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Assessment of human health hazards associated with the dietary exposure to organic and inorganic contaminants through the consumption of fishery products in Spain

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Abstract

In this work we have evaluated the potential carcinogenic and acutely toxic risks associated to the exposure to highly prevalent organic and inorganic contaminants through the consumption of fishery products by the Spanish population. The concentrations of 8 organochlorine pesticides (OCPs), 18 polychlorinated biphenils (PCBs), 7 polycyclic aromatic hydrocarbons (expressed as benzo[a]pyrene toxic equivalents (B[a]P_{eq})), and three inorganic toxic elements [arsenic (As), cadmium (Cd), and mercury (Hg)] were determined in 96 samples of the most consumed species of white fish, blue fish, cephalopods and seafood species, which were acquired directly in markets and supermarkets in the Canary Islands, Spain. The chemical concentration data were combined with the pattern of consumption of these foodstuffs in order to calculate the daily intake of these contaminants, and on this basis the risk quotients for carcinogenicity and acute toxicity were determined for Spanish adults and children. Our results showed that the daily intake of OCPs, PCBs and B[a]P_{eq} , which is associated to blue fish consumption was the highest within the fish group. The estimated intake of pollutants can be considered low or very low for the individual contaminants, when compared to reference values, except in the case of HCB and As. All the estimated intakes were below the reported Tolerable Daily Intakes. Considering the additive effects of multiple contaminants, the risk of acute toxic effects can also be considered as low or very low. However, our results reflect that the current consumption of white fish in adults and children, and also the blue fish in the case of adults, poses a moderate carcinogenic risk to Spanish consumers, mainly related to their concentrations of As. The conclusions of this research may be useful for the design of appropriate risk communication campaigns.

1. - INTRODUCTION

Organic and inorganic contaminants, such as legacy pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), mercury (Hg), arsenic (As), or cadmium (Cd) are commonly targeted contaminants for research and in monitoring programs. In the last decades, efforts have been made to raise knowledge about the adverse effects on humans and animals, worldwide distribution pattern, and new methods are developed to analyze these compounds in very different matrices and various environmental media (Luzardo et al., 2013b; Sharma et al., 2014). Thus, numerous studies have revealed that these toxic compounds, individually and in combination, may contribute to the development of severe health problems such as cancer, immune suppression or genotoxic effects in humans, even with long-term low-dose exposure (Bergman et al., 2012; Jarvis et al., 2014; WHO, 2003), and many of them have demonstrated endocrine disrupting effects in both animals and humans (Camacho et al., 2014; Kortenkamp et al., 2011). In fact, the use of organochlorine pesticides (OCPs) and PCBs is now banned in most developed countries, but they are still widespread in the environment (Almeida-González et al., 2012; Kakuschke et al., 2010; Luzardo et al., 2014).

Although there are different routes of exposure for humans to these pollutants, it has been established that ingestion of food contributes more than 90% of total human exposure, and that the fatty fraction of food represents the main entrance to the human body (Darnerud et al., 2006; Vazquez et al., 2015). In the last decade, studies on human dietary exposure to persistent pollutants have been carried out in various countries over the world and it has been reported that the dietary intakes vary considerably between countries. The dietary intakes are mainly influenced by the specific dietary habits of each country

(Domingo and Bocio, 2007; Storelli et al., 2011). The daily intake of contaminants needs to be calculated on the basis of the typical food basket consumed in the country obtained from surveys on consumers. The dietary exposure to a wide range of persistent organic and inorganic pollutants of Spanish consumers has been investigated by several authors in the past years for different food groups, such as milk and cheese (Almeida-González et al., 2012; Luzardo et al., 2012), eggs (Luzardo et al., 2013a), yogurt (Rodríguez-Hernández et al., 2015c), meat and processed meat (Rodríguez-Hernández et al., 2015a; Rodríguez-Hernández et al., 2015b), and seafood (Bocio et al., 2007; Domingo and Bocio, 2007; Falcó et al., 2006). Also several basket market studies have been performed in Spain including the major food groups (Bocio and Domingo, 2005; Bocio et al., 2005; Falco et al., 2003; Llobet et al., 2003a; Llobet et al., 2003b; Llobet et al., 2003c), and even the consumption of foods of animal origin has been investigated as a determinant of contamination by OCPs and PCBs (Boada et al., 2014). However, to date only few studies have estimated the carcinogenic risk associated to the exposure to contaminants associated to certain food groups in the Spanish population (Rodríguez-Hernández et al., 2015a; Rodríguez-Hernández et al., 2015b), and to our knowledge none has been developed for the seafood group.

Fish is an important supplier of high quality nutrients such as omega 3 fatty acids, which have been proven reduce the risk of stroke, lower blood pressure and improve arterial integrity, and even decrease the risks of certain cancers (Kris-Etherton et al., 2002). However, fish is also one of the main contributors of the total dietary intake of environmental pollutants (Bocio et al., 2005; Falco et al., 2003; Llobet et al., 2003b; Llobet et al., 2003c). Thus, on the one hand, the health benefits of sea foodstuff consumption have been proven but on the other hand there also exist an increasing concern of the potential risk arising from exposure to toxic pollutants through the intake of fishery products. Because of the growing public concern about the health effects of food borne diseases related to chemical pollutants, there exists the need carrying out studies on particular food groups (such as fish), based on their current pattern of consumption by a given population. In some guidance documents for environmental risk assessment, a reference point from toxicity testing is divided by a default assessment factor and the result compared to the predicted exposure by computing their ratio, which is known as the risk quotient (RQ) (EFSA, 2015; USEPA, 2000). It has been proposed that RQ is a good method to estimate the risk to carcinogenic and acutely toxic effects associated to food contaminants in a population and that is useful to establish exposure limits to those chemicals.

