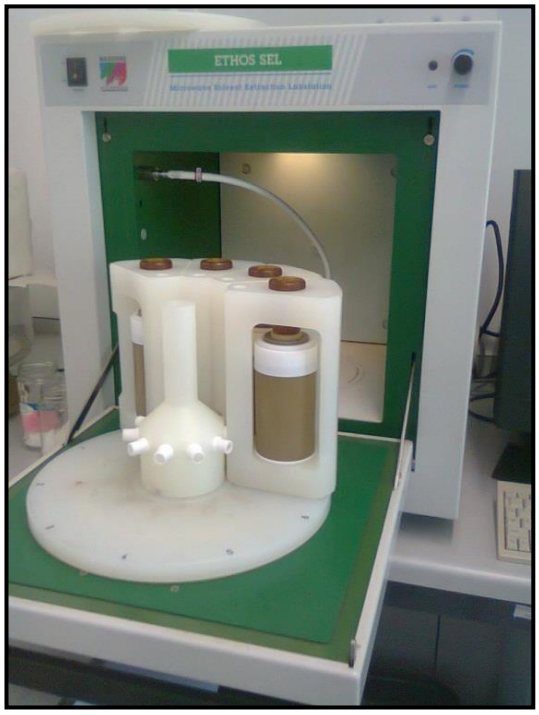

Figure 25. Microwave oven Milestone Ethos Sel model with representation of the loading of the samples

The Millestone microwave oven Ethos Sel model is equipped with a high-pressure rotor HPR 1000/10 with closed system at high pressure and it, has a maximum capacity of 10 samples at a time. It also comes with its own program, "organic up to 0.4" divided into 5 main stages (Fig.26):

1st stage: the temperature start to increase up to 85°C in 2 minutes, the digestion has already started;

2nd stage: the temperature increases from 85°C to 145°C in 5 minutes;

3rd stage: from 145°C to 210°C in 3 minutes;

4th stage: maintaining a fixed temperature of 210 ° C for 10 minutes, allowing to complete the mineralization of the entire biological material;

5th stage: gradual lowering of the temperature through ventilation of the containers, in order to lower the temperature of the same containers and of the system too.

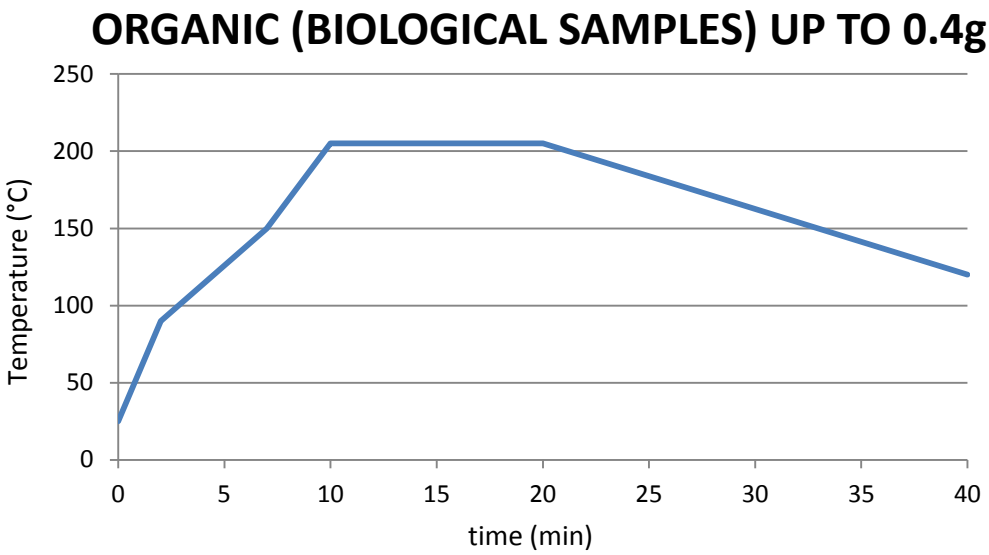

Figure 26. Graphical representation supported by the program easy WAVE3 of a complete cycle of digestion in a microwave oven Millestone

The cycle involves the complete oxidative digestion of the biological sample by adding a concentrated acid, and also the component temperature.

It is possible to follow graphically step by step the sequence of all the 5 stages of mineralization.

It is important to let stand the samples at the end of the cycle, allowing the lowering of the temperature, without opening the containers in order to avoid the loss of volatile mercury and vapours that may be harmful to human health if inhaled.

3.7.3 Analysis of the atomic fluorescence spectrophotometer (AFS)

The analysis of mercury, as previously mentioned, is carried out in cooperation with the Chemical Department of the University of Murcia through the atomic fluorescence spectrophotometer analysis (AFS) PSAnalytical Millennium System (MICROBEAM S.A.) (Fig.27).

Figure 27. Atomic fluorescence spectrophotometer PSAnalytical Millennium system and some samples subjected to analysis

The samples, previously digested (see par.3.7.2), are sent to the laboratory and subjected to spectrophotometer analysis and this give us all the results about mercury concentration of each sampling. The recorded data allow us to carry out a series of observations about the toxicity level of the mercury in these three species.

Mercury analyzers have become an important tool in laboratories, involving the analysis of this element that is highly toxic to human beings and contaminant throughout our environment. The analyzer is very versatile and measures the concentration of mercury in various matrices (organic ones such as food, blood, hair, etc. and inorganic ones such as groundwater, well water, in industrial and chemicals samples). This kind of analysis matches the advantages of steam generation techniques that remove chemical interference with the sensitivity and selectivity of the atomic fluorescence spectrometry, which allows searching concentrations up to ppb. The system control is carried out through Millenium software, which monitors the device and also records all the parameters of the device itself and the calibration, providing excellent results.

The analysis involves the use of pre-treated sample and includes stages as the vaporization and atomization of the individual sample, and there is an atomic fluorescence detector which reveals mercury concentration in the analyte, providing a detection time in less than 5 seconds. The atomization is an important phase, and involves the transformation of the sample that was previously sprayed into gaseous atoms and elementary ions. The

atomization takes place with a temperature between 1700°C and 3150°C. Obviously, this technique has in common with the previous one the formation of atoms that by a suitable heating, moving through a flame, reach a detector that records a linear response according to the concentration, so this allows the quantitative analysis of the sample. In this case, the intensity re-emitted is measured at a different wavelength from the one of the source, and subjected to the levels of the sample. In atomic fluorescence spectrometry the sample re-emits part of the absorbed radiation. The output is low, but selectivity and sensitivity are high.

In this technique, as in ICP-AES, the flame has the function to atomize the sample, while the other components of the device isolate the atomic spectral lines, measure the response according to the concentration and record the results.

It is clear that the analyte before being sprayed and atomized must be dissolved in a solution: so in this study, where our samples are solid, we have to properly dissolve them (see paragraph 3.7.2).

Then the absorbance of the sample is measured throughout its optical path and detected by a reading and recording system.

Before starting with the detection of the concentration of mercury in the different fish species, we have to build an appropriate calibration straight line that allows us to obtain a range where the tool could read the concentration of the metal; in this case we use five different concentrations of the standard solution.

Chapter IV: RESULTS AND DISCUSSION

4.1 The statistical analysis

In this work the results are obtained through the use of the statistical package SPSS version 19.0 for Windows (SPSS Inc. Chicago IL). The results are analysed from abroad view of all the results until we get to a more detailed analysis. The first analysis concerns the study of possible differences of all the results, determined by the variables “Time” and “Species” so we can see if there are significant changes in the nutritional composition, in the mineral composition and in the presence of contaminants in time and among the three species. We recorded values of minimum, maximum, average and standard error of each value. Then we continued with a variance analysis (ANOVA test) for each parameter. Once established with ANOVA test for each parameter has been carried post-hoc a Tuckey test to determine differences in the mean values of the different parameters according to variables “Time” and “Species”.

4.2 The results

In this work we wanted to carry out an animal life study on wild fish species of wide consumption in all the Spanish community. The search concerns the study of nutritional components (moisture, ash, total proteins and lipids), which offer a series of general information about the nutritional value of *M. merluccius, P. blennoides* and *M. barbatus*, the time trend of these characteristics during the sampling period and the possible interspecies variations. Then through the analysis with ICP-AES spectrophotometer and AFS spectrophotometer, we focused on the revelation of macro, trace elements, and toxic contaminants comparing the values obtained. As concerning the essential mineral elements, the second part of the study has enabled us to understand if there are significant differences between species, but mainly how much these species meet the requested daily nutritional needs. The analysis of heavy metals eventually turned out to be an excellent tool for assessing possible dangers to the consumer in the consumption of these fish products. Before starting with the results of the laboratory analysis, following we can view all the

average weight values of the samples (divided by date of collection) with the related number of analyzed specimens in each sampling (tab.9, 10 e 11).

