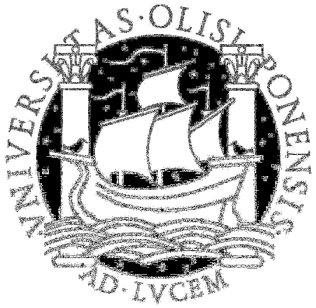
UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA



ESSENTIAL AND TOXIC ELEMENTS IN FISH PRODUCTS CONSUMED IN PORTUGAL

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DOUTORAMENTO EM BIOQUÍMICA
(Bioquímica Farmacêutica e Toxicológica)

2011

In memory of my beloved father

ACKNOWLEDGEMENTS

From the beginning to the conclusion of this work there were many persons who were on my side. I am particularly grateful to:

Dr. Maria Leonor Nunes, I show my deep appreciation for the careful and attentive supervision during all phases of this work, by dedication, clarifications, suggestions and cautious review conducted for this thesis. Thanks also for the sympathy and encouragement without which the realization of this work would not have been possible. My sincere and profound gratitude for all that you have done and continue to do for me.

Prof. Dr. Ana Rosa Lino, I thank all the support and availability that you have always provided in the supervision of this work and for clarification, advice and criticism given during a careful reading of this thesis. Thank you for your kindness and professionalism that you have always expressed during these years.

Dr. Claudia Afonso, my thank you for everything! By friendship, availability, encourage, support, expertise and company in the long nights of writing papers.

My colleagues Dr. Fernanda Martins, Dr. Susana Gonçalves, Mrs Fernanda Queiroz, Ms Margarida Muro, Mrs Fátima Augusto, Mrs Céu Reis and Mrs Silvina Joaquina, for all your support and for your friendship. Also like to thank the colleagues that have retired, Dr. Alcina Lopes, Dr. Teresa Álvaro and Mrs Carminda Ferreira for all their teaching in the area of Food Science.

Dr. Maria José Passos, Dr. José Manuel Bívar, Dr. Fernando Lage, Dr. Santos Pereira and Dr. Ilda Costa for helping me in one of the most difficult moments of my life. I would not have written this thesis without you...

My friends of all ages, from childhood, high school, college and work, for being present in my life. The list is long and hopefully you know who you are! Also to their children for helping me to unwind this period of my life so complicated and giving me the privilege of being "Aunt Lena".

Prof. Dr. Maria Luisa Carvalho of the Physics Department of the Science College of Lisbon University for the precious help in the determination of some studied elements in this present work and for the kindness with which she always received me and the team of her laboratory.

Dr. Patricia Anacleto, Dr. Cátia Pereira and Dr. Vanessa Ferraria for all your help during the course of this work.

Colleagues of the project "Aphacarbo", namely Prof. Dr. Leonel Serrano Gordo of the Biology Department of the Science College of Lisbon University and Dr. Valentina Costa of the Oceanography and Fisheries Department of the Azores University, by information of black scabbard fish, writing the paper on the same species and for the good times we spent together in the project meetings.

Dr. Amparo Gonçalves, Dr. Carlos Cardoso, Dr. João Aguiar and Dr. Cristina Ramos for valuable help in statistical and risk analysis, and also for computer problems. Thanks for your friendship!

All colleagues of the Unit of Upgrading of Fish and Aquaculture Products that somehow supported me at work or simply by friendliness demonstrated since 1990!

Colleagues from the Unit of Marine Resources and Sustainability, in particular to Dr. Graça Pestana, Dr. Ivone Figueiredo, Dr. João Pereira, Dr. Ana Moreno, Dr. Cristina Silva, Dr. Francisco Leote, Dr. Inês Farias, Dr. Ana Rita Vieira, Dr. Alexandra Silva, Dr. Aida Campos and Dr. Paulo Fonseca for having offered the samples of most species used in this thesis and information about biological and fisheries aspects.

Colleagues from the Unit of Aquatic Environment and Biodiversity, in particular to Dr. Miguel Caetano, Dr. João Canário, Dr. Rute Cesário, Dr. Joana Raimundo and Dr. Vasco Branco, by laboratory support when requested and also by their sympathy.

Colleagues from the Group of Bivalves, in particular to Dr. Maria Fernanda Castilho, Dr. Maria do Céu Viegas, Dr. Manuel Sobral, Dr. Maria Damásia Dias, Dr. José Oliveira and Dr. Rui Cachola to provide the samples of bivalve molluscs, and their expertise in these seafood species and also for the good times during the group meetings. I also thank Mr. Helder for having provided the samples from Peniche area.

INRB, I. P / L-IPIMAR for being the host institution.

Science College of Lisbon University for having accepted my application to the doctorate.

And last, but certainly the most important, to my dear mother for all her love, affection, friendship and unconditional support in good and bad times of my life. Would have been impossible without you!

ABSTRACT

Fishery and aquaculture products are considered indispensables in balanced diets, partly because they have high protein content and are an excellent source of almost elements, considered to be essential for man. However, such products can, in a certain extent, be contaminated with some chemicals, like toxic elements, coming from several sources. In Portugal these products assume particular importance since, within European Union countries, it is the one presenting the highest per capita consumption, about 56 kg / person / year. In this context, the primary aim of this work was to characterize the proximate and elemental profile of some seafood, commercialized in Portugal, in order to contribute for the evaluation of benefits and the hazards associated to their consumption.

All products presented a similar pattern in what regards the essential elements. Dominant constituents were potassium, chloride, sulphur, sodium, phosphorus, magnesium and calcium. Interesting contents of zinc, copper and iron were also found, mainly in the three cephalopods and the crustacean. The results lead to the conclusion that these species can be a major contribution to the recommended daily intakes of essential elements.

Appreciable amounts of total arsenic were detected in Norway lobster and octopus; however, as about 90 % of this element is mostly in organic form in marine organisms, these species do not constitute a hazard in terms of human consumption. The highest total mercury contents were found in deep-water fish species while the lowest were found in bivalve molluscs. Canned seafood reflected the profile of raw-material. Regarding lead and cadmium, the levels detected in all edible part of samples were below the EU limits with the exception of some mollusc samples.

Attending the results obtained, this study supports the importance of promoting consumption of fishery and aquaculture products, although some species have to be consumed moderately given the provisional tolerable weekly intake (PTWI) values for toxic metals recommended by FAO/WHO. The probability of exceeding the methyl-mercury PTWI, among the three cephalopods in Portugal, is considered minor.

Key-words: fishery and aquaculture products; essential macro and micro elements; toxic elements; risks assessment.

RESUMO

Os produtos da pesca e aquicultura são considerados indispensáveis num regime alimentar equilibrado, porque têm elevados teores de proteína e de ácidos gordos polinsaturados do tipo ómega-3, apresentam baixos níveis de colesterol e são uma excelente fonte de quase todos os elementos, considerados essenciais para o homem. Além disso, são um alimento de digestão fácil. No entanto, estes produtos podem, em certa medida, conter alguns contaminantes químicos, como os elementos considerados tóxicos (arsénio, cádmio, chumbo e mercúrio), provenientes quer de fontes antropogénicas quer de fontes naturais. Estes elementos podem ser assimilados, armazenados e concentrados pelos organismos vivos, através da cadeia alimentar, originando efeitos fisiológicos graves.

Em Portugal, pela posição priveligiada no conjunto dos países europeus, devido ao seu posicionamento geo-estratégico, frontal ao Oceano Atlântico, e pelo conhecimento acumulado ao longo de gerações das práticas e técnicas de capturas de espécies marinhas, da sua conservação e transformação e da sua utilização culinária, a pesca e o pescado sempre tiveram uma elevada importância sócio-económica. Daqui resulta que Portugal, do conjunto dos países da União Europeia, é o que apresenta o maior consumo *per capita*, cerca de 160 g/dia.

Neste contexto, o objectivo principal deste trabalho foi o de caracterizar o perfil centesimal e elementar de algumas espécies de pescado, comercializadas em Portugal, a fim de avaliar os benefícios e os riscos associados ao seu consumo. As espécies seleccionadas foram os cefalópodes (choco, lula e polvo), o lagostim e quatro espécies de peixe de aquicultura (dourada, pregado robalo e truta) por serem muito apreciadas e haver pouca informação sobre elas. Assim, para além dos quatro constituíntes principais, água, proteína, gordura e cinza, foram também quantificados 14 elementos considerados essenciais (cálcio, cloro, cobre, crómio, enxofre, ferro, fósforo, magnésio, manganês, níquel, potássio, selénio, sódio e zinco) e quatro considerados tóxicos (arsénio, cádmio, chumbo e mercúrio). No que se refere aos elementos tóxicos foram também analisadas algumas espécies de peixes de profundidade (cantarilho e peixe-espada preto), vários moluscos bivalves e algumas conservas de pescado. Foi ainda objectivo desta tese estimar o risco em função dos níveis de metil-mercúrio presente nos cefalópodes, ou seja, estimar a parte de uma determinada população em risco (probabilidade de exceder a dose tolerável de ingestão semanal de metil-mercúrio).