As fish is a staple food of the Spanish diet, with an average consumption of 26.4 kg/person/year (MAGRAMA, 2015) we have designed this study in which we assess the toxic potential of the current pattern of consumption of this food group by the Spanish population. We have acquired seafood samples directly at points of sale to the consumer, and the sampling was designed to follow the Spanish consumers' preferences. We have assessed two types of health risks associated with the consumption of seafood: the carcinogenic risk, and the acute toxicity potential. In this research we have calculated the RQs considering multiple contaminants in fishery products for both carcinogenic and acutely toxic effects, and on this basis we calculated the number of healthy meals per month for a safe consumption in the Spanish population. Obviously, the results of this study need to be considered in the context of the proven health benefits of the nutrients of fish, but may serve for the design of appropriate risk communication campaigns in order to reduce the consumption of certain types of seafood with the aim of optimizing the risk-to-benefit balance.

2. - MATERIAL AND METHODS

2.1. Sampling

We selected for this study the most consumed species of seafood: fish (white fish and blue fish), cephalopods, crustaceans and bivalve molluscs in Spain, according to the data available (AECOSAN, 2006; AECOSAN, 2011). A total of 93 samples from the main commercial species (MAGRAMA, 2015; Martín Cerdeño, 2010) were randomly acquired from multinational retailers settled in the Canary Islands (Spain) between September and November of 2014.. The samples purchased were transported to the Laboratory of Toxicology of the University of Las Palmas de Gran Canaria (ULPGC) and processed immediately upon arrival at the laboratory. We processed and analyzed only the edible parts of seafood (muscle + skin, depending on how the species are consumed). Each sample was constituted by five individual subsamples for each species of fish and cephalopods (fillets, small fishes, or parts of octopus and squids), and six subsamples of each species of crustaceans and mollusks to give pooled samples (using a stainless steel domestic food processor). Thus, 5 to 6 of these composites were used to obtain the data of each species. After that, all samples were frozen at – 80ºC (until analysis).

The species of white fish included in this study were: wreckfish (*Polyprion americanus*), megrim *(Stephanoiepis hispidus*), sole (*Solea vulgaris*), seabass (*Dicentrarchus labrax*), hake (Merluccius merluccius), toothed sparus (*Dentex dentex*), parrot fish (*Sparisoma cretense*), gilt head fish (*Sparus aurata*) and iridiscent shark (*Pangasius hypophthalmus*).

The selected species of blue fish were: tuna (*Thunnus thynnus*), salmon (*Salmo salar*), sardine (*Sardina pilchardus*), and trout (*Salmo trutta*). Additionally, we included those most consumed species of crustaceans, cephalopods, and mollusks: shrimp (*Parapenaeus spp.*), prawn (*Penaeus spp.*), mussel (*Mytilus galloprovincialis*), octopus (*Octopus vulgaris*), and squid (*Theutida spp.*).

2.2. Chemicals, reagents and analytes of interest

All the organic solvents (dichlorometane, hexane, ethyl acetate, and cyclohexane) were of mass spectrometry grade (VWR International, PA, USA). Ultrapure (UP) water was produced in the laboratory using a Milli-Q Gradient A10 apparatus (Millipore, Molsheim, France). The inert desiccant (Celite ® 545) was purchased from Sigma-Aldrich (St. Louis, USA). Bio-Beads SX-3 were purchased from BioRad Laboratories (Hercules, USA). Standards of OCPs, PCB congeners, and internal standards (ISs, PCB 202, tetrachloro-m-xylene, p,p'-DDE-d8, heptachloro epoxide cis, and phenanthrene-d10), were purchased from Dr Ehrenstorfer, Reference Materials (Augsburg, Germany). Standards of PAHs were purchased from Absolute Standards, Inc. (Connecticut, USA). All standards were neat compounds. Stock solutions of each compound at 1 mg/mL were prepared in cyclohexane and stored at -20 °C. Diluted solutions from 0.05 ng/mL to 40 ng/mL were used for calibration curves (9 points).

All samples were screened for the presence of the following anthropogenic contaminants: (a) 8 OCPs: the four isomers of hexachlorocyclohexane (α-, β and γ-, and δ- HCH), p,p′-DDT and its metabolites (p,p′-DDE, and p,p′-DDD) and hexachlorobenzene (HCB); (b) a total of 18 congeners of PCBs: the six marker PCBs (M-PCBs), and the 12 dioxin-like

PCBs (DL-PCBs), which were numbered according to the International Union of Pure and Applied Chemistry (IUPAC): IUPAC numbers # 28, 52, 77, 81, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 180, 189; (c) the 7 PAHs listed as carcinogens by the United States Environmental Protection Agency: benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene and indeno[1,2,3,-cd]pyrene. Finally, we also included the analysis of 3 inorganic toxic elements, which have been reported to be very abundant in fish: arsenic (As), cadmium (Cd), and mercury (Hg).