Table 9. Number of samples analyzed of *M. merluccius* and average weight (g) for each collection

Merluccius merluccius		
Dates	**number samples**	**average weight (g)**
10.04.13	4	**311.39**
17.04.13	3	**458.41**
24.04.13	4	**374.97**
08.05.13	2	**586.30**
15.05.13	4	**365.67**
29.05.13	3	**447.72**
05.06.13	3	**396.32**
19.06.13	5	**184.50**
total samples	28	390.66

Table 10. Number of samples analyzed of *P. blennoides* and average weight (g) for each collection

Phycis blennoides		
Dates	**number samples**	**average weight (g)**
10.04.13	9	**110.78**
17.04.13	11	**111.37**
24.04.13	8	**115.61**
30.04.13	10	**103.87**
08.05.13	11	**110.33**
15.05.13	10	**112.98**
29.05.13	10	**108.11**
05.06.13	10	**102.96**
19.06.13	7	**133.18**
total samples	86	112.13

Table 11. Number of samples analyzed of *M. barbatus* and average weight (g) for each collection

Mullus barbatus		
Dates	**number samples**	**average weight (g)**
10.04.13	13	**95.07**
17.04.13	12	**96.47**
24.04.13	12	**100.71**
30.04.13	13	**90.7**
08.05.13	11	**100.96**
15.05.13	13	**90.83**
05.06.13	10	**101.88**
19.06.13	13	**73.86**
total samples	97	93.82

In 2013 (April-June) 211 exemplar were totally analyzed: 28 correspond to *M. merluccius*, 86 to *P. blennoides* and 97 to *M. barbatus.* The samples are wild exemplars and they are all caught in the same area, Santa Pola harbour (Autonomous Valencian Community) and then sold in Alcantarilla fish market (Murcia Region).

The large difference in the number of samples among *M. merluccius* and the other two species is in the weight: in our animal life study we intended to analyze 1kg of fish for each species. The hake has a heavier weight than the other two species, for that weekly a fewer number is purchased to be analyzed (to reach the desired weight).

In paragraph 3.1.2 are shown the average numbers of specimens picked up and analyzed weekly, and they correspond to 4-6 pieces of hake, 6-8 of greater forkbeard and 12-15 of red mullets.

The total average weight for each species, as we can view in tables 9, 10 and 11 is 390.66g for *M. merluccius*, 112.13g for *P. blennoides* and 93.92g for *M. barbatus*. These figures may be considered in case we observe significant differences in some values that depend on intrinsic factors, but they may be also useful to carry out a series of calculations regarding heavy metals.

In the first part of the results we consider only the “Species” variable. This allows us to build a set of tables showing all the total average data and the possible significant differences between the species (tab.12, 13, 14 and 15). Following, we consider also the “Time” variable, only for some observations regarding the trend of nutritional composition and some toxic elements (particularly those that have a limit set by law). We add the “Time” variable to

build charts remarking: the trend of nutritional composition for all the specimens analyzed (moisture, ash, total proteins and total lipids); moreover other two charts, in particular about Hg and Pb, underlining concentration peaks and considering the fact if they are below the limits set by law (Fig. 28, 29, 30 and 31, 32 and 33).

The following table 12 contains the total average values of chemical components for the three species analyzed (moisture, ash, proteins and lipids). It is possible to view the possible significant differences marked with the letters a-c.

Table 12. Descriptive statistics results of nutritional components of *M. merluccius, P. blennoides* and *M. barbatus*

CHEMICAL COMPOSITION (%)			
	M. merluccius	*P. blennoides*	*M. barbatus*
Humidity ***(Min-max.)***	79.97 ± 0.11[a] (79.08-80.88)	78.84 ± 0.13[b] (77.03-79.82)	76.58 ± 0.31[c] (73.17-78.38)
Ash ***(Min-max.)***	1.28 ± 0.05 (0.7-1.61)	1.24 ± 0.05 (0.7-1.83)	1.32 ± 0,05 (0.56-1.92)
Protein ***(Min-max.)***	14 ± 0.01 (12.5-14.5)	15 ± 0.01 (14-15.5)	14 ± 0,01 (12.5-14)
Lipid content ***(Min-max.)***	0.21 ± 0.02[b] (0.05-0.45)	0.09 ± 0.01[b] (0.01-0.18)	3.51 ± 0.57[a] (0.19-8.61)
N	**24**	**27**	**24**

a-c = different letters indicate significant difference at $p < 0.05$

Following there are 4 charts (one for each value) reporting the values obtained for each sampling (Fig.28, 29, 30 and 31). In this way it is possible to view the time trend of each feature analyzed during all the sampling period. We may observe the possible significant differences throughout the entire analysis process and possible peaks and sudden changes of values. From the charts we see a constant trend of moisture values (Fig.28), some variations not high ones though, relating to the ash and protein content (Fig.29 and 30), while the chart on the lipids (Fig.31) denotes sudden variations especially with regard to *M. barbatus* (the only "semi-oily fish" among the three species).

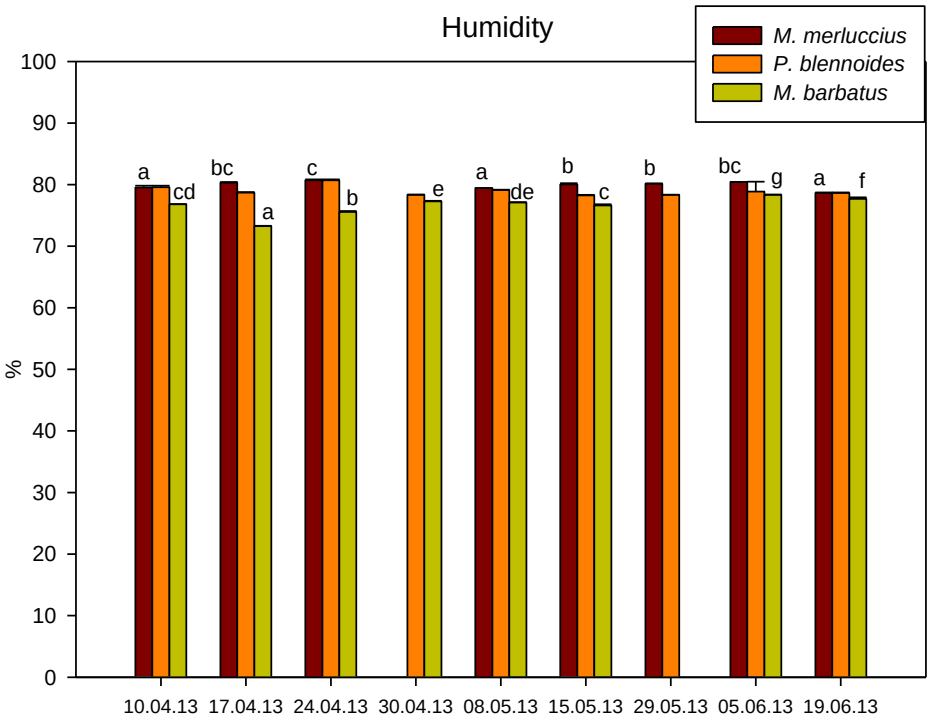

Figure 28. Representation of moisture time trend in *M. merluccius*, *P. blennoides* and *M. barbatus* with related significant differences p <0.05 indicated by the letters a-f

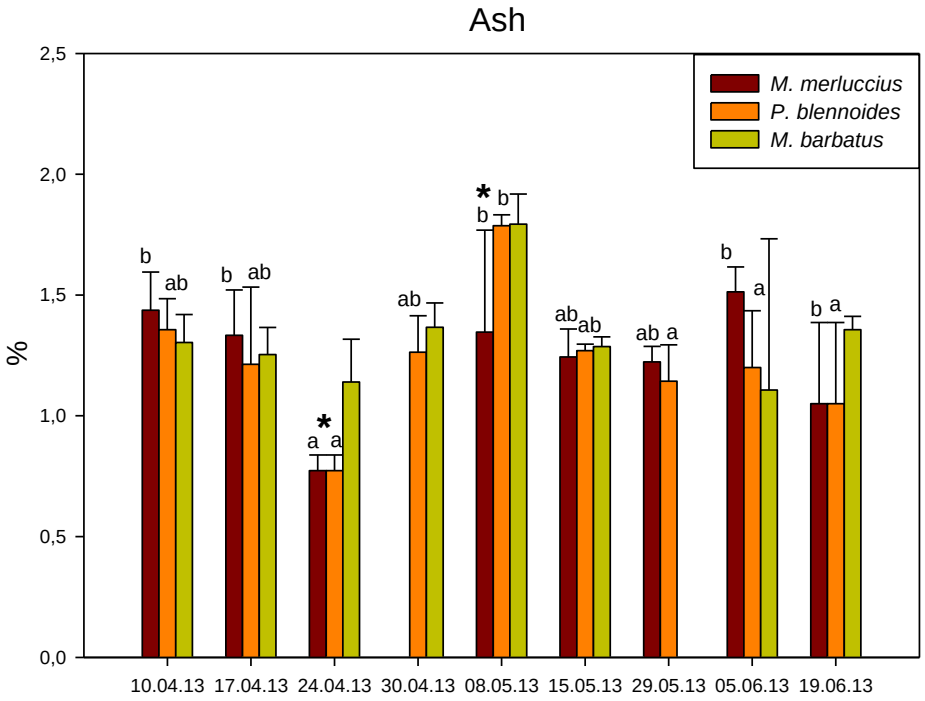

Figure 29. Representation of time trend for ash values recorded in *M. merluccius*, *P. blennoides* and *M. barbatus* with related significant differences p <0.05 indicated by the letters a-b