Foram utilizadas várias técnicas para realizar as análises elementares - espectrometria de absorção atómica (chama, em fase de vapor frio e por combustão directa), fluorescência de raios X por energia dispersiva (EDXRF) e espectrometria de absorção molecular (UV-Vis). Para quantificar as probabilidades de risco associadas ao consumo de cefalópodes, recorreu-se a um tratamento matemático-estatístico, utilizando os valores de metil-mercúrio.

De um modo geral, os resultados obtidos demonstram que as espécies estudadas têm um elevado teor proteico e podem ser consideradas magras, à excepção das espécies de aquicultura que apresentaram teores de gordura superiores aos valores usuais nas congéneres selvagens.

Em regra a distribuição dos elementos essenciais nas espécies estudadas era semelhante. Os elementos predominantes foram potássio, cloro, enxofre, potássio, sódio, fósforo, magnésio e cálcio seguidos do zinco, ferro, cobre, crómio, selénio, manganês e níquel. No entanto, os perfis observados nos três cefalópodes e no lagostim diferiam do dos peixes. Assim, o potássio era mais abundante nos peixes do que nas outras espécies e o teor de cálcio foi mais elevado no crustáceo, possivelmente devido ao processo de biomineralização da carapaça. Também foram encontrados teores de zinco, cobre e ferro, mais elevados nos três cefalópodes e crustáceo do que nas espécies de aquicultura. O lagostim também apresentou níveis elevados de manganês e de níquel. Estas diferenças são devidas principalmente às características intrínsecas de cada espécie, habitats e dietas. Em geral, de entre os três cefalópodes, a lula foi a que apresentou menores teores dos vários elementos essenciais. No que se refere aos peixes de aquicultura observou-se que o pregado revelou os menores teores da maioria dos minerais. Os resultados obtidos indicam que as diferentes espécies podem contribuir para as doses diárias recomendadas de vários elementos essenciais, como enxofre, fósforo, zinco e cobre no caso dos cefalópodes e de fósforo, zinco, cobre magnésio e selénio no lagostim. Por seu lado as espécies de aquicultura estudadas são uma boa fonte de fósforo e potássio.

Em relação aos elementos tóxicos, o universo das espécies foi alargado a peixes de profundidade, moluscos bivalves e conservas de pescado, pelo facto de existir pouca informação sobre a sua eventual contaminação e também serem importantes do ponto de vista do consumo. Os níveis de arsénio total no lagostim e no polvo eram elevados, no entanto, como cerca de 90 % deste elemento se encontra sob a forma orgânica nos organismos marinhos, estas espécies não constituem um perigo para o consumo humano. No que respeita ao mercúrio total, os teores mais elevados foram encontrados no músculo das espécies de peixes de profundidade, como o cantarilho e o peixe-espada-preto, excedendo alguns exemplares (70 % e 20 %, respectivamente), os limites propostos pela União Europeia. Este facto decorre destas espécies terem uma grande longevidade, serem carnívoras e habitarem zonas que as podem predispor à exposição de contaminantes químicos. Também se verificaram para estas duas espécies correlações significativas entre os níveis de mercúrio total e alguns dados biométricos e região de captura. O mercúrio orgânico representou no cantarilho e no peixe-espada-preto cerca de 86 % do mercúrio total o que indica que o consumo destas espécies deve ser parcimonioso (no máximo uma refeição por semana). Os níveis de mercúrio total encontrados no lagostim indicaram alguma contaminação, mas tendo em conta o baixo consumo, *per capita,* desta espécie, não parece representar um risco para o consumidor português. Os bivalves foram as espécies que apresentaram as menores concentrações médias de mercúrio total (< 0,05 mg/kg). Por seu lado, as conservas, reflectem o perfil das matérias-primas, assim, por exemplo, as de atum apresentaram valores médios mais elevados de mercúrio total (0,28 mg/kg) enquanto que nas de sardinha e de mexilhão os valores médios eram muito baixos (< 0,05 mg/kg), o que está de acordo com os valores habitualmente obtidos para estas espécies.

Em geral, os níveis de chumbo e de cádmio foram, para todas as espécies e exemplares, sempre inferiores aos limites indicados pela União Europeia, sendo as de aquicultura as que apresentaram os valores mais baixos (≤ 0,05 mg/kg). Contudo, existiram algumas excepções em amostras de moluscos, quer de bivalves quer de cefalópodes e em algumas conservas, nomeadamente nas de lula. Os teores mais elevados nos moluscos resultam não só da sua morfologia mas também do tipo de habitat, como os estuários, que os tornam mais predispostos à acumulação e concentração de metais tóxicos como o cádmio e o chumbo. Assim, o limite proposto pela União Europeia de 1,0 mg/kg para o cádmio foi ultrapassado em 10 % das amostras de cefalópodes, 18 % das amostras de conserva de lula e em 9 % de ostra portuguesa do estuário do Sado. Relativamente ao chumbo verificou-se que 50 % das amostras de lambujinha do estuário do Tejo excederam o limite proposto pela União Europeia (1,5 mg/kg).

No sentido de avaliar o risco de ingestão de metil-mercúrio através do consumo de cefalópodes em Portugal, foi estimada a probabilidade de ultrapassar a ingestão semanal tolerável provisória desse composto pela combinação dos níveis de contaminação nos três cefalópodes estudados com a construção de cenários de consumo ou com uma distribuição hipotética do consumo para a população em geral portuguesa. Verificou-se que a lula não apresenta nenhum problema de saúde com relação ao metil-mercúrio, mas, para o choco e polvo, o consumo não deve exceder duas refeições por semana.

Atendendo aos resultados obtidos, esta tese corrobora a importância de promover o consumo dos produtos da pesca e da aquicultura, devido sobretudo ao seu elevado teor em proteína e em elementos essenciais suficientes para satisfazer as doses diárias recomendadas. No entanto, algumas espécies devem ser consumidas moderadamente, dados os valores de ingestão semanal tolerável provisória (PTWI) para os metais tóxicos recomendados pela Organização Mundial de Saúde e pela Organização das Nações Unidas para a Agricultura e Alimentação (WHO/FAO).

Palavras-chave: produtos da pesca e aquicultura; macro e micro elementos essenciais; elementos tóxicos; avaliação de risco.

FOREWORD

This thesis is the result of a research project that was accomplished during the first years of this century at INRB, I. P. / L-IPIMAR and FCUL. It is divided into two parts: the first contains eight chapters and an annex and the second included nine scientific papers. The first chapter gives a brief general introduction of the world fisheries and the present situation in Portugal, in particular about the species studied in this thesis. The aims appear in the second chapter. The third chapter presents a background on the nutritional composition of seafood, particularly on the essential and toxic elements, as well as about the risks and benefits regarding in the human diet. The design of the study is presented in chapter four. All the methodologies used are described in the chapter five. The main results, reported in detail in the second part of this thesis, are summarized in chapter six. The major conclusions and possible future research in the same area of study are addressed in chapter seven. Finally, the references throughout the document are listed in the last chapter and in the Annex A, it is described the biology of all studied seafood species.

The nine papers included in the second part are the result of the experimental work performed on several seafood species: three cephalopods (cuttlefish, octopus and squid), one crustacean (Norway lobster), four farmed fish (gilthead seabream, European seabass, trout and turbot), two deep-water fish (blackbelly rosefish and black scabbardfish), some bivalve molluscs and canned seafood (details in design of the study).

The author wishes to state that the entire content of this thesis is of your responsibility, although, with papers in which it is co-author, it just attended a part. Thus, in the paper IV, analysis of α-tocopherol, as well as all data on the species *Lophius* spp., *Aphanopus carbo* and *Lepidorhombus* spp were not their own. With respect to paper V, the levels of mercury were performed by colleagues from the University of the Azores. Regarding paper VI, the author provided data of mercury and methyl mercury and risk analysis was carried out by first author. In the case of paper VII only data from the cephalopods, crustacean and gilthead seabream are of the author.