2.3. Extraction and clean-up

Prior to the extraction procedure, samples were lyophilized for 72 hours. For the extraction of organic pollutants from fishery products samples, we firstly extracted the fat because all these chemicals are completely lipid-soluble and therefore found bound to the lipid fraction. 5 g of each lyophilized sample were spiked with the ISs mix (10 µg/ml) in acetone to yield a final concentration of 20 ng/g and mixed with 30 grams of Celite® to absorb all humidity. The method of extraction and purification followed that recommended by the European Standard for the determination of pesticides and PCBs in fatty food (EN, 1996a; EN, 1996b), whose validity has been previously proven in our laboratory for fatty samples (Camacho et al., 2014; Camacho et al., 2013a; García-Álvarez et al., 2014a). This method combines an automated Soxhlet extraction method (FOSS Soxtec Avanti 2055) with a purification step using gel permeation chromatography (GPC), and gives acceptable recoveries that range between 74.5 % and 104.7 %. Briefly, the Soxtec™ 2055 Auto Fat Extraction (Foss® Analytical, Hilleroed, Denmark) apparatus consisted of an extraction unit, a control unit and a drive unit. The samples, prepared as described above, were inserted into the extraction unit, 40 ml of solvent (dichloromethane) were added to the extraction cups in a closed system and the cups were heated with an electric heating plate. The three-step extraction consisted of boiling, rinsing and solvent recovery. The recovered solvent was evaporated in a rotary evaporator (Hei-VAP Advantage™, Heidolph Instruments®, Schwabach, Germany) at 40 °C to prevent analytes loses. Using a precision balance, the fat obtained was carefully weighted into a zeroed glass tube in order to be able of correcting the results and express them against fresh weight of product. 100 mg of the Soxhlet extracted fat were dissolved in 2 ml of cyclohexane/ethyl acetate (1:1) and subjected to purification by gel permeation chromatography (BioBeads SX-3) using cyclohexane/ethyl acetate (1:1) at a constant flow of 2 ml/min as the eluent. The first 25 minutes of elution, containing the great majority of lipids (> 98%), were discarded. The 25-90 minutes elution volume (130 ml), containing all of the analytes that were co-extracted with the fat, was collected. The sample was concentrated using a rotary evaporator, and finally the solvent was evaporated to dryness under a gentle nitrogen stream. The analytes were re-dissolved in 1 ml of cyclohexane without any further purification and these extracts in cyclohexane were used for the gas chromatography/triple quadrupole mass spectrometry (GC-MS/MS) analysis.

For the analyses of inorganic contaminants, 0.5 g aliquots of lyophilized samples were mineralized with 6 mL of nitric acid (HNO_3) and 50 µl of Yttrium (Y) was added as an internal standard. Vessels were then placed inside a microwave oven (Milestone ETHOS ONE) and heated up to 190°C for 50 minutes. All of the reagents used were of high quality, for analysis of trace elements (Suprapur, Merk, Darmstadt, Germany). After cooling, digested samples were filtered with 1 µm strainer and diluted to a final volume of 50 mL with distilled water into a conical polypropylene tube.

2.4. Procedure of chemical analysis, quality assurance (QA) and quality control (QC)

Gas chromatography analyses of organic contaminants were performed in a single run on a Thermo Trace GC Ultra equipped with a TriPlus Autosampler and coupled to a Triple Quadrupole Mass Spectrometer Quantum XLS (Thermo Fisher Scientific Inc., Waltham, MA, USA), as previously described (Bucchia et al., 2015; Formigaro et al., 2014), and identifications were done using an electron ionization (EI)-MS/MS based on the retention time and the relative ion ratios of each of the analytes. Quantifications were performed against calibration curves as mentioned above. The LOQs of organic pollutants ranged from 0.008 to 0.028 ng/g wet weight, as previously described (García-Álvarez et al., 2014b) (Supplementary Table 1).

All the measurements were performed in triplicate, and we used the means for the calculations. In each batch of samples, four controls were included for every 18 vials (6 samples): a reagent blank consisting of a vial containing only cyclohexane; a vial containing 2 ng/ml of each of the pollutants in cyclohexane; and an internal laboratory quality control sample (QCs) consisting of fish oil spiked at 20 ng/ml of each of the analytes, which was processed using the same method as the seafood samples. The results were considered to be acceptable when the concentration of the analytes determined in the QC sample was within 15% of the deviation of the theoretical value.

Inorganic elements (As, Cd, and Hg) were quantified with inductively coupled plasma-optic emission spectrometry technique (ICP-OES) using a Perkin Elmer Optima 2100 DV instrument coupled with a CETAC U5000AT + ultrasound nebulizer for mercury. A calibration curve and two blanks were run during each set of analyses to check purity of the chemicals, and the blank reading was subtracted from all of the experimental readings. The sample readings (two replicates for each sample and three readings for each replicate) were performed using axial plasma, which provides increased sensitivity, lower background, and improved the limits of detection (LODs) compared to traditional radial plasma. This sensitivity enhancement results in a 5- to 10-fold improvement in the detection limits compared with radially viewed plasma. The concentration values were obtained from the mean of each three readings. The accuracy of the method was verified using reference materials (CRM 278: lyophilized mussel, Community Bureau of Reference, BCR, Brussels). All values of reference materials were within the certified limits. LODs, expressed by wet weight (w.w.), were 0.1 ng/g for As; 1.8 ng/g for Cd; and 0.061 ng/g for Hg. The LODs were determined following the protocol described by Perkin Elmer ICP application study number 57.