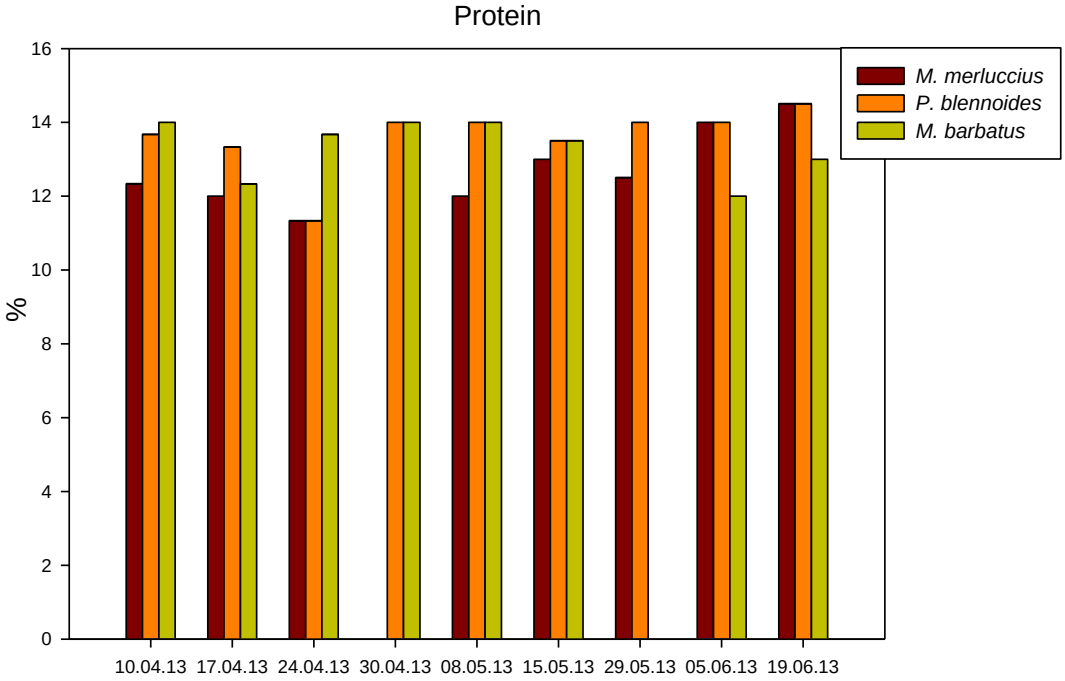

Fig. 30 Representation of time trend for total protein content in *M. merluccius*, *P. blennoides* and *M. barbatus*. Significant differences in values were not observed.

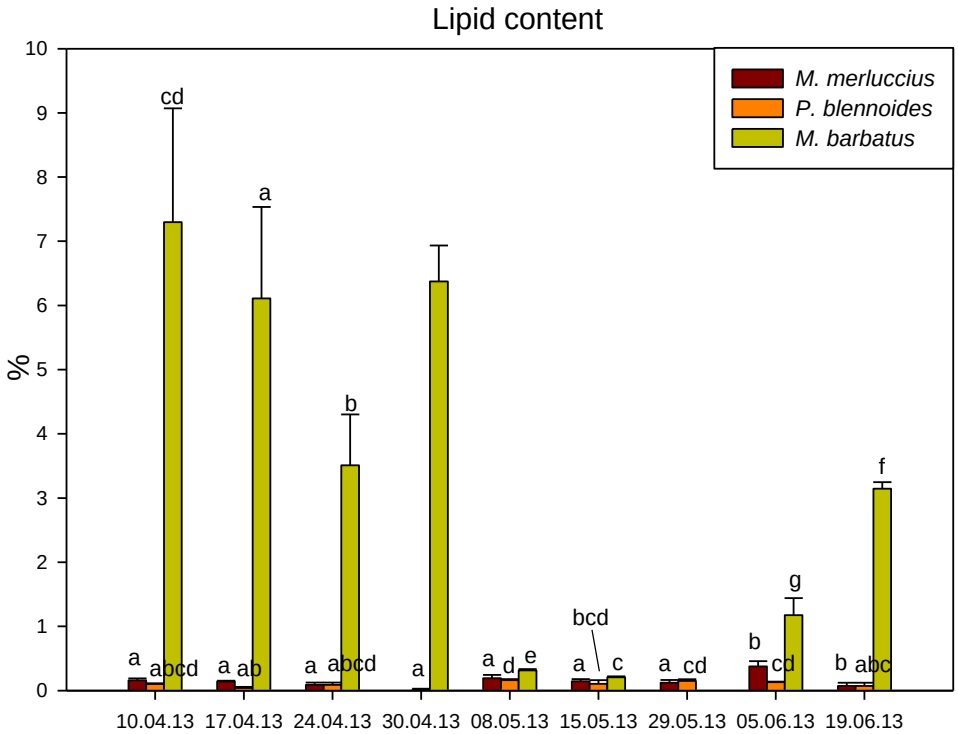

Fig. 31 Representation of time trend for lipid total content in M. merluccius, P. blennoides and M. barbatus with related significant differences p <0.05 indicated by the letters a-g.

Following there are other three tables (Tab. 13, 14 and 15) showing the total average data of: macro and micro elements in mg/100g and heavy metals in mg/kg, highlighting the significant differences among species, in case there are any.

On these tables we can observe, on average, which species contains the highest concentration of a mineral element rather than another, and in total throughout the period of sampling and which accumulates on average the highest concentration of toxic elements.

In Tab.13 we notice significant differences among the species for all the elements except Na. Ca has quite different values in all the three species, while for K there are differences between the greater forkbeard and the other two; there are significant differences for Mg in the values for hake and greater forkbeard, while the red mullet has an average value that is halfway between the two. At the end, P differs significantly in the values for the three species. Generally we can state that the hake has a greater total average concentration of K, the greater forkbeard of Ca, Mg and Na and finally the red mullet a greater concentration of P.

Table 13. Descriptive statistics results of macroelements of *M. merluccius*, *P. blennoides* and *M. barbatus.* Average values and typical error of Ca, K, Mg, Na and P expressed in mg/100g of fresh weight

MACROELEMENTS (mg/100g)			
	M. merluccius	*P. blennoides*	*M. barbatus*
Ca **(Min-max.)**	22.83 ± 2.57^{c} (9.5-59.81)	43.98 ± 3.09^{a} (20.20-82.61)	34.08 ± 2.93^{b} (19.28-79.22)
K **(Min-max.)**	404.88 ± 8.97^{a} (290.01-460.86)	364.63 ± 10.71^{b} (238.8-443.72)	401.08 ± 7.94^{a} (330.81-501.09)
Mg **(Min-max.)**	34.22 ± 0.39^{b} (31.27-38.98)	36.13 ± 0.37^{a} (31.93-39.38)	35.53 ± 0.48^{ab} (31.61-40.66)
Na **(Min-max.)**	128.31 ± 7.21 (70.92-205.27)	148.32 ± 7.4 (78.17-244.10)	132.22 ± 7.24 (71.46-206.55)
P **(Min-max.)**	230.96 ± 3.31^{b} (204.14-253.17)	207.55 ± 3.24^{c} (169.25-240.10)	242.59 ± 3.19^{a} (207.08-271.68)
N	**24**	**27**	**24**

a-c = different letters indicate significant difference at $p < 0.05$

In the following table we notice significant differences only for Fe and Mn. The first mentioned element shows significant differences in the values between the red mullet and the other two species; while for Mn the differences among the species are minimal, although significant between the greater forkbeard and the other two species.