ABBREVIATIONS

AA	Azores Archipelago
AAS	Atomic absorption spectrometry
AI	Adequate intake
AMSE	Asymptotic mean squared error
AOAC	Association of Official Analytical Chemists
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BCR	Bureau Communitaire de Référence
BENPER	Project Acronym " Benefícios e perigos associados aos produtos da pesca"
BHT	Butylated hydroxytoluene
BSE	Bovine spongiform encephalopathy
BW	Body weigh
CRM	Certified reference material
CRM-421	Milk powder (certified reference material from IRMM)
CRM-422	Cod muscle (certified reference material from IRMM)
CRM-463	Tuna fish (certified reference material from IRMM)
CRN	Council for Responsible Nutrition
CVAAS	Cold vapour atomic absorption spectrometry
δ -ALAD	δ-aminolevulinic acid dehydratase
DGPA	Direcção Geral das Pescas e Aquicultura
DGV	Direcção Geral de Veterinária
DHA	Docosahexaenoic acid
DI	Daily intake
DL	Detection Limit (also detectable limit)
DNA	Deoxyribonucleic acid
DOI	Digital Object Identifier
DOLT-2	Dogfish liver (certified reference material from NRC-CNRC)
DOP-IMAR	Departamento de Oceanografia e Pescas - Instituto do Mar
DORM-2	Dogfish muscle (certified reference material from NRC-CNRC)
DRI	Dietary Reference Intake
DSIP	Direcção de Serviços e Investigação das Pescas (Madeira)
dw	Dry weight
EAR	Estimated average requirement
EC	European Commission
EDI	Estimated daily intake
EDXRF	Energy dispersive X-ray fluorescence
EPA	Eicosapentaenoic acid / Environmental Protection Agency

ESFA	European Food Safety Authority
ETFE	Ethylene tetrafluoroethylene
EU	European Union
EUROSTAT	European Statistics
EVT	Extreme value theory
F	Female
FAAS	Flame Atomic Absorption Spectrometry
FAO	Food and Agriculture Organization
FAPAS	"Food Analysis Performance Assessment Scheme" managed by the Food and Environment Research Agency
FC	Funchal
FCUL	Faculdade de Ciências da Universidade de Lisboa
FCT	Fundação para a Ciência e Tecnologia
FDA	Food and Drug Administration
FEP	Fluorinated ethylene propylene
FMD	Footh-and-mouth disease
FSA	Food Standards Agency
GI	Gastrointestinal
GLM	Univariate general linear models
GOODFISH	Project acronym " Benefícios e riscos associados ao consumo de produtos da pesca: Uma análise de benefício-risco baseada na abundância e bioacessibilidade de n-3 PUFA e Selénio, Mercúrio e Arsénio em produtos crus e cozinhados"
GSH	Glutathione peroxidase
Hg_{Org}	Organic mercury
Hg_T	Total mercury
HPLC	High Performance Liquid Chromatography
IAEA	International Atomic Energy Agency
IAEA-A-13	Freeze dried animal blood (certified reference material from IAEA)
INA-PG	Institut National Agronomique **Paris**-Grignon
INE	Instituto Nacional de Estatística
INIAP-IPIMAR	Instituto Nacional de Investigação Agrária e das Pescas - Instituto de Investigação das Pescas e do Mar (former name of current INRB Institute.
INRB, I.P./L-IPIMAR	Instituto Nacional de Recursos Biológicos/ Laboratório de Investigação das Pescas e do Mar
IOM	Institute of Medicine
IPAC	Instituto Português de Acreditação
IRMM	Institute for Reference Material and Measurements
ISO	International Organization for Standardization

JECFA	Joint FAO/WHO Expert Committee on Food Additives
LL	Legal limits
LSD	Least significant difference test
LUTS-1	Non defatted lobster hepatopancreas (certified reference material from NRC-CNRC)
M	Male
MA	Madeira Archipelago
MA-A-2	Fish flesh (certified reference material from IAEA)
MAFF	Ministry of Agriculture, Fisheries and Food
Max	Maximum
3-MCPD	3-monochloropropane-1,2-diol
MeHg	Methyl mercury
Min	Minimum
MS	Maturity stages
MT	Metallothioneins
n / N	number of individuals
NAS	National Academic of Sciences
Nd	Not detected
ND	Not determined
NE	Northeast
NIST	National Institute of Standards and Technology
NPN	Non protein nitrogen
NRC-CNRC	National Research Council Canada
NS /ns	Not significant
NW	Northwest
PC	Pico Island
PCBs	Polychlorinated biphenyl compounds
PCDD	Polychlorinated dibenzodioxins
PCDF	Polychlorinated dibenzofurans
PI	Plug-in
PNAB/EU	Programa Nacional de Amostragem Biológica / Recolha de dados
POCTI	Programa Operacional Ciência, Tecnologia, Inovação
PTWI	Provisional tolerable weekly intake
PUFA	Polyunsaturated fatty acids
QALIBRA	Project acronym "Quality of life - integrated benefit and risk analysis"
QCA III PO MARE	Quadro Comunitário de Apoio III - Programa para o Desenvolvimento Sustentável do Sector da Pesca
QL	Quantification Limit
QQ plot	Quantile-quantile plot

RELACRE	Associação de Laboratórios Acreditados de Portugal
RDA	Recommended dietary allowance
RSD	Residual standard deviation
RV	Ranged Values
R/V	Research Vessel
S	Slope
SD / sd	Standard Deviation
SE	Southeast
SM	Santa Maria Island
SMRD - 2000	Canned matrix meat (certified reference material from Swedish Meats R&D and Scan Foods/National Food Administration)
SRM 1566	Oyster tissue (certified reference material from NIST)
SRM 1571	Orchard leaves (certified reference material from NIST)
SZ	Sesimbra (mainland Portugal)
TE	Tail estimation
THg	Total mercury
TORT-2	Lobster hepatopancreas (certified reference material from NRC-CNRC)
TWI	Tolerable weekly intake
UAç	Azores University
UL	Tolerable upper intake level
U-REMS	Unidade de Recursos Marinhos e Sustentabilidade
U-VPPA	Unidade de Valorização dos Produtos da Pesca e Aquicultura
UV-Vis	Ultraviolet-visible absorption spectrometry
WHO	World Health Organization
ww	wet weight

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LIST OF PUBLICATIONS

The summary part of this thesis is based on work reported in the following six publications, referred to in the text by their Roman numerals:

I - Elemental composition of cephalopods from Portuguese continental waters.

Lourenço, H.M., Anacleto, P., Afonso, C., Ferraria, V., Martins, M.F., Carvalho, M.L., Lino, A.R., Nunes, M.L. *Food Chemistry*, 113:1146-1153 (2009) (IF: 2.696)

II - Chemical characterisation of *Nephrops norvegicus* from Portuguese coast.

Lourenço, H.M., Anacleto, P., Afonso, C., Martins, M.F., Carvalho, M.L.. Lino, A.R., Nunes, M.L. *Journal of the Science of Food and Agriculture,* 89:2572-2580 (2009) (IF: 1.333)

III - Proximate composition and mineral content of farmed fish.

Lourenço, H.M., Afonso, C., Anacleto, P., Martins, M.F., Nunes, M.L., Lino, A.R. (*Submitted to Journal of Trace Elements in Medicine and Biology* - IF:1.404)

IV - Total and organic mercury, selenium and α-tocopherol in some deep-water fish species.

Afonso, C., Lourenço, H.M., Pereira, C., Martins, M.F., Carvalho, M.L., Castro, M., Nunes, M.L. *Journal of the Science of Food and Agriculture,* 88:2543-2550 (2008) (IF: 1.333)

V - Mercury, cadmium and lead in black scabbardfish (*Aphanopus carbo* Lowe, 1839) from mainland Portugal and the Azores and Madeira archipelagos.

Costa, V., Lourenço, H.M., Figueiredo, I., Carvalho, L., Lopes, H., Farias, I., Pires, L., Afonso, C., Vieira, A.R., Nunes, M.L., Gordo, L.S. *Scientia Marina,* 73S2:77-88 (2009) (IF: 1.075)

VI - Risk assessment of methylmercury intake through cephalopods consumption.

Cardoso, C., Lourenço, H. M., Ferraria, V., Afonso, C., Nunes, M. L.
(Submitted to *Risk Analysis* - IF: 1.831)

Three other publications also reflected the work done in this thesis:

VII – Total arsenic content in seafood consumed in Portugal

Anacleto, P., Lourenço, H.M., Ferraria, V., Afonso, C., Carvalho, M.L., Martins, M.F., Nunes, M.L. *Journal of Aquatic Food Product Technology*, 18(1-2):32-45 (2009) (IF: 0.444)

VIII – Concentrations of mercury, lead and cadmium in bivalves from the Portuguese coast

Lourenço, H.M., Lima, C., Oliveira, A., Gonçalves, S., Afonso, C., Martins, Nunes, M.L. *In:* Seafood research from fish to dish, Quality, safety and processing of wild and farmed fish, J. B. Luten, C. Jacobsen, K. Bekaert, A. Saeb∅, J. Oehlenschläger (Eds.), Wageningen Academic Publishers, The Netherlands, pp 497-502 (2006)

IX – Levels of toxic metals in canned seafood

Lourenço, H.M., Afonso, C., Martins, M.F., Lino, A.R., Nunes, M.L. *Journal of Aquatic Food Product Technology*, 13(3):117-125 (2004) (IF: 0.444)

1 INTRODUCTION

1.1 Historical development of fisheries in the World

Since ancient times fishing, hunting and harvesting of fruits were the first conscious and organized manifestations of man to fight hunger and survive. According to Cave Painting archaeologies man caught the fish in the rivers and lakes or along the beaches by hand or with small instruments, or with rudimentary buildings armed in the sand (Thomazi, 1947). As the fisheries grew, there was a need to store and to maintain these products for longer periods which led to the concept of handling and storage.