2.5. Dietary intake estimates and calculations

For the assessment of the contaminants' exposure through the consumption of fishery products, we first grouped the results of contaminants in food as white fish, blue fish, cephalopods, and seafood (mean values, expressed in ng/g fresh product), and then multiplied these values by the average daily consumption rate of each one of these types of food (expressed in grams/day). Following the recommendations of the EFSA we have used also the percentile 97.5^{th} of consumption to calculate the estimated daily intakes (EDIs) using the upper-bound approach. These assessments (middle-bound (MB) and upper bound (UB)) were done for both adults and children (average body weight: 68.48

and 34.48 kg, respectively). A zero value was assigned to all the compounds below the LOD, and for those compounds below the limit of quantification (LOQ) but above the LOD, the value was assumed to be ½ LOQ (Camacho et al., 2013b; Luzardo et al., 2013b). Food consumption data of the Spanish population were obtained from the Spanish Diet Model for the Determination of the Consumer's Exposure to Chemicals of the Spanish Agency for Consumer Food Safety and Nutrition (AECOSAN, 2006; AECOSAN, 2011).

In this research, for the calculations we considered the total value of DDTs (ΣDDTs) as the sum of the measured values of p,p′-DDT, p,p′-DDE and p,p′-DDD; the total value of HCH residues (ΣHCH) as the sum of the 4 HCH isomers (α-,β-,γ- and δ-HCH); the HCB as an independent contaminant; the value of the PCB congeners that are considered markers of exposition (ΣM-PCB: #28, 52, 101, 138, 153 and 180); the value of the PCB congeners that are similar to dioxins (ΣDL-PCBs: #77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169 and 189). For the risk estimation, we calculated the potential toxicity for the DL-PCBs (in terms of toxic equivalence to dioxins; $\sum TEQ_{DL\text{-}PCBs}$) using the OMS 2005 TEQs (Van den Berg et al., 2006). Finally, we also considered the total content of carcinogenic PAHs (Σc-PAHs) following the EFSA recommendations (EFSA, 2008). Benzo[a]pyrene is the most widely known and studied compound of this group due to its importance as one of the most potent carcinogenic hazards. Thus, the carcinogenic risk of a PAH mixture is often expressed by its BaP equivalent concentration ($B[a]P_{eq}$). Thus, for the risk estimation, we used toxic equivalency factors (TEFs), which are established for the carcinogenic PAHs (Nisbet and LaGoy, 1992), to express the results in the form of benzo[a]pyrene toxic equivalents ($B[a]P_{eq}$).

2.6. Risk characterization

We applied a risk quotient (RQ) to estimate whether the intake of contaminated sea foodstuff. We calculated this intake RQ as the ratio between the consumption of a given foodstuff (in this case seafood expressed in grams/day, R_{fish}) and the maximum tolerable consumption of that foodstuff (CR_{lim}), which is calculated taking into account their concentrations of contaminants. We have used this index both, for the calculation of the carcinogenic risk, and also for the risk of acutely toxic effects associated to the consumption of that food.

Thus, in the present study, the carcinogenic effects of multiple contaminants were evaluated using the methodology previously used for different food groups (Rodríguez-Hernández et al., 2015a; Rodríguez-Hernández et al., 2015b; Yu et al., 2014), according to the following formulas:

(Equation 1)

$$RQ = \frac{R_{fish}}{CR_{\lim single}} \text{ (for a single contaminant)}$$

(Equation 2)

$$RQ = R_{fish} \cdot \sum_{m=1}^{x} \frac{1}{CR_{\lim multiple}} \text{ (for multiple contaminant groups)}$$

(Equation 3)

$$CR_{\lim(single\ or\ multiple)} = \frac{ARL \cdot BW}{\sum_{m=1}^{X} C_m \cdot CSF_m}$$

Where CR_{lim} is the maximum allowable consumption rate for a particular fishery product (kg/day), and may be calculated either for a single contaminant or for various chemicals belonging to the same chemical group, and assuming they share the toxicological properties; ARL is the maximum acceptable individual lifetime risk level, which is dimensionless and a value of 10^{-5} (one-in-100.000) was used in this study, base on the literature (Yu et al., 2014); BW is the body weight (kg); C_m is the median concentration of contaminant *m* in a particular fishery product (mg/kg); and CSF_m is the cancer slope factor of contaminant *m* for a carcinogenic hazard (mg/kg/day)-1. In the case of multiple contaminants with the same CSF, their concentrations in a particular type of seafood were summed (from m = 1 to m = X).

In addition, we evaluated the acutely toxic effects of multiple contaminants using the following equation:

(Equation 4)

$$CR_{lim} = \frac{BW}{\sum_{m=1}^{X} \frac{C_m}{RfD}}$$

where RfD_m is the reference dose of contaminant *m* for acute toxic effects (mg/kg/day).