Table 14. Descriptive statistics results of microelements of *M. merluccius*, *P. blennoides* and *M. barbatus*. Average values and typical error of Cr, Cu, Fe, Mn, Mo and Zn expressed in mg/100g of fresh weight

MICROELEMENTS (mg/100g)			
	M. merluccius	*P. blennoides*	*M. barbatus*
Cu **(Min-max.)**	0.06 ± 0.008 (0.00-0.16)	0.06 ± 0.009 (0.03-0.20)	0.06 ± 0.004 (0.03-0.12)
Fe **(Min-max.)**	0.86 ± 0.12 (0.15-2.94)	0.85 ± 0.05 (0.35-1.70)	1.47 ± 0.46 (1.01-1.93)
Mn **(Min-max.)**	0.23 ± 0.005[b] (0.19-0.27)	0.26 ± 0.003[a] (0.21-0.29)	0.24 ± 0.005[b] (0.2-0.28)
Mo **(Min-max.)**	0.006 ± 0.003 (0.00-0.05)	0.02 ± 0.009 (0.00-0.21)	0.01 ± 0.008 (0.00-0.13)
Zn **(Min-max.)**	0.45 ± 0.03 (0.32-1.00)	0.42 ± 0.02 (0.31-0.71)	0.44 ± 0.02 (0.29-0.64)
N	**24**	**27**	**24**

a-c = different letters indicate significant difference at $p < 0.05$

M. merluccius, in tab. 14, as for trace elements, shows a greater concentration in the total average values of Zn, while *P. blennoides* contains a higher concentration of Cu, Mn and Mo. *M. barbatus* can be considered the kind of fish that has a higher concentration of Fe, with a value which is almost double compared to the other ones (1.47mg/100g compared to 0.85 in *P. blennoides* and 0.86mg/100g in *M. barbatus*).

The last table, Tab.15, shows the total average values of mineral elements: we notice significant differences among the species in the values of As, Hg and Pb. Arsenic in particular has a higher value in the greater forkbeard and it has also significant differences between the hake and the other two species. Hake has the lowest total concentration of arsenic. Mercury is higher again in the greater forkbeard and we find significant differences between *P. blennoides* and *M. barbatus*, while *M. merluccius* shows a value that is halfway between the two species. Also in this case the hake has a lower total concentration of Hg. Finally Pb shows significant differences between the hake and the red mullet, with a greater concentration in the first mentioned species. As for the other heavy metals, the hake is the species containing the higher concentration of Al; Ni in the greater forkbeard, while for cadmium it wasn't possible to point out any peaks because all the values recorded were below the LOQ of ICP-AES spectrophotometer (0.01ppm).

Table 15. Descriptive statistics results of contaminants of *M. merluccius*, *P. blennoides* and *M. barbatus.* Total average values of all the analyses performed and typical error of Al, As, Cd, Hg, Ni and Pb are expressed in mg/kg

	CONTAMINANTS (ppm)		
	M. merluccius	*P. blennoides*	*M. barbatus*
Al **(Min-max.)**	0.82 ± 0.19 (0.63-1.01)	0.78 ± 0.06 (0.72-0.84)	0.76 ± 0.12 (0.64-0.88)
As **(Min-max.)**	0.06 ± 0.01[b] (0.05-0.07)	0.17 ± 0.02[a] (0.15-0.19)	0.15 ± 0.03[a] (0.12-0.18)
Cd **(Min-max.)**	<0.01	<0.01	<0.01
Hg **(Min-max.)**	0.192 ± 0.05[ab] (0.142-0.242)	0.336 ± 0.04[a] (0.296-0.376)	0.131 ± 0.03[b] (0.101-0.161)
Ni **(Min-max.)**	0.01 ± 0.003 (0.007-0.013)	0.02 ± 0.005 (0.015-0.025)	0.02 ± 0.003 (0.017-0.023)
Pb **(Min-max.)**	0.01 ± 0.004[a] (0.006-0.014)	<0.01	0.004 ± 0.002[b] (0.002-0.006)
N	**24**	**27**	**24**

a-b = different letters indicate significant difference at $p < 0.05$

Below we have made up other two tables (Tab.16 and 17) where we gathered all the values of macro and micro elements analyzed in this study. The average concentration for each mineral element has been calculated in 150g of fresh fish, which is the average weight of a fish fillet for a normal meal. The table also shows the recommended daily values (RDA) for each mineral element expressed by mg/die.

	total average mg/150g			RDA (mg/die)	
Macroelements	*M. merluccius*	*P. blennoides*	*M. barbatus*	Men (30-55 years)	Women (30-55 years)
Ca	34.245±3.85	65.97±4.63	51.12±4.39	900-1200	800-1000
K	607.32±13.45	546.945±1.06	601.62±11.91	3100 - 3500	3000 - 3200
Mg	51.33±0.58	54.195±0.55	53.295±0.72	420	320
Na	192.465±10.82	222.48±11.1	198.33±10.86	4000 - 6000	4000 - 6000
P	346.44±4.96	311.325±4.86	363.885±4.78	800 - 900	800 - 900

Table 16. Macro elements average concentration in 150g of fish fresh product and related RDA mg/die

	total average mg/150g			RDA (mg/die)	
Microelements	*M. merluccius*	*P. blennoides*	*M. barbatus*	Men (30-55 years)	Women (30-55 years)
Cu	0.09±0.01	0.09±0.01	0.09±0.006	1 – 2.3	0.9 – 1.8
Fe	1.29±0.18	1.275±0.07	2.205±0.69	8 - 10	15 - 20
Mn	0.345±0.007	0.39±0.004	0.36±0.007	1 - 10	1 - 10
Mo	0.009±0.004	0.03±0.01	0.015±0.01	0.075 - 0.25	0.075 - 0.25
Zn	0.675±0.04	0.63±0.03	0.66±0.03	9.5	7

Table 17. Micro elements average concentration in 150g of fish fresh product and related RDA mg/die

Clearly through the values shown on tables 16 and 17 it was then possible to talk about the nutritional value of *M. merluccius, P. blennoides* and *M.barbatus*, and also to see in percentage how much these values meet the recommended daily nutritional needs (RDA).

Regarding the contaminants, the results of some of them are also graphically shown (as previously said), considering, in addition to the variable “Specie”, as well the variable “Time”. It is possible to view the time trend of mercury and lead and the possible peaks or linearity situations (Fig.32 and 33), and also if the values exceeded the limits prescribed by law or how much these values are below the limit set by the Community Regulation and therefore they reflect a situation of safety for the consumer. Cadmium was not reported in the chart because the values have never exceeded the LOQ of 0.01ppm.

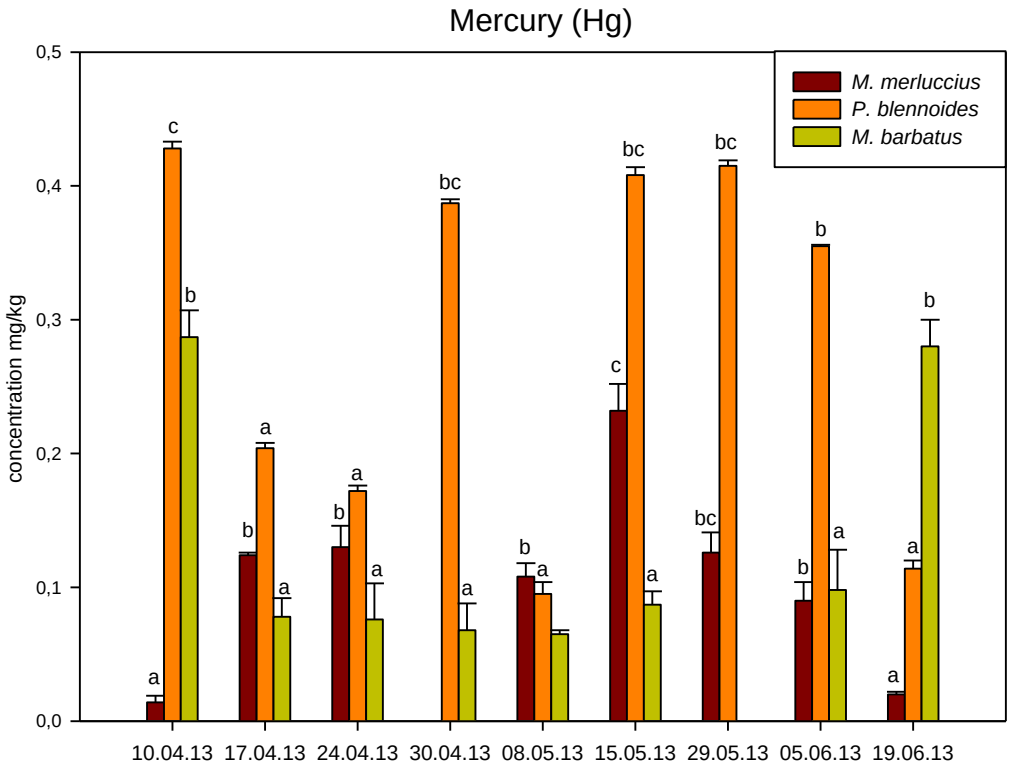

Figure 32. Graphic representation of time trend of mercury concentration in *M. merluccius, P. blennoides* and *M. barbatus* (mg/kg)