Ancient civilizations like the Egyptian, Assyrian, Phoenician or Chinese were those that contributed to major progress in activities related to fisheries. It is thought that the Egyptians were the first to use salt as a process of preservation, the Phoenicians, for example, increased the trade of fishery products, since they were great navigators and the Chinese have contributed to a remarkable development in fisheries, using different fishing techniques (Thomazi, 1947). Later, in classic civilizations, the Greeks already had a great knowledge regarding the biology and behaviour of marine species and fishing technology. The main species consumed at that time was tuna, which was used as fresh, salted or marinated in oil. Upon extension of the Roman Empire there was a widespread of fishing methods in the Mediterranean by the central and northern Europe. The strong sea trade regime between the various Roman colonies due to the sophisticated taste food of the Romans, led to the installation of tanks and ponds on board or in the case of transport by land, the use of ice or snow, which caused the arrival of fresh fish products to the destination (Thomazi, 1947).

In the Middle Ages with the fall of the Roman Empire, there was an almost total disappearance of industry and trade of fishery products in Europe, and this activity was only held locally. The most important sites where the man preserved the techniques of fishing and preservation were the religious institutions. The main species caught at that time were herring and cod (Thomazi, 1947)

The fisheries in international waters appeared in the sixteenth century and was strongly continued over the seventeenth after the improvement of boats and navigation techniques. Due to business expansion, the fisheries and trade of seafood products were developed. With the invention of the steam engine and its application to fishing vessels from 1865, there was an increase in the capture of cod and herring (Thomazi, 1947). The introduction of new materials in fisheries and the discovery of new methods of preservation based on the industrial production of ice favoured a substantial development of the fisheries and trade dependent on it. In the early twentieth century was an extraordinary increase in catches due to the introduction of mechanized fishing activity, that was then stopped over the first and second world wars, however several new technological applications have been enhanced fishing, during this period. The appearance of the first steel ships using diesel, equipped with new facilities (more efficient nets, tracking devices, freezers) have allowed a better storage and processing of fishery products.

Recently, the world total production of fish, crustaceans and molluscs has continued to increase and in 2007, reached up to 140 million tonnes. Since 2001, capture production has stayed around 90 millions tonnes level and aquaculture production has continued to grow, attaining 50.3 million tonnes in 2007 (FAO, 2009). At the present, aquaculture is an important alternative to traditional forms of supplying the fishery products, According to Food and Agriculture Organization (FAO) estimates, for the year 2030, aquaculture will dominate the market and it is likely that less than half of fish products consumption will come from traditional fisheries (FAO, 2003). The most caught species at the global level has been the Peruvian anchoveta, Alaska Pollock, skipjack tuna, Atlantic herring and Chilean jack mackerel. Global capture production of marine crustaceans and shell molluscs remained fairly stable, whereas total catch of cephalopods has continued its constant growth with an overall 35 percent increase during the last 5 years (FAO, 2009). Among the most important farmed fish species in the European Union are gilthead sea bream, European seabass, trout, turbot and salmon and even shellfish such as mussels, clams and oysters, whose production has been increasing in recent years and are often sold whole and fresh. About 81 percent of total fishery production (113.7 million tonnes in 2007) was used for direct human consumption. The remaining 19 percent were destined for non-food products, mainly for the manufacture of fishmeal and fish oil (FAO, 2009). In **Figure 1.1** it can be observed the evolution of World fish utilization and supply.

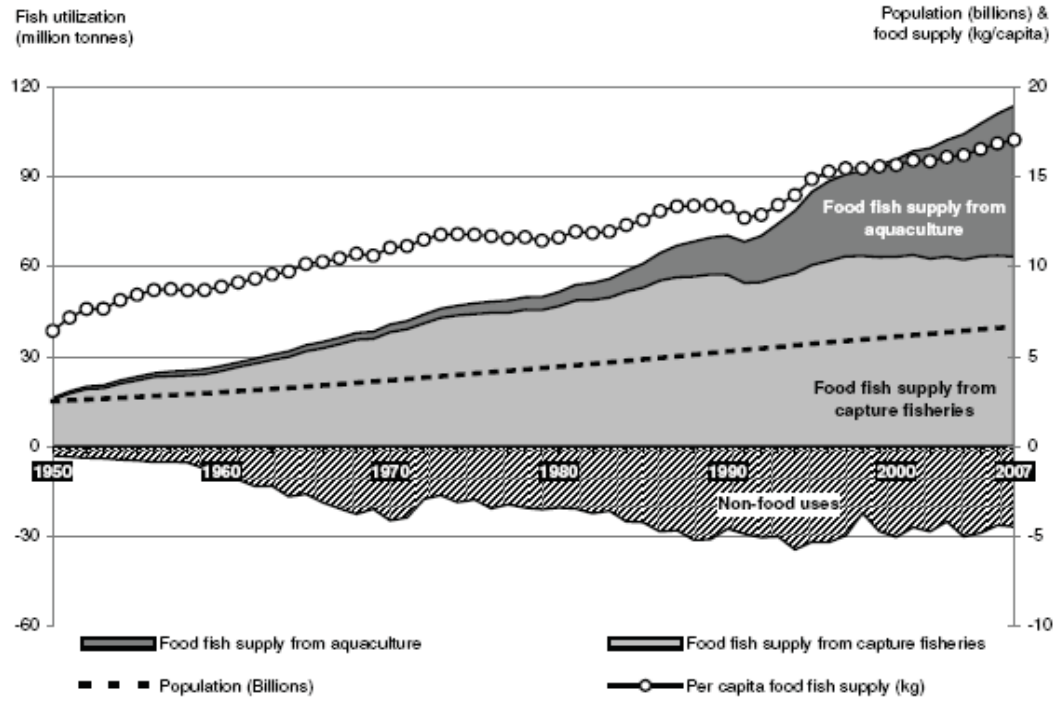


Figure 1.1 – Evolution of World fish utilization and supply. (Source: FAO, 2009).

1.2 Fish consumption in Portugal

Portugal presents a privileged position comparing to the other European countries due to its geo-strategic front to the Atlantic Ocean, and the accumulated knowledge of generations of

practical and technical catch of marine species, their preservation and processing and use as food. With an exclusive economic zone currently around 170,0000 Km^2 and a coastline of 2,830 km on the mainland and two island areas, the fishing has been always an important source of livelihood, especially for coastal communities, many of which are almost totally dependent on fisheries and other related activities (DGPA, 2007 a, b).

In 2009, 144,792 tonnes of seafood, were landed in Portugal (**Figure 1.2**), which were unloaded as fresh or refrigerated in the auctions, accounting for a decrease of 14.9 % relatively to 2008 (INE, 2010). Behind this decrease was decisively the lower catch of marine fish (sardine and mackerel) and cephalopods, especially octopuses (INE, 2010). Relatively to crustaceans there was a rise due to an increase in the catch of shrimps (INE, 2010). Total aquaculture production in 2008 reached to 7,987 tonnes (**Figure 1.3**). This value implies an increase from the previous year of about 7.3 % of total. The production has been focusing on European seabass, gilthead sea bream and clam in sea water and trout in fresh water.

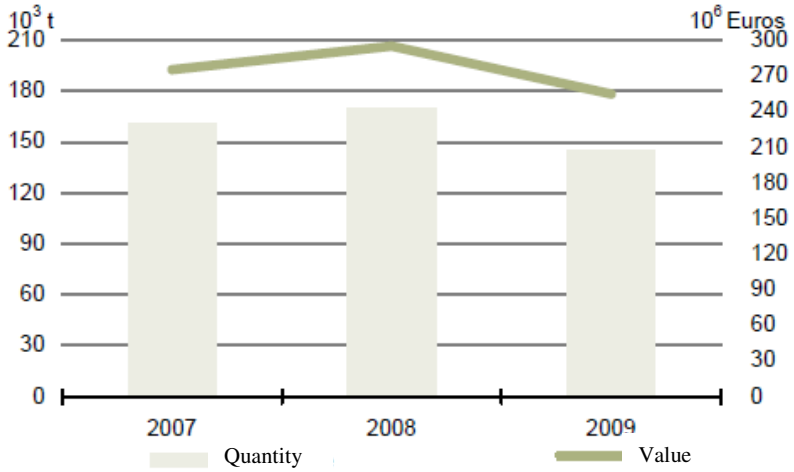


Figure 1.2 – Landings (tonnes and value) of fresh/chilled fish in Portuguese ports (2007-2009). (Source: INE, 2010).