The RfD and CSF values of contaminants for carcinogenic and toxic effects were taken from the Integrated Risk Information System (IRIS) of the USEPA (http://www.epa.gov/IRIS/).

According to the previous reports it is considered that if the RQ value is equal or less than 1 then it can be considered that the risk is low (< 10^{-5}) via fishery products consumption. However, the population is considered to be at health risk when RQ is greater than 1. (Yu et al., 2014).

2.7. Meal suggestions for the consumption of seafood.

Once we determined the concentrations of pollutants in seafood we considered very useful for the consumer and the health authorities to calculate the maximum intake of these foods that can be considered safe. The USEPA notes that daily fish consumption limits may be more conveniently expressed as the allowable number of fish meals (of a specified meal size) that may be consumed over a given time period (USEPA, 2000; Yu et al., 2014). For the consumer to express this as the number of allowable meals per month is more practical. Therefore, we calculated the number of allowable meals per month considering multiple contaminants for carcinogenic and acute toxic effects according to the following equations:

(Equation 5)

$$C_{mm} = \frac{R_{fish} \cdot TP}{MS}$$

(Equation 6)

$$RC_{mm} = \frac{C_{mm}}{RQ}$$

where C_{mm} is the current number of meals per month for each type of fishery product; MS is the meal size (225 g for fish, and 120 g for cephalopods and seafood); TP is the averaged time period (month = 30.44 days); and RC_{mm} is the recommended maximum number of serving of each food per month.

2.8. Statistical analysis

Database management and statistical analysis were performed with PASW Statistics v 20.0 (SPSS Inc., Chicago, IL, USA). Because the data did not follow a normal distribution, the statistical analyses involved the use of non-parametric tests. The differences of contaminants between two independent groups were tested with the Mann–Whitney U-test and Kruskal Wallis test. *P* values of less than 0.05 (two-tailed) were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Occurrence of chemical pollutants in fishery products

Table 1 shows the concentrations of the toxic contaminants included in this study in the different groups of fishery products: blue fish, white fish, cephalopods and other seafood (crustaceans and molluscs). We also present in this table the statistical comparison between the two classes of fish, and also the comparison between cephalopods and seafood. In addition, we also considered interesting to present the comparison between total seafood (including cephalopods) in a graphical manner as supplementary material (Suppl. Figure 1).

We found great differences in the levels of contaminants among the different groups of fishery products (Table 1), and also among the different species within each group (data not shown). This is logical, because the distribution of the pollutants in the aquatic organisms is highly dependent on the environment that they live, as well as on other many factors, such as the trophic levels, the feeding habits of the species, differences in metabolism due to different abilities of biotransformation, the excretion rate of these compounds from the body, etc… (Liao et al., 2016). Moreover, it is well know that most of the contaminants included in this study are lipid soluble and therefore it is reasonable to find a direct relationship between their concentration and the lipid content of each species. Thus, as seen in Table 1, we found that blue fish (which contains at least 5% of lipids in the edible part) has higher levels of organic pollutants than white fish: ΣDDTs (median: 1.5 vs. 0.21 ng/g); ΣHCHs (median: 0 ng/g in both groups; mean: 0.4 vs. 0.1 ng/g); HCB (median: 0.6 vs. 0.1 ng/g); M-PCBs (median: 2.6 vs. 0.3 ng/g); $\sum TEQ_{DL\text{-}PCBs}$ (0.006 vs. 0.0009 pg/g), and $B[a]P_{eq}$ (0.2 vs. 0.03 ng/g). These findings are consistent with other studies that found that higher levels of contamination occur in blue fish (Mezzetta et al., 2011). We also found that fish in general (blue and white fish) presented higher levels of organic pollutants than cephalopods, molluscs, and crustaceans, which may be also related with the lower percentage of fat of these foods. This is also consistent with the data published previously (Bayarri et al., 2001; Carubelli et al., 2007). The only group in which these differences were not observed was PAHs, (expressed as $B[a]P_{eq}$), as we found that the levels in cephalopods were similar to those in blue fish. Other authors have also previously reported high levels of PAHs in molluscs, even higher than in fish

(Martí-Cid et al., 2007), probably due to the fact that most edible sea molluscs are filter feeders.

With regards to inorganic pollutants, we included in this study the determination of As, Cd, and Hg due to the concerns on human health of these elements, and that it has been reported these metals are the most abundant in sea foodstuff (EFSA, 2009a; EFSA, 2009b; EFSA, 2012). There are many studies, which have determined their contents in the edible parts of commercial seafood species, since the monitoring of metal concentrations in fish meat is very important to ensure compliance with food safety regulations and consequent consumers' protection (Bosch et al., 2016). In the present study the pattern of contamination observed for organic pollutants in which blue fish species are the most contaminated is not maintained. Except in the case of Hg, we found that cephalopods, crustaceans, and molluscs exhibited the highest levels of these elements (Table 1), which probably relates with their different feeding habits. White fish also had higher concentrations of As than those detected in blue fish species. We considered this especially of concern since white fish is the most consumed fish by the Spanish population, and several studies have shown that the intake of As, particularly the inorganic forms of this metalloid, is related with an increase incidence of cancer (Carlin et al., 2015; Di Lorenzo et al., 2015). Although we could not perform the speciation of As and only the total content of As was measured, it is accepted that in the edible parts of marine fishes, ~ 10% of As is generally present in inorganic forms (Rahman et al., 2012). Assuming that this ratio is maintained in the samples of aquatic organisms included in this study we considered only 10% of the values depicted in Table 1 in the further risk assessment, which is detailed in the following sections.