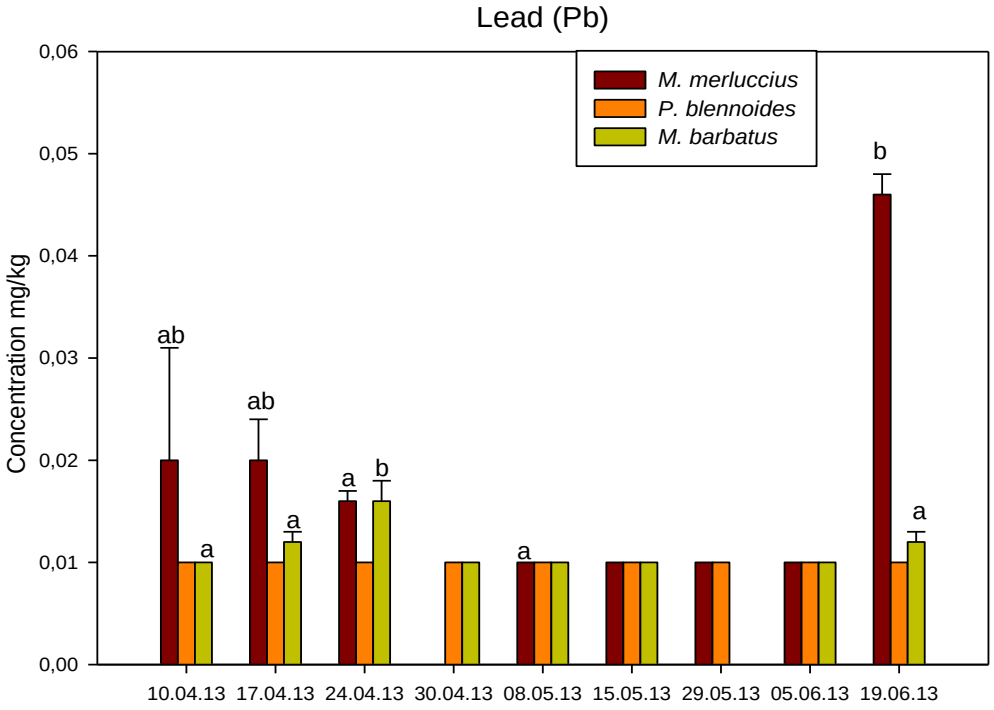

Figure 33. Graphic representation of time trend of lead concentration in *M. merluccius, P. blennoides* and *M. barbatus* (mg/kg)

Figure 32 shows the time trend of mercury. It is quite irregular in the three species. *P. blennoides* shows in almost all the analysis values significantly higher than the other two species (except in dates 08.05.13 and 19.06.13). A third of the analysis reported values exceeding 0.4ppm, but they have never got closer to safety limit. Concerning *M. merluccius* and *M. barbatus* the values are clearly lower and are almost all below 0.2ppm (except the analysis of *M. merluccius* in date 15.05.13).

Figure 33 shows a chart of lead values: on the contrary in this case in half of all the analysis *M. merluccius* reports the highest values even though generally no value gets closer to the limits set by law nor exceeds them (0.3ppm). *P. blennoides* doesn't show any value above the LOQ of ICP-AES spectrophotometer, while a third of the analysis of *M. barbatus* shows values between 0.01ppm and 0.02ppm, in all the other analysis the values are below LOQ.

4.3 Discussion

4.3.1 Chemical composition

Most part of the analyses performed in the Department of Food Technology, Nutrition and Food Chemistry (Bromatology) of University of Murcia concerns especially the aspect, broadly speaking, of chemical composition of fish species for nutrition interest. In this project we analyzed the content of moisture, ash, total protein and total lipids of three wild fish species of wide commercial consumption: *M. merluccius, P. blennoides* e *M. barbatus.* In this first part of the analyses, we wanted to focus on both the total average chemical content of the three species, and on the temporal view of all the sampling performed through proper bar charts. Obviously these data are a great tool to understand the real quality of fish, since its muscle is the main edible part and the change of its composition in terms of chemical-nutritional factors is something crucial in the intrinsic quality of the product (Grigorakis & Alexis 2005; Testi *et al.* 2006; Santaella 2011).

Fishes are biologically important foods in terms of low satiety and high nutritional value (Arinc & Nermin 2012). According to the majority of the researchers, the yield and chemical composition of muscle shows differences among and within the species (FAO 2002; Grigorakis *et al.* 2002 and Luzia *et al*. 2003). This change is mainly dependent on several intrinsic and extrinsic factors such as diet, energy expenditure, migration, sexual changes during the breeding period, water temperature and salinity, the season of catch, age, size and the external environment (FAO 2002; Grigorakis *et al.* 2002; Luzia *et al.* 2003; Nakamura *et al.* 2007; Hünkar & Esra 2009; Santaella 2011). Obviously being aware of these factors is important for a conscious and proper nutrition.

Nowadays the importance of fish consumption is well known because of its digestible proteins and lipid sources (Brown 2000). *M. merluccius, M. barbatus* and *P. blennoides* have a high economic and commercial value in Spain, especially in the Region of Murcia where we carried out our study (Fernández *et al.* 2005; García-Rodríguez *et al*. 2005, 2006b), therefore their study turns out to be very useful to understand the nutritional value as wild fresh fish.

Tab.12 (par.4.2) shows all the total average results of nutritional components of the three species analyzed (moisture, ash, proteins, lipids). We performed a Tuckey test of these values with $p<0.05$ to detect if there are significant differences among the species. Talking

about Tab.12 we note in the total values of moisture a higher content of water in *M. merluccius* with an average value of 79.97%, follows *P. blennoides* with an average value of 78.84% and at the end for *M. barbatus* 76.58%. We know from the literature that the percentage of water content is inversely proportional to the total lipid content (Wheaterley *et al.* 1983) and this can be confirmed observing the table. The highest content of total lipids is in *M. barbatus* (defined a semi-oily fish) with an average value of 3.51% and exactly he stand within the semi-oily category fish that is between 2.5% and 6%. The other two species, which are within non-oily fish, have a total average content lower than 2.5%. In other studies carried out by different authors at different times the results, obtained about species of commercial interest in the group of fish called "low-fat content", (that could be compared with *M. merluccius* and *P. blennoides*) are: *M. hubbsi* (Gadidae) 79.5% (Méndez & Gomez, 1997), *M. aeglefinus* (Gadidae) 79.4%, *M. Merlangius* (Gadidae) 80.4% (Holland *et al.* 1993), *S. aurata* (Sparidae) 76.72% (Kyrana & Lougovois 2002). In comparison with other studies we note a certain similarity in water content values, as these species have a very low fat content in muscle.

The total average content of ash in the three analyzed specie is not so different and it doesn't show any significant difference. The total ash content (which represents the content of inorganic elements and mineral salts in the analyzed edible portion) greatly depends on the size and the samples weight, and also from the sexual maturity, food source and external environment (Watanabe *et al.* 1997; Roy & Lall 2006; Ye C.X. *et al*. 2006; Santaella 2011). In this case the hake, having a significantly larger size than the other two species, should have a higher content of inorganic elements and mineral salts, but this is not confirmed by the results in the table. However, the values do not differ much and do not show significant differences. This could be due to the type of sampling, as this animal life study does not include the analysis of each sample taken at the fish market, but gathers all the edible portion of each fish and analyzes the average content of each weekly sampling. This leads us to state that the precision in the analysis could be lost. The slight difference of the values may come from some extrinsic or intrinsic conditions to the species. It would be interesting to check how the values differ when these specimens were subjected to processing (smoking, pickling, freezing, etc.).

Since these species live in the wild, the total protein content mainly depends on the type of diet, and according to Ros *et al.* (2010) varies between 13% and 20%. Also in this case there are no differences statistically significant for all the three species and the values range between 12.5 and 15.5%. Low values could be caused by the loss of protein content during the digestion or distillation phase, or the incorrect titration in case we used the chemical indicator instead of the pH meter. Furthermore also the sampling could make uniform the values and, in case of errors, we find clear decreases.

Fish lipid content has a beneficial effect on human health, in particular regarding the polyunsaturated fatty acids of ω3 type (Grigorakis *et al.* 2002; Mnari *et al.* 2007), but in this study we considered only the search for the total lipid content, which is an important factor to make the consumer aware of what he consumes. The lipids generally are not evenly distributed in the body of a fish, but the greatest accumulation is expected especially in the abdominal cavity (peritoneal and perivisceral fat) (Santaella 2011). On the contrary, the muscle or edible part contains proportionally less fat than the rest of the fish portion (Rasmussen & Ostenfeld 2000; Regost *et al.* 2001; Santaella 2011), being evenly distributed within the fillet, where the ventral portion is the part that accumulates the greatest amount. In this study we performed homogenisation of the entire edible part, in order to minimize errors during sampling and to obtain a general value of the edible portion available to the consumer. It is clearly evident in our study the inverse proportional relation between moisture and lipids content, as previously mentioned (Weatherley & Gill 1983).