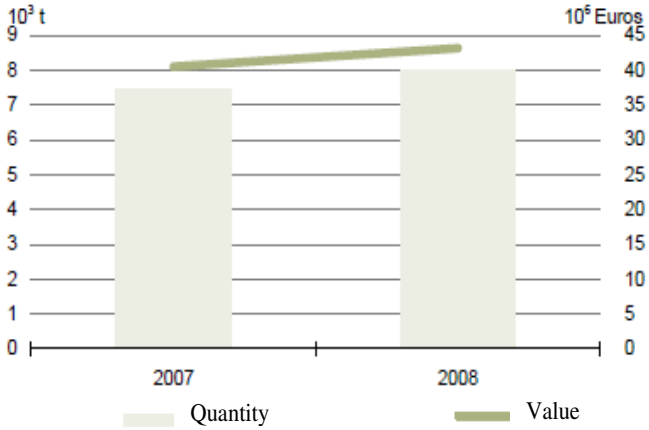


Figure 1.3 – Portuguese aquaculture production (2007-2008). (Source: INE, 2010).

Per capita consumption of seafood in Portugal is one of the highest among European Union (EU) countries and one of the largest worldwide, being estimated at 56 kg / person / year (FAO, 2009). It should be noted that for example in Iceland this consumption is estimated at more or less 90 kg while in the United States is less than 25 kg. While it has seen in recent years the increase of meat consumption, it is expected that in future, the consumption of fishery products will grow due to diseases such as bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD) in meat. Moreover, a growing number of consumers prefer these products to consider that they are an healthier alternative to meat consumption or by dietary choices related to an easier digestibility. Fishery products fulfil an important role in human nutrition and they have economic advantages. The nutritional benefits originate in their valuable proteins, high content of ω3 fatty acids, low cholesterol level, mineral profile and contents as well as in some vitamins (Belitz *et al*., 2004; Simopoulos, 1997; Oehlenschläger, 1997).

1.3 Importance of species used in this study

From an economic point of view, some species are interesting due to the large quantities available at the moment and also because they are much appreciated in Portuguese cuisine. Among these species can stand out the cephalopods (octopus, squid and cuttlefish), some species of shellfish or black scabbardfish. Currently, farmed fish are also very important in the Portuguese economy mainly because they are highly valued species but at a lower price when compared to the same species of wildlife habitat. The most appreciated farmed fish are gilthead seabream and European seabass. Another fishery product very appreciated in Portugal are canned fish such as tuna or sardines in various sauces.

Although, there is a huge amount of data available in seafood, most studies are concerned to North Atlantic or Mediterranean species. Regarding species and products in Portugal such as cephalopods, bivalves, deep-water species, farmed fish species as well canned products the available data it is not so abundant. Therefore, to ameliorate the seafood composition database, to better inform the consumers, data of that seafood are needed. Thus, this work focused on the various species described above, in particular, three cephalopods (cuttlefish, squid, octopus), a crustacean (Norway lobster), two deep-water fish (blackbelly rosefish and black scabbardfish) and four farmed fish (gilthead seabream, European seabass, trout and turbot). Also have been studied several species of bivalves molluscs produced in Portugal and some canned seafood.

According to Instituto Nacional de Estatística (INE) (2010), in 2009, the most important wild species studied in the present work present about 15 % of the total seafood landed in Portugal (**Figure 1.4**). With respect to farmed fish studied in this work, they present about 50 % of the total produced in 2008 in Portugal (**Figure 1.5**). Bivalve molluscs represent about 2 % of the seafood landed and about 49 % of the total produced in aquaculture in 2008 (**Figure 1.6**). In what concerns average production of canned seafood in Portugal, the number is situated around 40, 000 tonnes, since 2001, being the most relevant canned sardine and canned tuna (**Figure 1.7**).

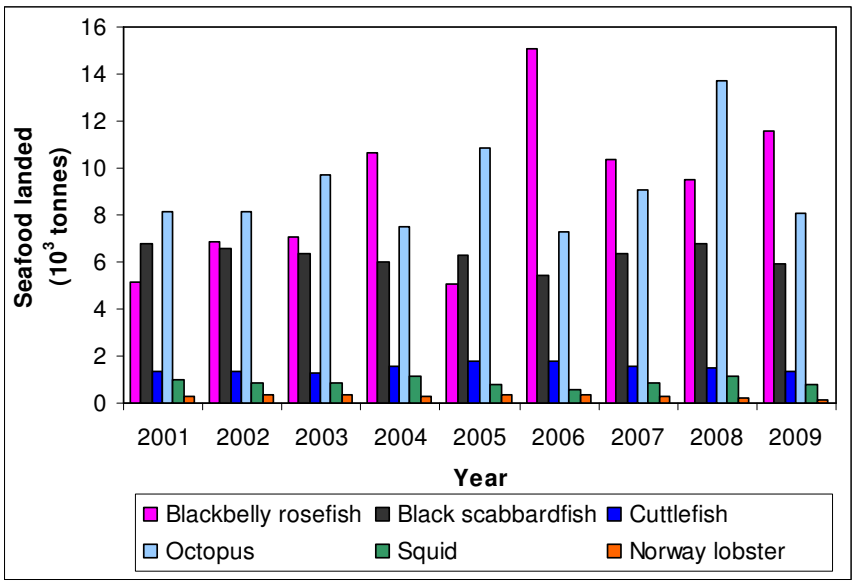


Figure 1.4 – Studied seafood species landed in Portugal (Source: INE, 2002 - 2010).

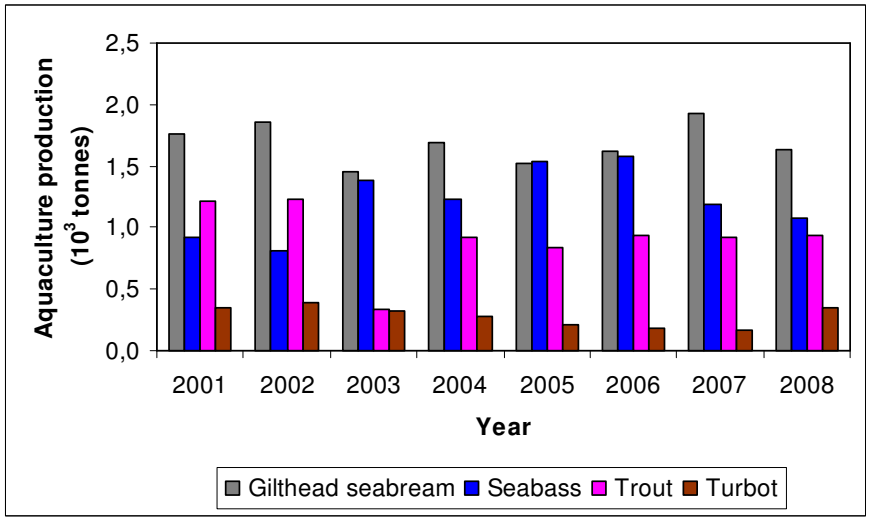


Figure 1.5 – Some of the most representative farmed fish produced in Portugal, since 2001. (Source: INE, 2002 - 2010).

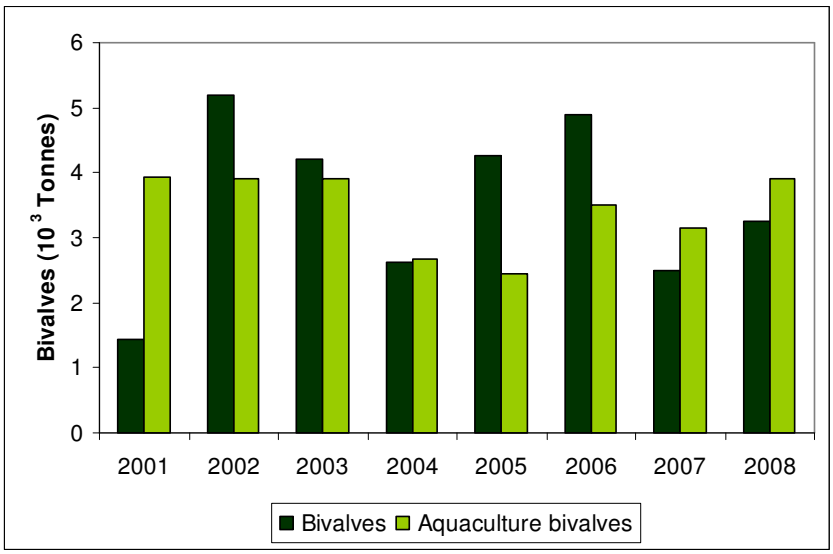


Figure 1.6 – Bivalve molluscs in Portugal, since 2001. (Source: INE, 2002 - 2009).