3.2. Daily intake of toxic contaminants through the consumption of fishery products

The estimation of the daily intake (EDI) of pollutants through the consumption of fishery products was obtained by combining the results of contamination of the samples and the pattern of consumption of these products as reported by the Spanish authorities (median and percentile 97.5^{th}, in ng/day) (AECOSAN, 2006; AECOSAN, 2011). The results of these estimations (MB and UB approaches) for both adults and children are presented in Table 2.

3.2.1 Organic contaminants

According to our results the greater contribution to the EDI of organochlorine compounds (in both adults and children) occurs through the consumption of blue fish (68.4% and 50.8%, respectively) followed by white fish (25.3 and 40.1%, respectively), seafood (4.2% and 5.0%, respectively), and cephalopods (2.3 and 4.0%, respectively). This pattern was observed for all the individual compounds, and this had been also reported for these pollutants by several authors (Mezzetta et al., 2011; Moon et al., 2009). According to our calculations the EDI of ΣM-PCBs was the highest, followed by ΣDDTs. To adequately evaluate the exposure to contaminants by means of the consumption of a given food group it is necessary to compare the values with the previously calculated reference values, such as the Tolerable Daily Intake (TDI). Regarding this we have to note that none of the OCPs exceeded their respective TDIs (JECFA, 2000), and even did not surpass 1% of those values, nor in the MB nor in the UB approach (TDI ∑DDTs = 10000 ng/kg b.w., TDI ∑HCHs = 5000 ng/kg b.w., TDI HCB = 160 ng/kg/day) (ATSDR, 2002; Luzardo et al., 2013a). To be able of comparing the exposure to PCBs with some reference values it is necessary to use the approximation of toxic equivalence to dioxins as defined by the WHO (Van den Berg et al., 2006), as the TDI for PCBs has been set in the context of dioxin exposure (2 pg WHO-TEQ/kg b.w/day (SCF, 2000)). Once the results were transformed using the corresponding TEFs, our results indicate that the exposure to dioxin-like PCBs through the consumption of fishery products only accounts for 1.08% of that TDI in the worst scenario (adults, UB approach, Table 2).

Regarding to the other group of organic pollutants included in this research – the PAHs – the EDI of $\Sigma B[a]P_{eq}$ was estimated to be 9.34 ng/day and 5.30 ng/day in Spanish adults and children respectively, and fivefold when the UB approach is considered. Similarly to organochlorine pollutants, blue fish species were the main contributors to the exposure to these carcinogenic pollutants within this food group (57.4% in adults and 46.9% in children, Table 2). Although the TDI for the carcinogenic PAHs has not yet officially established, we used the TDI for $B[a]P_{eq}$ of 20 ng/kg b.w. day, as recommended for the Contaminated Land Exposure Assessment of UK (CLEA-UK, 2008). The EDIs of $B[a]P_{eq}$ calculated in this study account for less than 3% of this reference value in both adults and children, in the worst-case scenario (Table 2).

3.2.2. Inorganic contaminants

When we consider the intake of inorganic contaminants, contrary to what is described above, we found that white fish is the main contributor. Arsenic is considered one of the most dangerous elements for health and all the studies conducted so far show that the foods that are the richest in inorganic arsenic are seaweed, fish, other seafood and cereals (EFSA, 2009b). According to our estimations the daily intake of total As through fishery products could be as much as 1.96 µg/kg/day (adults, worst case scenario (UB approach), Table 2), which would represent almost 94% of the established TDI (2.1 µg/kg/day, (JECFA, 2010)), which is of very much concern. If we take into account that the most dangerous As is that which is in inorganic form, and we assume that 10% of total As in fishery products is inorganic As (Rahman et al., 2012), the average intake would represent around 14%-60% of the estimated average inorganic As exposure from food and water across 19 European countries (0.13 to 0.56 µg/kg b.w./day, (EFSA, 2009b)). Moreover, the EFSA CONTAM Panel has identified a range of values for the 95% lower confidence limit of the benchmark dose of 1% extra risk ($BMDL_{01}$) for each endpoint of a wide range of key epidemiological studies (0.3 to 8 µg/kg/day, (EFSA, 2009b)), and recommended that the overall range is used as reference instead of a single reference value. Thus, the lowest values, which correspond with the risk of lung cancer, are well below the MB-EDI of 0.78 µg/kg/day reported in this study, which would mean that theoretically the current pattern of fish consumption in Spain would not be exempt of risk (even more if the UB approach is taken into account, Table 2).