All the 4 charts in par.4.2 (28, 29, 30 and 31) show average values obtained in each sampling and the x-axis reports the time, in order to highlight temporally possible changes in chemical content of each species.

Chart 28 arranges all the values of moisture content, showing an almost constant trend as we could imagine. Obviously there are significant differences within species during the sampling period, but they do not have an effect by changing the quality of the fresh product as they all remain in a percentage between 76% and 80%.

Chart 29 shows the trend of ash content that remains in the range between 0.8% and 1.9%. By analyzing each case, *M. merluccius* has values concentration that exceed nearly always those of the other two species, except the sampling in date 24.04.13 and 08.05.13 that are marked by an asterisk because there was an error during the analysis (part of the incinerated

sample was lost due to operator mistake). Overall, however, there aren't big differences, for this reason also in this case we can confirm that the total ash content does not vary much among and within the species.

Chart 30 is about protein content trend which shows neither significant differences within the species, nor great standard deviations in values (these are very low values that we can't clearly note in the chart). Total protein content ranges between 12% and 14.5%. The hake is the species that on average has a low content of total protein, while the greater forkbeard and the red mullet have higher average values. These data make the consumer think about the choice of product according to his needs. The values are generally very low compared to what is said in the litterature according to Ros *et al.* 2010. The protein content greatly depends on the presence of red muscle, the kind of diet, the age and especially the fish size (Santaella 2011). All the specimens analyzed have a very small size (see tab. 9, 10 and 11), so individually they can't have a high protein content, since also the edible portion is not remarkable. As for *M. merluccius*, there is a contrast in the results: being the sample of larger size it should have the highest total protein content. The results of nearly the entire analyses record lower values than *P. barbatus* and *M. blennoides* (certainly of smaller size): this can be explained because this specimen is in fact the young cod. So this low content might be due to its age, a crucial factor for the development of the product chemical characteristics, beside the type of diet that is different from the adults diet.

The last chart shows the trend of the total lipid concentration and reflects what is said in the literature with regard to the two specimens belonging the group of non-oily fish: *M. merluccius* and *P. blennoides* with very low concentration in the edible portion and always below 0.5%. We note a unique time trend in *M. barbatus*, which in the beginning shows high concentrations (between 4% and 8%), then it decreases drastically in the period of May-beginning of June (below 1%), and in the end it goes back to normal values in the last stages of the analysis. We think that this is due to the accumulation of reserve substances in the abdomen during the reproductive period that in the case of red mullet is from April to August. During sampling we didn't make any distinction between males and females, but in this case it is likely that we analyzed many more females in their reproductive age. This explains the sudden change in lipid concentration of these specimens. There are statistically

significant differences within the species among almost all the values for the performed analyses.

4.3.2 Mineral content

The analysis on the macro elements content are displayed in tables 13 and 16. These tables show the average values of macro elements analyzed (Ca, K, Mg, Na and P) expressed in mg/100g. In this table we can note significant differences with $p<0,05$ for all macro elements except Na.

Fish is known, with regard to mineral salts, as an excellent source of potassium, phosphorus, iodine and selenium (Holland *et al.* 1993), but there are also, although in very low quantities, also sodium, magnesium and calcium. From tab. 13 we notice that there is a very high concentration of phosphorus and potassium and that confirms our statement above(phosphorus: 230.96±3.31mg/100g in *M. merluccius*, 207.55±3.24mg/100g in *P. blennoides* and 242.59±3.19mg/100g in M. barbatus; potassium: 404.88±8.97mg/100g in *M. merluccius*, 364.63±10.71mg/100g in *P. blennoides* and 401.08±7.94mg/100g in *M. barbatus*), then follows a reasonable concentration of sodium (128.31±7.21mg/100g in *M. merluccius*, 148.32±7.4mg/100g in *P. blennoides* and 132.22±7.24mg/100g in *M. barbatus*) and in the end there are, although in smaller quantity, calcium and magnesium (calcium: 22.83±2.57mg/100g in *M. merluccius*, 43.98±3.09mg/100g in *P. blennoides* and 34.08±2.93mg/100g in *M. barbatus*). Overall the data show that *M. Merluccius* has on average a higher content in K compared to the other two, *P. blennoides* has a higher content in Na and Ca and in the end *M. barbatus* has a higher concentration in P. Altogether the average data obtained from these analyses don't differ much considering the same mineral elements in the three different subjects.

Several studies showed that the concentration of minerals in the fish species is highly influenced by many factors, both intrinsic and extrinsic, such as: season, chemical composition, temperature, water salinity, contaminants concentration, place, nutrient availability, size of the fish, age, sex, biological cycle, sexual maturity stage, correlation between white and red muscle and the analyzed portion of the fish (Pérez-Martìn 1986; Orban *et al.* 2002). According to a study by Martìnez-Valverde *et al.* in 2000 the concentration of Mg, Ca and P increases if there are splinters or small bones inside the fish

samples analyzed. So their presence, due to minor mistakes or oversights of the operator during the stages of dissection and shredding (see par.3.1.2 and 3.1.3), may have led to an increase of these values. In this same study it was found that concerning K and Na the recorded values don't show any differences in results whether the presence or not of bones and splinters. So during the review of the results it could be possible to take into consideration a concentration excess in the fillet in Mg, Ca and P in all the three species analyzed.

Taking into account the data in tab.1 and 13 it has been calculated the daily intake percentage of Ca, K, Mg, Na and P in a portion of fish fillet (150g). Tab.16 shows the values of macro elements concentrations in 150g of edible portion with the related value of RDA required to meet the needs of a middle age man/woman (35-55 of age). Through this set of data it was possible to identify how much in percentage these fish products, considered here, meet the daily needs required to maintain a good health (Tab.18):

	DAILY INTAKE %					
Macroelements	***M. merluccius***		***P. blennoides***		***M.barbatus***	
	Men (30-55 years)	***Women (30-55 years)***	***Men (30-55 years)***	***Women (30-55 years)***	***Men (30-55 years)***	***Women (30-55 years)***
Ca	3.3	7.3	6.3	7.3	4.9	5.7
K	18.4	19.6	16.6	17.6	18.2	19.4
Mg	12.2	16.9	12.9	16.9	12.7	16.7
Na	3.8	3.8	4.4	4.4	4.0	4.0
P	40.8	40.8	36.6	36.6	42.8	42.8

Table 18. Daily percentage intake for Ca, K, Mg, Na and P in *M. merluccius* , *P. blennoides* and *M. barbatus*

The table confirms what we have said previously, phosphorous and potassium are the elements that give a greater contribution to meet the daily nutritional needs (respectively for P: 40.8% in *M. merluccius*, 36.6% in *P. blenn*oides and 42.8% in *M. barbatu*s; while for K 18.4% and 19.6% in *M. merluccius*, 16.6% and 17.6% in *P. blennoides* and in the end 18.2% e 19.4% in *M. barbatus*). Following in descending order we find Mg (respectively 19.6% in *M. merluccius*, 17.6% in *P. blennoides* and 19.4% in *M. barbatus*), we have then Ca (7.3% *in M. merluccius* and in *P. blennoides* while in *M. barbatus* 5.7%), and finally Na (3.8% in *M. merluccius*, 4.4% in *P. blennoides* and 4% in *M. barbatus*).

Considering the values of P the percentages obtained seem to be too high compared to what has been obtained in other sources, stating that an average serving of fish provides an average daily intake of about 20% or a little bit more (Pèrez llamas *et al.* 2005). These results

suggest the possible presence of splinters and bones that could have distorted the results increasing to a great extent the recorded values (Martinéz-Valverde *et al.* 2000).

Hereafter we will take into consideration further scientific works obtaining from the recorded data the percentage of RDA given from other wild species in order to compare the different results (Tab.19). All the scientific studies analyzed have in common the same method of detection of mineral elements, so this variable can't be taken into consideration in case of detection of conflicting data.