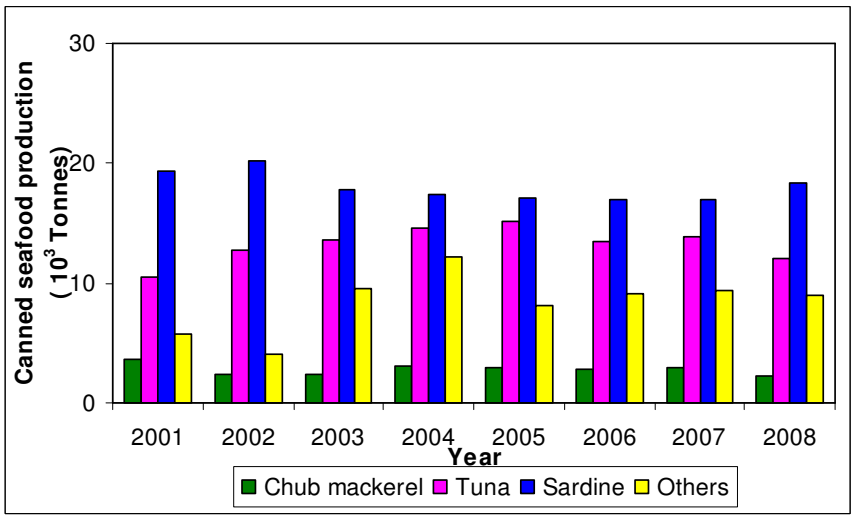


Figure 1.7 – Canned seafood production in Portugal, since 2001. (Source INE 2002-2010).

2 AIMS

The aim of this work was to characterize the proximate and elemental content of some seafood, commercialized in Portugal, in order to evaluate the benefits and the risks of consumption of these species, not so well studied, but very appreciated in Portuguese cuisine.

The specific aims were:

- To characterize three cephalopods (cuttlefish, octopus and squid), crustacean (Norway lobster) caught off Portuguese coast and farmed fish species produced in Portugal (gilthead seabream, European seabass, trout and turbot) regarding centesimal composition and essential elements;

- To evaluate the levels of toxic elements, as arsenic, cadmium, mercury and lead in the three cephalopods, one crustacean and four farmed fish. In addition, the levels of cadmium, mercury and lead were also studied in deep-water fish, bivalve molluscs and canned seafood products;

- To quantify the probabilities of risks associated to seafood products consumption in Portugal and methyl-mercury contents, using cephalopods as model.

3 BACKGROUND

3.1 Proximate composition and nutritional value of seafood

The knowledge of seafood proximate composition is a key aspect to assess their nutritional value. However, this composition varies among species and depends on age and sex of individuals, as well as the environment and the spawning season. This is observed in wild fish from open sea or inland waters. The farmed fish also presents variations on proximate composition, having factors such as diet composition, environment, fish size and genetic characteristics an impact on the composition (Huss, 1995). Seafood meat is readily digestible as mammals but seafood is digested substantially faster than mammal ones and has therefore a much lower nutritive saturation value (Belitz *et al.*, 2004). Like other food products, seafood contains water, proteins and non protein-nitrogen compounds, lipids, carbohydrates, vitamins and minerals. Apart from water, proteins and lipids are the major constituents while carbohydrates are the minor ones, although in molluscs can attained 5 %. **Table 3.1** presents the proximate composition of some of the most consumed species in Portugal.

Table 3.1 Proximate composition (g) and energy value (kcal/kJ) of some seafood species (100 g of edible portion). (Source: Nunes *et al.*, 2008).

Seafood	Water	Protein	Fat	Minerals	Energy
Cod	80.0	17.8	0.5	1.4	76/317
Gilthead seabream	68.9	19.7	9.8	1.4	167/699
Hake	81.1	17.0	0.8	1.1	75/315
Horse mackerel	75.6	19.7	2.9	1.4	105/439
Salmon	60.5	16.2	21.9	1.3	262/1096
Sardine	63.4	18.4	16.4	1.7	221/926
Clam	81.1	11.7	0.9	1.0	58/243
Octopus	83.1	15.6	1.2	0.9	73/306
Shrimp	79.2	17.6	0.6	1.5	77/322

From the **Table 3.1**, it can be observed that a considerable normal variation can occurred for the constituents of seafood edible portion.

3.1.1 Water

Water is the most abundant constituent in the muscle of seafood, ranging usually between 50-85% (Nunes *et al.*, 2008). Within the same species water and fat contents are inversely related constituting around 80 % of seafood edible portion (Huss, 1995). This constituent plays a key role as a solvent for organic and inorganic solutes, and is an integral part in most of the reactions, having a major impact on the conformation and reactivity of proteins. Changes in the

amount of water present in the muscle affect their rheological properties, nutritional value and sensory quality. These changes affect the shelf life of fish (Sikorski *et al.*, 1990).

3.1.2 Proteins and other non protein nitrogen compounds

As a rule, seafood contains 17-20 % of crude protein corresponding to 2-3 % of protein nitrogen (Nunes *et al.*, 2003) These percentages are rather constant in most species although some variations can occurred during spawning (Huss, 1995). The proteins in seafood muscle tissue can be divided into the following three groups:

- Structural proteins (ex: actin, myosin, tropomyosins), which constitute 70-80 % of the total protein content. These proteins are soluble in neutral salt solutions of fairly high ionic strength (0.5 M);
- Sarcoplasmic proteins (myoalbumin, globulin and enzymes), which constitute 25-30% of the total protein. These proteins are soluble in neutral , salt solutions of low ionic strength (< 0.15 M);
- Connective tissue proteins (collagen and elastin), which constitute approximately 3 % of the protein in teleosts fishes and about 10 % in elasmobranches (Huss, 1995; Belitz *et al.*, 2004).

The proteins of fishery products have a high biological value because they have all essential amino acids and is also recognized its great digestibility. They play a key role in the growth and maintenance of vital bodily functions (Nunes *et al.*, 2008). In **Table 3.2** it can be observed the aminoacid profile of some species of seafood. Proteins collectively influence all the seafood sensorial attributes (colour, flavour, texture), and the post harvest deterioration of seafood meat. These compounds in seafood meat are also important because they contribute to chemical and physical changes during its processing (Haard, 1995).

Table 3.2 - Essential aminoacid composition (g) of some seafood species (100 g of edible portion). (Source: Nunes *et al.*, 2008).

Aminoacid	Gilthead seabream	Sardine	Clam	Octopus	Shrimp
Arginine	1.1	1.1	0.9	1.1	2.2
Isoleucine	0.9	0.8	0.6	0.5	0.7
Leucine	1.5	1.5	0.9	1.0	1.5
Lysine	1.8	1.8	1.0	0.9	0.4
Methionine	0.5	0.5	0.1	0.3	0.6
Phenylalanine	0.8	0.8	0.5	0.5	0.8
Serine	0.7	0.8	0.5	0.6	0.8
Threonine	0.8	0.8	0.6	0.6	0.7
Valine	1.0	1.0	0.6	0.5	0.8

The non protein nitrogen compounds (NPN) of seafood are significantly higher than that in other foods, about 9-18 % of total nitrogen in teleosts and 33-38 % in elasmobranches. The major classes of sarcoplasmic components are free amino acids, peptides, guanidine compounds, urea, nucleotides and quaternary ammonium compounds. These compounds are important because they influence the delicate taste of seafood and they contribute to the spoilage of fishery products (Simopoulos, 1997).

3.1.3 Lipids

The fat content can vary between 0.1 % and about 30 %. According to Belitz *et al*. (2004) seafood can be classified in lean (0.1 - 0.4 % of lipids), fatty (≥ 16 %) and semi fatty (0.4 % < lipids < 16 %). Generally, lean fish store lipids mostly in the liver and the fatty ones store in fat cells distributed in other body tissues. Semi-fatty species store lipids in limited parts of their body tissues only, or in lower quantities than classic fatty species. Typical lean species are the bottom-dwelling ground fish like cod, saithe and hake. Fatty species include the pelagic like herring, mackerel and sardine. Mullet, tuna and swordfish can be considered semi-fatty species. Despite of this classification, seafood is generally regard as a low fat food. The majority of seafood species contain less than 2.5 % of total fat, and only a short number of species has more than 16 %. The fat content of seafood is strongly influenced by season, geographical area and age. Hence, lipid content tends to be higher when fish are intensively feeding, frequently during summer period (northern hemisphere), and in older and healthier specimens. In addition, fat contents tend to be lower during spawning or reproduction and when food is less abundant (Nunes *et al*., 2003). Another important fact is that the dark muscle is fattier than the white one. This different lipid composition is related to the biological adaptation of different species, white muscle is used, particularly in sudden movements, while the dark is used in constant movement. Thus, species that show white muscle do not make major shifts to feed or reproduce (lower energy consumption), contrary to what happens to species that have dark muscle that utilize constant movement to achieve the same objectives, which implies increased energy consumption (Huss, 1995).