The Cd has also been extensively studied due to its toxic properties (EFSA, 2009a), being considered primarily toxic to the kidney, where it accumulates over time and may cause renal dysfunction. Besides, the International Agency for Research on Cancer has classified Cd as a probable human carcinogen on the basis of occupational studies, and recently epidemiological studies have revealed an increased risk of lung, endometrium, bladder, and breast cancer in relation with the environmental exposure to this metal (EFSA, 2009a; Menon et al., 2015; Vilahur et al., 2015; Weidemann et al., 2015). However, basically all the carcinogenicity data available are related to inhalation exposure, and there are no studies of orally ingested cadmium suitable for quantitation, so we did not further considered this metal as a carcinogen in the present study. Nevertheless, as many other toxic effects (other than cancer) have been described for Cd, a Provisional Tolerable Weekly Intake (PTWI) of 7 μg/kg has been established. According to our estimations the average intake in Spanish population through the consumption of fishery products does not reach 2% of its PTWI (9% when the UB approach is considered). The EFSA has determined from the analyses of more than 140000 food samples that seafood are the commodities where the highest Cd levels are detected, and besides it has also been determined that only 3-5% of this metal is absorbed after dietary exposure (EFSA, 2009a). Considering this and the estimations done in this research, we can conclude that the dietary exposure to Cd in Spain is currently very low, and very far away from being worrying.

Finally, regarding to the Hg, it has also been established the foods in the group "Fish and other seafood" have the highest values of this highly toxic heavy metal in comparison to all other food groups, although the different surveys available indicate that the total Hg content varies widely among different fish species, and is highest in predatory fish (JECFA, 2004; JECFA, 2006). The toxic properties of Hg are well known, especially for kidney and the developing nervous system. Therefore the EFSA's CONTAM Panel has established a PTWI of 4 μg/kg (EFSA, 2012) for this metal. According to our results, the dietary exposure to total Hg from fishery products of an average Spanish consumer is 0.37 μg/kg/week in adults and 0.53 μg/kg/week in children (Table 2). These values are more than tripled for both age groups when the UB approach is considered. As is estimated that approximately 90% of the total mercury in fish and shellfish is present in the form of methyl mercury (MeHg) (EFSA, 2005), our results would indicate that Spanish adults would be exposed to 0.33 μg/kg/week, and Spanish children to 0.48

µg/kg/week of this extremely toxic form of Hg, in the Mb approach (1.13 µg/kg/week and 1.47 µg/kg/week, respectively in the UB approach). However, it should be also noted that one of the major risks that have been associated to Hg, and in particular to MeHg, is developmental toxicity, where a brief exposure to the foetus can lead to permanent damage. Various organizations have estimated the daily intake of mercury (as MeHg) that is unlikely to be harmful. The World Health Organization has estimated that 0.22 µg/kg/day is unlikely to be harmful, with pregnant women identified for concern (Wise, 2004). Considering this, our estimates indicate that in the upper bound approach a Spanish pregnant woman could be exposed to 73% of this reference value (0.16 µg/kg/day), only via seafood consumption, and the children, which are high consumers of seafood would almost reach this threshold (96%). These results can be considered of very much concern.

The estimates of this study regarding Hg are consistent with the exposure estimates in the European Union (EU) as calculated by the EFSA using the middle bound approach, which range from the lowest minimum of 0.14 µg/kg/week in very elderly to the highest maximum of 5.05 µg/kg/week in adolescents. If we additionally consider that it has been estimated that Hg in fish would represent approximately 37% of total dietary intake (36.8% of food product coverage) (EFSA, 2012), a bulk calculation indicate that Spanish adults would be exposed to 25% of the PTWI through their total diet (9.2% from fishery products), and that this exposure would reach 35.7% of PTWI in the case of children (13.2% from fishery products). Therefore, the estimated exposure to total Hg in Spain from the diet alone would not exceed the PTWI, as it has also been reported for the rest of EU’s countries (EFSA, 2012).

3.3. Health risk assessment via multiple contaminants associated to the consumption

of fishery products in Spain

Although according to the above calculations none of the individual TDIs are exceeded for any of the contaminants, the consumption of fish implies the exposure of the consumer to multiple contaminants, and antagonistic, synergistic, and additive interactions among the contaminants can occur. For the adequate human health risk assessment the USEPA recommends that the additive model be used for multiple contaminants that cause similar toxicological effects (USEPA, 2000; Yu et al., 2014). Using the calculated acute reference doses (RfDs) and cancer slope factors (CSFs) for the contaminants included in this study (USEPA, 2014) we have considered two types of health risks: acute toxicity and carcinogenic (genotoxic) potential of fish consumption. For each of these endpoints, we first calculated the individual CR_{lim} to estimate the exposure limits to these chemicals through the consumption of fishery products, as previously reported (Yu et al., 2014). Secondly, from the calculated CR_{lims} we calculated the individual RQs. The RQ evaluation has been proposed as a convenient method of estimating population risk and to provide a plausible worst-case scenario for initial screening of potential risk (USEPA, 2000; Yu et al., 2014). Finally, the RQs of each type of pollutant were summed and presented as the overall risk associated to each subgroup of food (blue fish, white fish, cephalopods, and other seafood) (Figure 1).