Reference	Species	Ca	K	Mg	Na	P
(Santaella et al. 2011)	*S. aurata*	2.28%	22.3%	13.29%	1.38%	46.7%
(Martìnez et al. 2000)	*M.merluccius (little)*	5.47%	20.27%	13.1%	3.72%	43.7%
(Martìnez et al. 2000)	*M. merluccius*	3.65%	14.54%	13.17%	0.768%	40.1%
(Martìnez et al. 2000)	*S. vulgaris*	11.4%	13%	1.,17%	4.8%	42.2%
(Santaella et al. 2007)	*D. labrax*	2.96%	13.59%	7.08%	2.04%	31.58%
	M. merluccius (little)	**3.3%**	**1.4%**	**12.2%**	**3.8%**	**40.8%**
	P. blennoides	**6.3%**	**16.6%**	**12.9%**	**4.4%**	**36.6%**
	M. barbatus	**4.9%**	**12.7%**	**12.7%**	**4%**	**42.8%**

Table 19. Comparison in several scientific studies of different daily intake in % of Ca, K, Mg, Na and P

Based on a study carried out by Santaella in 2011 in *Sparus aurata*, have been found out the concentrations of Ca, K, Mg, Na and P in 150g of fillet and have been calculated the values in percentage of the daily intake for this species. As expected, comparing the results, these don't differ much. It is obvious that the percentages don't match, the small variations are due to several factors as: species, age, size, sex, and a whole range of ecological factors that cause the different accumulation of mineral elements in the edible portion of the fish.

In another study by Martìnez *et al.* in 2000 it was possible to perform the same data comparison for other species of sea water, and all of them of commercial interest, in particular for *Merluccius merluccius* (small), *Merluccius merluccius* e *Solea vulgaris*. Taking into consideration the data obtained from this study, we can state that the percentages correspond, except for the Ca value of *S.vulgaris*. This difference may be due to several factors (different diet and geographical location, age, weight, size etc.). Considering the results for the hake, the results correspond (daily need of 3-5% regarding the Ca, 18-20% of K, 12-13% of Mg, 3.7-3.8% of Na e 40-43% of P).

Santaella *et al.* in 2007 analyzed the concentrations of Ca, K, Mg, Na and P of another wild species of interest, the European sea bass also known as sea dace (*Dicentrarchus labrax*). In this case too it is possible to compare the data since the method used to obtain the concentration of mineral salts was the same. The data differ, but not that much, compared to the data recorded in this study, even if in the case of Mg and P the value is significantly lower in comparison with all the three species we are interested in. In this case too the reasons affecting the difference in the values could be both intrinsic and extrinsic as we mentioned above.

The analysis related to the content in trace elements in the three different species are displayed in Tab.14 and 17. These tables show the average values of Cu, Fe, Mn, Mo and Zn expressed in mg/100g. The values go with the analysis of variance performed with ANOVA ($p<0.05$) in this way we highlight if there are significant differences in the content of trace elements in *M. merluccius, P. blennoides* and *M. barbatus*. In Tab. 14 there are significant differences among species for Fe and Mn.

Concerning trace elements, in general, the fish species are excellent sources of selenium and iodine (Holland *et al.* 1993; Olmedo *et al.* 2013). There are also amounts of iron and zinc (Olmedo *et al.* 2013) and other trace elements, even if in small quantities. Having a look at tab 18 and proving what expressed in other scientific works (Martìnez-Valverde *et al.* 2000; Alasalvar *et al.* 2002), Fe and Zn are two mineral elements with highest concentrations in the edible fish serving (iron: 0.86±0.12mg/100g in *M. merluccius*, 0.85±0.05mg/100g in *P. blennoides* and 1.47±0.46mg/100g in *M. barbatus;* zinc: 0.45±0.03mg/100g in *M. merluccius*, 0.42±0.02mg/100g in *P. blennoides* and 0.4±0.02 in *M. barbatus*). Considering that our three species don't come from aquaculture, the higher content of iron and zinc may be caused by a predominance of red fibre in respect to white fibre and this could come from the lifestyle of wild sea species and their greater swimming activity. In fact, the red muscle is characterized by a higher content in trace elements (Schricker *et al.* 1982; Lal 1995).

Considering again the study by Martìnez-Valverde *et al.* in 2000 beside Ca, Mg and P also Fe and Zn are elements to take into consideration concerning the increase of their concentration in case there are splinters and bones in the samples to be analyzed: if these parts are added by mistake, then the results could be altered, increasing the real

concentration in the edible part of the fish. In this study, concerning Cu and Mn it was proved that the accidental presence of bones during the sampling doesn't cause a change in the results.

Tab.17 shows the values of average concentration for Cu, Fe, Mn, Mo and Zn expressed in mg/150g with its related RDA either for a man of middle age and average weight or a woman of middle age and average weight (35-50 years and 70kg). The data obtained from Tab.1 and 14 were used to calculate the percentage of daily nutritional intake of trace elements that these three different fish products supply:

	DAILY INTAKE %					
Microelements	*M. merluccius*		*P. blennoides*		*M.barbatus*	
	Men (30-55 years)	*Women (30-55 years)*	*Men (30-55 years)*	*Women (30-55 years)*	*Men (30-55 years)*	*Women (30-55 years)*
Cu	3.9	5.0	3.9	5.0	3.9	5.0
Fe	12.9	6.5	12.8	6.4	22.1	11.0
Mn	3.5	3.5	3.9	3.9	4.0	4.0
Mo	3.6	3.6	12.0	12.0	6.0	6.0
Zn	7.1	8.4	6.6	8.0	6.9	8.3

Table 20. Percentage of daily intake for Cu, Fe, Mn, Mo and Zn in M. merluccius, P. blennoides and M. barbatus

The table shows the same results in the case of Cu, very similar results for the three species in the case of Mn and Zn. While looking at the data of Fe and Mo we find differences among the species in the percentages obtained and differences both between the daily intake in men and women. It is clear that the different daily intake, concerning a male or a female, regards the fact that the human being (woman-man) has a different need of iron and molybdenum (see Tab.1).

According to the literature data fish is a good source of iron and zinc (Holland *et al.* 1993). The study by Holland *et al.* (1993) informs us that iron gives a daily intake of 10-20% while zinc approximately 5%. In this same study different species have been analyzed (are mentioned mackerel, tuna, herring etc.) compared with the daily intake given by bivalve molluscs and crustaceans. The results in percentage don't differ much from the ones identified in our study: in fact here in percentage the hake, the greater forkbeard and the red mullet give a daily intake between 6 and 13% with a peak of 22.1% in the case of red mullet. This confirms the results recorded in the work we carried out.

4.3.3 Heavy metals

First of all we notice from the results of tab. 15 that of all the 211 specimens analyzed none of them recorded values that exceeded the concentrations of mercury, cadmium and lead established by Regulation (EC) n.629/2009 Commission of the 2nd of July 2008 amending Regulation (EC) no. 1881/2006 of the 19th of December 2006 which sets out the maximum levels for certain contaminants in food (see tab. 2, 3 and 4).

For hake (*M. merluccius*), regarding cadmium, the maximum content allowed by law is 0.05 mg/kg of fresh product (see par.1.4.1). For all the analyses performed cadmium value turned out to be below the LOQ (limit of quantification) of ICP-AES, so with this method it is 0.01 ppm, so there is no doubt: it corresponds to a value that is far below the limit allowed by law. For the greater forkbeard (*P. blennoides*) and the red mullet (*M. barbatus*) too the maximum amount allowed by the Regulations is 0.05 mg/kg and also in this case the results of all the analyses with the spectrophotometer ICP-AES showed values below LOQ (0.01 ppm). Even in the case of these two species the values are quite below the maximum allowed limit. Whereby in all the three species it wasn't possible to detect neither a maximum value nor a minimum one, so it is useless to compose a time chart as we won't notice any change. All the values obtained in the individual analysis regarding cadmium are far below the limit set by law, so the representative fish samples of the entire animal life of Santa Pola port allow us to state that the three species of our study caught during the period April-June 2013 in Santa Pola port (Es) are not a detriment to the health of human beings.

As for lead contents of the hake (*M. merluccius*) the maximum amount allowed by law is 0.3 mg/kg, which is the same value allowed for the greater forkbeard (*P. blennoides*) and for red mullet (*M. barbatus*) (see par.1.2.1). Analyzing the data gained from the hake samples we recorded a maximum value for lead of 0.046±0.005 mg/kg of fresh weight in the analysis carried out on samples taken in date 19.06.2013, while the lowest value recorded corresponds to a value that is below the LOQ, that is <0.01 mg/kg, registered exactly in the following dates 08.05.13, 15.05.13, 29.05.13 and 05.06.13 (Fig.33). Anyway all the values recorded are far below the limit set by the law. At the same way also for the greater forkbeard (*P. blennoides*) the lead values are quite below the LOQ, therefore they are lower than 0.01mg/kg for fresh fish weight. For such reasons it wasn't possible to identify neither a

maximum nor a minimum value in the recorded results during the whole study. At the end, considering the red mullet (*M. barbatus*) and observing the chart in Fig.33 the maximum value worked out goes back to the analysis carried out on the sample in date 24.04.13 and that was a lead concentration of 0.016±0.02ppm, while, concerning the minimum value, we recorded a set of values below the LOQ in all the analyses, except the sampling in date 19.06.13 that recorded a slightly higher value. So even in this case, the entire values do not represent any risk to the consumer, which means that the fishing products of Santa Pola port are to be considered safe for the food market over this sampling period.