The lipids present in seafood species may be divided into major groups: the phospholipids and the triacylglycerols. The phospholipids make up the integral structure of the unit membranes in the cells, frequently called structural lipids. The triacylglycerols are lipids used for storage of energy in fat depots, usually within special fat cells surrounded by a phospholipids' membrane and a rather weak collagen network. Fatty fish lipids are constituted especially by triacylglycerols, whereas in lean fish the phospholipids are almost 90 % of the total lipids in muscle (Huss, 1995).

3.1.3.1 Fatty acids

Seafood lipids are composed by a wide variety of saturated, monounsaturated and polyunsaturated fatty acids (consists of a chain of carbon atoms with a carboxyl group at one end and a methyl group at the other) (**Figure 3.1**), being the percentage of fatty acids with 20 or

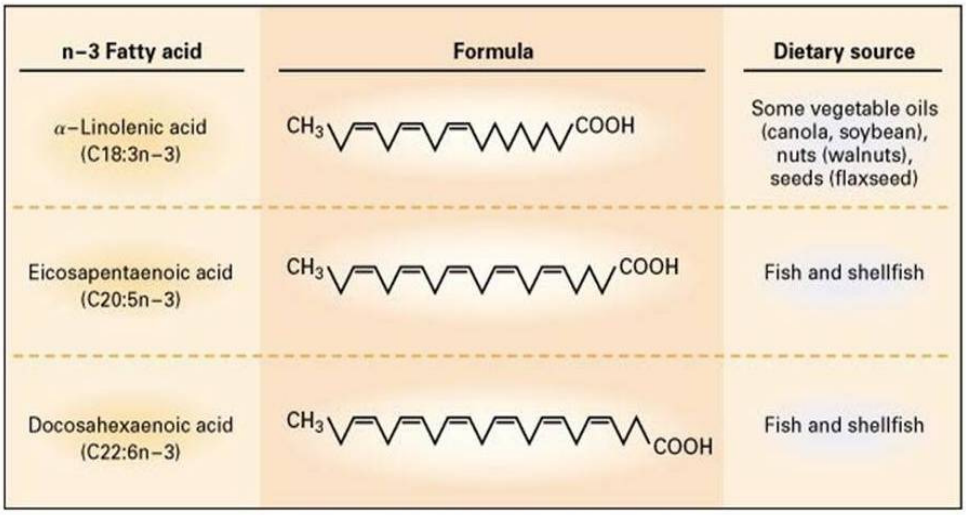


Figure 3.1 - Structure of some polyunsaturated fatty acids. (Picture source site: Nephropal, 2009)

more carbon atoms and more than four double bonds significantly higher than that found in vegetable oils and in mammalian fat (Huss, 1995). Many of these compounds belong to the omega-3 fatty acids family. Fish and shellfish ingest and accumulate such compounds through the food chain from algae and phytoplankton, which are the primary producers (Nunes *et al.*, 2003). The proportion and amounts of saturated, mono- and polyunsaturated and omega-3 fatty acids vary considerably from one species to another, and in farmed species is strongly dependent on the diet. As a rule, the fattier seafood contains more omega-3 fatty acids than the leaner. In human nutrition fatty acids such as linoleic and linolenic acid are regarded as essential since they cannot be synthesized by the organism. In seafood, these fatty acids constitute only around 2 % of the total lipids, which is a small percentage when compared with many vegetables oils (Huss, 1995). However, fish oils contain other polyunsaturated fatty acids which are also considered essential. The most important omega-3 fatty acids found in seafood are eicosapentanoic acid (EPA) and docosahexaenoic acid (DHA) that are beneficial to health. The effects of fish consumption on growth and development and in health and disease have been extensively studied in human life. Epidemiologic studies, clinical investigations, animal experiments, and in vitro studies have been carried out to determine the role of omega-3 fatty acids in coronary heart disease, hypertension, diabetes, cancer, rheumatoid arthritis, psoriasis, ulcerative colitis and other autoimmune disorders with encouraging results (Simopoulos, 1997, Kolakowska *et al.*, 2003). Some of the typical values in the species most consumed in Portugal are found in **Table 3.3**.

3.1.4 Carbohydrates

The carbohydrate content in muscle of fresh fish is insignificant (< 0.3 - 0.5 %) and generally lower than in mammalian muscle tissue (Belitz *et al.*, 2004); however, certain shellfish store some of their energy reserves as glycogen, which contributes to the characteristic sweet taste of their products. Some bivalve molluscs contain 3-5 % glycogen while lobsters have around 1 % (Simopoulos, 1997).

Table 3.3 - Total fat, total of polynsaturated and EPA and DHA fatty acids in some seafood species (g of 100 g of edible portion). (source: Nunes *et al*., 2008).

Seafood	Total fat	Total of Polynsaturated	Fatty acids omega-3	
			EPA	DHA
Cod	0.5	0.2	0.1	0.1
Gilthead seabream	9.8	2.8	0.4	1.2
Hake	0.8	0.3	0.1	0.2
Horse mackerel	2.9	0.9	0.2	0.6
Salmon	21.9	5.1	1.2	1.8
Sardine	16.4	5.6	1.4	3.3
Octopus	1.2	0.6	0.2	0.3
Shrimp	0.6	0.3	0.1	0.1

3.1.5 Vitamins

Seafood is not considered a significant source of vitamins and its content are species specific. They can vary considerably within one species with age, size, sex, season, diet, state of health and geographic location. In farmed fish the contents of vitamins reflect the composition of the corresponding components in the fish feed. Therefore, the vitamin content of wild and farmed fish can be different (Rehbein & Oehlenschläger, 2009). According to the solubility of the vitamins, they are grouped into fat soluble and water soluble. In the first group are included the vitamins A, D and E, while in the latter are included the B complex vitamins. The levels of vitamin B are comparable to those found in many other foods with high protein content; some fatty species can be a reasonable source of vitamins A and D, although most fish species is not considered to be a good source of fat-soluble vitamins (Nunes *et al*., 2003). Also present are vitamins E and K. Vitamin E, which is present in foods in different forms, is presented in seafood mainly as α-tocopherol that is the most efficient natural antioxidant in living organisms. This vitamin is found in significant amounts in seafood species that can provide about 15% of the recommended daily dose. Besides its role in prevention of lipid oxidation is also known its antioxidative action in the oxidative stress reactions originated by the rupture of mercury organic forms in target organs, being its action synergetic with selenium. In this type of food there is little vitamin C. In **Table 3.4** it can be found mean levels of vitamins A, D and E in some seafood species.

Table 3.4 - Mean levels of vitamins A, D and E in some seafood species (100 g of edible portion). (source: Nunes *et al.*, 2008).

Seafood	A (µg)	D (µg)	E (mg)
Cod	3.8	4.5	0.3
Gilthead seabream	11.0	12.0	0.8
Hake	10.0	1.4	0.5
Horse mackerel	15.0	4.1	0.4
Salmon	33.0	11.0	4.0
Sardine	47.0	21.0	0.7
Octopus	3.0	0.7	0.7
Shrimp	≈0	≈0	0.7

3.1.6 Minerals

Mineral content of seafood is in average between 1.2 - 1.5 % The constituents are almost the 90 elements that occur in nature (Lall,1995).

Since this thesis is mainly concerned with elemental composition of seafood this matter will be presented more detailed in the next sub-chapter.

3.2 Elemental composition and main benefits and hazards

Since some years ago the discussion on nutritional benefits in seafood was also focused on the mineral content, especially on the amounts of essential elements presented. In a simplified definition, essential elements are elements which are necessary required for the maintenance of life (Oehlenschläger, 1997). The major constituents of their body tissues are some bulk structural elements, present in amounts of percents, carbon, hydrogen, nitrogen, oxygen, sulphur (S) and phosphorus (P). In addition, five macro elements, potassium (K), sodium (Na), chloride (Cl), magnesium (Mg) and calcium (Ca) are present in gram per kilogram quantities. The remaining elements occur in seafood in much lower concentrations (mg or µg per kilogram quantities), like the trace elements copper (Cu), iron (Fe) and zinc (Zn) and the ultra trace elements like fluoride (F), iodine (I), selenium (Se), silicon (Si), manganese (Mn), molybdenum (Mo), cobalt (Co), chromium (Cr), vanadium (V) and nickel (Ni). All these elements are considered essential for human and animal life. Other elements presented in seafood are bromine (Br), strontium (Sr) and rubidium (Rb). They have also been claimed essential, but the physiological functions of these trace elements have not been clearly demonstrated or remain to be confirmed. The main functions of essential elements include formation of skeletal structure, maintenance of colloidal systems and regulation of acid-base equilibrium. They are important components of hormones, enzymes and enzyme activators (Lall, 1995). Generally, species have the following descending order of concentration: K>P>Na>Mg>Ca>Zn>Fe>Cu>Mn. In

Table 3.5 it can be observed mean levels of some essential elements in various seafood species.