3.3.1. Acute toxicity potential of the consumption of fishery products

When the acute toxic effects of the contaminants were considered, the maximum allowable consumption rates (CR_{lims}) (Table 3) of blue fish in children were from 350 times higher (for $\sum TEQ_{DL\text{-}PCBs}$) to 5185 times higher (for $B[a]P_{eq}$) than the current consumption rate of this type of food (Table 2), and these values were more than double in adults. For white fish the CR_{lims} were from 7 times higher (for As) to 15801 times higher (for $B[a]P_{eq}$); for cephalopods the CR_{lims} ranged from 25 (As) to 127000 times higher (ΣHCHs); and for seafood from 12 (As) to 91145 times higher (HCB) than the current consumption rates of these food subgroups by Spanish children. Again, in all the cases the estimations of maximum allowable consumption for Spanish adults were more than double than in children (Table 3). Therefore, the individual RQs ranged from 0 to 0.06 in adults and 0 to 0.13 in children for the individual contaminants (Table 3), and at most 0.2 for all contaminants (white fish, children) (Figure 1A). Thus, as all the RQ values were much lower than 1 we can conclude that the consumption of the fishery products would not pose risk of producing acute toxicity associated to their content in chemical contaminants.

3.3.2. Carcinogenic potential of the consumption of fishery products

In a similar manner we also calculated the maximum allowable consumption limits and the RQs associated to the current consumption of this group, but considering the carcinogenic potential (Table 4).

Based on the contamination and the consumption data of fishery products, our calculations indicate that again all the CR_{lim} of the individual pollutants were higher than the pattern of current consumption (which would not indicate obvious health risks due to the intake or uptake of contaminants via fish consumption would be experienced) except in the case of inorganic As (using the current CSF value of 1.5 mg/kg/day on IRIS, (USEPA, 2014)), for which the current consumption of all the subgroups of fishery products would exceed the maximum allowable rate. When we considered the additive effect of all contaminants by food subgroups (Figure 1B) the RQs were higher than 1 for blue fish, white fish, and seafood in Spanish adults, and for white seafood in Spanish children, mainly due to the contribution of As. This means that the current dietary intake of fishery products would represent a risk of carcinogenicity, especially associated to the consumption of white fish. These results are consistent with those recently reported in the Mediterranean region, where the highest risk of carcinogenicity of the fish consumption pattern was associated with the content in As of these foods (Copat et al., 2013). In that study Copat et al. (2013) suggested a modification of the pattern of consumption of these foods, as we also do in the following section.

3.4. Meal recommendation for consuming fishery products

The USEPA has suggested that the CR_{lim} for carcinogenic and acute toxic effects (whichever value is lower) should be used to calculate the maximum number of meals of fishery products per month, and thus be able of giving advise to consumers to protect the human health (USEPA, 2000; Yu et al., 2014). As in this study we found that the CR_{lims} for carcinogenic effects were lower than those of acute toxicity, we used these values to calculate the maximum number of meals of each food subgroup that would no pose obvious health risks due to the intake or uptake of contaminants via fish consumption (this is, consumption which that would allow a RQ $\leqq$ 1 for all products). In Table 5 we summarize these recommendations for adults and children (RC_{mm}), and compare these recommended maximum number of meals with the current pattern of consumption (C_{mm}). According to our calculations, and strictly considering the results of our study, the Spanish population should reduce the consumption of fishery products in general terms, but more importantly in adults. Since the white fish involves greater risk, as detailed in this research, its consumption should be further reduced, to around one-third of the current consumption rate (that is, no more than one meal every two weeks). Adults should also slightly reduce consumption of blue fish and cephalopods, crustaceans and mollusks (Table 5). However, it is also necessary to consider that the health benefits of the high value nutrients from seafood have been deeply studied (PUFa as well as vitamin D_3, iodine, vitamin B12, etc.), and Therefore, it is not advisable to recommend abruptly reducing fish consumption (EFSA, 2014). Nevertheless, the results of this study should be taken into account for the design of appropriate risk communication campaigns aimed to reduce the consumption of certain types of seafood; the aim should be an optimal risk-to-benefit balance.

4. CONCLUSIONS

In this research we have estimated the daily intake of contaminants through the consumption of fishery products. When these intakes are individually considered we found that none of the reference values (tolerated daily intakes) were exceeded, although the case of As, HCB, and $B[a]P_{eq}$ could be somewhat of concern. However, when we estimated the risk associated to multiple contaminants acting together we found a moderate risk of carcinogenicity. Therefore, a decrease in the consumption of fish and seafood is recommended to avoid the carcinogenic risk associated to these pollutants, especially in the case of white fish, whose consumption should be reduced to one-third of the current level. It seems necessary to maintain surveillance programs that monitor the trend of persistent pollutants in sea foodstuffs, and especially of the concentrations of toxic elements, such as arsenic. The results of this study may be taken of utility for risk managers in the design of appropriate risk communication campaigns aimed to reduce the consumption of certain types of seafood with the aim of obtaining an optimal risk-to-benefit balance of fish consumption.

5. FIGURE CAPTIONS

Figure 1. Hazard ratios of the contaminants for acutely toxic effects **(A)** and carcinogenic effects **(B)** in adults and children via consumption of fishery products. The red line indicates the threshold for toxic effect (RQ = 1).

Supplementary Figure 1. Comparison of the levels of organic pollutants (A) and inorganic pollutants (B) in fish (blue + white fish) and seafood ((cephalopods + seafood) * P < 0.05; ** P < 0.01; *** P < 0.005

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