In the analysis for mercury which was carried out through spectrophotometer AFS (see par. 3.7.3), we recorded a set of values which are all below the limit allowed by law, as shown in tab. 15. The limit value allowed for hake and greater forkbeard was set to 0.05mg/kg while for the red mullet the maximum limit is 1.0 mg/kg of product (see par.1.2.1). As for the analysis performed on *M. merluccius*, the maximum concentration peak of mercury in the edible portion was registered in date 15.05.13 and it was 0.232±0.02 mg/kg, while the lowest value was recorded in date 24.04.13 and it was 0.014±0.016 mg/kg (see Fig.32) Analyzing the data of mercury in the greater forkbeard the average values reported in Tab.19 are all below the toxicity limit: looking at the chart of Fig. 32, we may observe maximum and minimum values recorded and the time trend of the specimens sampled; the maximum value was found in date 17.04.13 with a concentration of 0.428±0.005 mg/kg, while the lowest value recorded was in date 08.05.13 and corresponded to 0.095±0.09 mg/kg. Then considering the concentration values of mercury in the red mullet, a peak was detected in date 17.04.13 of 0.287±0.02 mg/kg and a minimum value of 0.06±0.03 mg/kg in samples taken in date 08.05.13 (see Fig.32). Concerning the mercury too, the fishes sampled during the period April-June 2013 may be considered safe to human health.

Analyzing the average results of all the contaminants besides those mentioned above, that is, the elements for which it was established a tolerable maximum limit for human health; we wanted to see if there are important differences among species for aluminium, arsenic, cadmium, mercury, nickel and lead (Tab.15).

In tab. 15 we can notice that among all the contaminants aluminium is the one with the greatest amount, in *M. merluccius*, *P. blennoides* and also *M. barbatus* (respectively: 0.82±0.19mg/kg; 0.78±0.06mg/kg e 0.76±0.12mg/kg). It is well known that aluminium is one

of the elements most found in the biosphere (Poleo 1995; Weng *et al.* 2002). It is an element that gets into the water through the cycle of acid rain, reaching a fairly high level in soil and water (Reitz *et al.* 1996). The presence of aluminium is influenced by a number of factors such as the geology of the area, mineralization and the pH of the river (Rosseland *et al.* 1990). One of the major causes of water pollution with negative effect on the sea food chain is the industrial wastes. It is clear from these results that Santa Pola port can be considered an area polluted by aluminium and this is undoubtedly due to the high industrial discharges from the surrounding areas and rivers inputs flowing into the port. We can consider Santa Pola port as an area to keep under control regarding the aluminium.

Taking again into consideration the mercury, we preferred to perform an additional calculation, taking into account the parameter PTWI (Provisionally Tolerable Weekly Intake) set by WHO/EU (JECFA, Joint FAO/WHO Expert Committee on Food Additives) (Quero Llor *et al.* 2011). For Hg this parameter corresponds to 5µg/kg of body weight (WHO 2007; Quero Llor *et al.* 2011). For the calculation are taken into account the results of tab. 15, that is the total average results of Hg in the three species: 0.192 mg/kg for hake, 0.336 mg/kg for greater forkbeard and 0.131 mg/kg for red mullet. According to WHO the weekly tolerable intake (PTWI) of Hg for a person of average weight (70kg) is 350µg. To get to a mercury intake of 0.35 mg per week, then a person should ingest a total of 1.822 kg of hake, 1.041 kg of greater forkbeard and 2.671 kg of red mullet. Considering that the weight value of the fillet on the total body weight ranges between 45-50%, then a person should eat about 10 hakes weekly to exceed the fixed provisional weekly dose; while regarding the greater forkbeard the weekly consumption should exceed 20 servings. Finally as for the red mullet that on average is the species with the lowest weight; a person should eat at least 56 of them to exceed the PTWI. Obviously this calculation should be carried out even in the worst case that is the maximum concentrations recorded for mercury: for the hake the maximum value recorded, previously mentioned, corresponds to 0.232 mg/kg, while for the greater forkbeard is 0.428 mg/kg and 0.287 mg/kg for the red mullet. Evaluating these mercury values the results change: the weekly consumption to exceed the limit will be 1.508 kg of hake, 0.817 kg of greater forkbeard and 1.219 kg of red mullet. So a person should eat approximately 8 hakes, 14 greater forkbeards and 24 red mullets.

Of course, to say whether or not we are in a safe situation from a health point of view, we should consider the average weekly consumption of fish products in a population. These data suggest that the average weekly consumption of these species certainly doesn't mean a health risk as for the mercury (excluding the greater forkbeard that could be a possible risk for those who weekly consume large quantities of fish).

Chapter V: CONCLUSIONS

Coming to a conclusion concerning this project, all the analyses carried out, the results obtained and the observations made, as regards both the nutritional components and toxic contaminants in *M. merluccius, P. blennoides* and *M. barbatus*, we may state that:

- Altogether the analyzed fishery products, concerning the nutritional value, have a high and constant water component, a constant content of inorganic elements, mineral salts and protein, and a low lipid content (except for *M. barbatus* that is a "semi-oily" fish and has a higher concentration, but as we noticed, with variable trend);

- We made the consumer acquainted with mineral salts content for species of high economic and commercial interest in Spain: in general all the specimens contain a great concentration of P and K compared with other elements, but in particular: *M. merluccius* among the three species has a higher content of K and Zn in the edible portion; *P. blennoides* of Ca, Cu, Mg, Mn, Mo and Na; while *M. barbatus* of Fe and P. Considering trace elements there are very subtle differences and in the case of Cu and Zn the differences are almost minimal among the three species.

- *M. merluccius, P. blennoides* and *M. barbatus* consumed as fresh products meet a good percentage of the individual daily needs, especially for K (about 20%) and P (40%). Regarding trace elements Fe is the most higher in *M. barbatus*, meeting the 22% of expected RDA for a male of average age and weight.

- The data about heavy metals (in particular those with limits set by EU Regulation) tell us that all these specimens are not a danger for the consumer, as we have never recorded any data exceeding the maximum limits, so the marketing of these fresh products from Santa Pola port don't represent a risk for the human being's health.

- Santa Pola port (Es) is mainly polluted by aluminium rather than the other elements analyzed in the entire analyses;

- Concerning the chemical composition (moisture, ash, protein, lipid, macro elements and essential trace elements) the recorded data may be considered a good source to carry out further studies about specimens of the same species, but grown up in different conditions, comparing the intrinsic features of nutritional interest for an exemplar growing in the wild compared to one from aquaculture, and making the consumer acquainted with the nutritional value of a product rather than another one (in terms of growth in a different environment and conditions);

- These data are important because they could also be valuable if we wanted to compare the nutritional value of fresh products with processed ones (for instance smoked, frozen, marinated) pointing out the possible loss of the biological properties;

- The obtained data about heavy metals are a great source for future studies, in particular for biomonitoring activities in order to understand the environmental impact that heavy metals have on sea environment. Comparing the concentration of toxic elements with studies from other fishing areas we could come to further conclusions regarding the food safety and in which area there are fewer risks for the consumer safety.

Chapter VI: BIBLIOGRAPHY

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Ringraziamenti

Desidero ringraziare coloro i quali hanno contribuito materialmente ma soprattutto moralmente alla realizzazione di questo elaborato. Un ringraziamento affettuoso va alla mia co-relatrice Marina Santaella Pascual dell'Università di Murcia, per avermi dato la possibilità d'intraprendere questa bellissima esperienza e per avermi indirizzata nel lavoro, senza di lei questa tesi non esisterebbe.

Ringrazio Vichi per il suo grandissimo aiuto nell'avermi insegnato tutte le tecniche di laboratorio, per la sua tranquillità e simpatia.

Ringrazio Marìa Jesùs per avermi fatta entrare in questa grande "famiglia" e per l'aiuto finale nella parte dei risultati e della statistica, "la mami" Toñi per la carica e l'energia che mi ha trasmesso tutti i giorni, l'affetto, la gentilezza e la generosità mostratami.

Un grazie infinito a tutto il personale del laboratorio di nutrizione e bromatologia, professori e dottorandi, per avermi sempre fatto sentire a mio agio e avermi fatto sentire una di loro.

Grazie a tutti gli amici di sempre e di università che mi sono stati vicino, incoraggiandomi e credendo in me e condividendo con me momenti felici ma anche momenti difficili.

La più profonda riconoscenza va alla mia famiglia, in particolare alla mamma che con il suo semplice sostegno morale mi ha supportata, ha avuto un'immensa pazienza e mi ha incoraggiata a non mollare mai e grazie anche al sostegno economico ha permesso che questo obiettivo si realizzasse.