Table 3.5 - Mean levels of some essential elements in various seafood species (mg per 100 g of edible portion). (source: Nunes *et al*., 2008).

Seafood	K	P	Na	Mg	Ca	Zn	Fe
Cod	362	200	65	26	15	0.5	0.3
Gilthead seabream	383	252	59	28	15	0.8	0.4
Hake	408	219	69	26	15	0.7	0.5
Horse mackerel	403	263	80	33	69	1.2	1.2
Salmon	301	209	38	23	12	0.5	0.5
Sardine	367	314	65	31	72	1.6	1.0
Octopus	236	165	259	43	13	1.3	0.7
Shrimp	179	150	194	30	87	0.3	1.8

All the trace and ultra trace essential elements can be toxic when in high concentrations (Belitz *et al*., 2004). On the other hand, some elements like cadmium (Cd), lead (Pb) and mercury (Hg) show no known essential function in life and are toxic even at low concentration when ingested over a long period; therefore many consumers regard any presence of these elements in seafood as a hazard to health. These elements were present in the aquatic environment long before human beings existed (Oehlenschläger, 2002). Others can be toxic in a determined chemical form or presented varied toxicity depending on biological system (Fraústo da Silva, 1985). As the other seafood components, minerals concentration is species-specific and can furthermore vary with biological aspects (size, age, sex, state of maturity) but also with diet, season and the environment. Most aquatic organisms accumulate and retain minerals from the environment. In what concerns farmed fish, the composition of commercial feeds used also influences their elemental composition.

3.2.1 Essential elements

3.2.1.1 Macro elements

Electrolytes (K, Na and Cl)

Sodium and Cl are the principal extracellular cation and anion, respectively, in the human body. Sodium is important in osmoregulation, acid-base balance and the membrane potential of cells, as well active transport across cell membranes (Lall, 1995). In addition, it activates some enzymes, such as amylase (Belitz *et al*., 2004). Chloride is essential in the maintenance of electrolyte balance and is also the most important anion in gastric juice (Lall, 1995). In diets, Cl and Na are necessary in equivalent levels on a molar basis. Potassium is localized mostly

within the cells. It regulates the osmotic pressure within the cell, is involved in cell membrane transport and also in the activation of a number of glycolytic and respiratory enzymes (Belitz *et al*., 2004). According to Institute of Medicine (IOM) (2005), in average, the dietary reference intake (DRI) for these three elements are 1500 mg/day for Na, 4700 mg/day for K and 2300 mg/day for Cl. The intake of too little or too much of these three elements can result in serious disorders. From a nutritional point of view an excessive dose of Na is more plausible than its deficiency. Somme symptoms caused by an high consumption of Na are hypertension, weakness and edema (IOM, 2004). Chloride deficiency or excess are associated to Na. Generally, a deficiency of K do not show symptoms but a severe deficiency of this element can cause hypokalemia, characterized by muscle weakness, cardiac arritmia and glucose intolerance. An excess may enhance the appearance of hyperkalemia (IOM, 2004).

Potassium is the most abundant mineral in seafood (Lall, 1995) and its concentration is always higher than that of Na (Belitz *et al*., 2004). Since the level of Na in seafood is relatively low, this kind of food is good for those consumers who need a low Na diet (Oehlenschläger, 1997, Martinez-Valverde *et al*., 2000). Levels of K in marine fish are around 3500 mg/kg, however some species can reach 5000 mg/kg (Lall, 1995). Shellfish show the lowest K values, for example shrimps only attained a mean concentration of 1790 mg/kg (Nunes *et al*., 2008). Wide variations in the Na content of fishery products can be observed. Freshwater and marine fish present a Na average of 600 mg/kg while most shellfish have Na in the range of 1200-4000 mg/kg. Data on distribution of Cl in seafood are scarce (Lall, 1995). In the study performed by Oehlenschläger (1997) on16 marine fish species from the North-East Atlantic, the levels were in the range of 400-1300 mg/kg.

Structural elements (P and S)

Phosphorous is an integral part of bone and tooth mineral as well as part of the structure of every cell (Pigott & Tucker, 1990). It is directly involved in energy-producing cellular reactions. Therefore, P plays an important role in overall metabolism as well as in various metabolic processes involving buffers in body fluids (Lall, 1995). Sulphur it is especially important for humans because it is part of the amino acid methionine, which is an absolute dietary requirement. The amino acid cysteine also contains S. This element also plays an important role in the health effects production of insulin, which keeps the blood sugar levels balanced. According to IOM (2005), in average, DRI for P is 700 mg/day. Dietary reference intake for S is about 800-1000 mg/day (Acu-cell Nutrition, 2010a). As P and S are found in most foods, deficiencies are uncommon.

The best source of P and S are high-protein foods such as seafood. In general, the content of P in seafood is around 2000 mg/kg. Usually, equal amounts of these two elements were found in marine species (Oehlenschläger, 1997). Data collected by some authors (Lall, 1995; Oehlenschläger, 1997; Nunes *et al*., 2008) show a P mean concentration range of 1400-3000 mg/kg being the small fatty pelagic fish the best source.

Calcium and Magnesium (Ca and Mg)

Approximately 99 % of Ca is present in bones as calcium phosphate and hydroxyapatite. The remaining portions of Ca are found in extracellular fluids and intracellular structures and cell membranes. Calcium is involved in the structure of the muscular system and controls essential processes like muscle contraction, blood clotting, activity of brain cells and cell growth (Belitz *et al*., 2004). The distribution of Mg is similar to the distribution of Ca and P, the major proportion is being located in bone. The remainder is found within the cells of soft tissues (Lall, 1995). Magnesium is a prosthetic group in enzymes that hydrolyse and transfer phosphate groups. Consequently, it is essential for energy-requiring biological functions such as membrane transport, generation and transmission of nerve impulses, contraction of muscles and oxidative phosphorylation. It is also essential for the maintenance of ribosomal structure and thus protein synthesis (Lall, 1995). According to IOM (2005), in average, the DRI for these two elements are 1000 mg/day for Ca and 420 mg/day for Mg. Symptoms of Ca deficiency are bone weakness, neuromuscular excitability and muscle spasms. Calcium excess can provoke kidney stones. Magnesium deficiency can cause neuromuscular irritability, muscle spasms and cardiovascular accidents. Furthermore, an excess of Mg can cause diarrhoea, anaesthesia and depression of central and peripheral nervous system (Fraústo da Silva, 1985).

Despite of seafood muscle are considered poor source of these two elements, generally, seafood contains more Mg than Ca (Oehlenschläger, 1997). The average concentrations of Ca in most fish and shellfish muscle are in general inferior to 400 mg/kg but some species show wide differences due to variable amounts of bone and shell. Crustaceans contain more Ca than fish (Lall, 1995). The concentrations of Mg in the edible portion of most seafood ranged between 200 - 500 mg/kg. However, molluscs and crustaceans can showed levels around 2000 mg/kg of Mg (Jodral-Segado *et al*., 2003).

3.2.1.2 Trace elements

Copper (Cu)

Copper is a component of a number of oxidoreductase enzymes. As a cofactor for enzymes it is involved in glucose metabolism and the synthesis of haemoglobin, connective tissue and phospholipids (Lall, 1995). In blood plasma, it is bound to ceruloplasmin, which catalyzes the oxidation of Fe^{2+} to Fe^{3+}. This reaction is of great importance since it is only the Fe^{3+} form in blood which is transported by the transferrin protein to the iron pool in the liver (Belitz *et al*., 2004). Copper can have toxic effects in all alive organisms; however, it is not toxic for humans at low concentrations (Celik & Oehlenschläger, 2004). The average DRI for Cu is 0.9 mg/day (IOM, 2005). This value is normally supplied in a normal diet. A deficient diet in Cu may cause in humans, anaemia, Menke's syndrome in children, deficient keratinisation and pigmentation (insufficient production of melamine). An excess, in turn, can cause, among other problems, Wilson's disease, hepatic necrosis, cirrhosis and haemolytic crises (Fraústo da Silva, 1985).

The overall concentration of Cu in most seafood is around 2 mg/kg, with the exception of molluscs and crustaceans where the concentration can attain average values of 10 mg/kg, in crabs, or 80 mg/kg in oysters (Lall, 1995).

Iron (Fe)

Iron is present in all cells of living organisms and plays a vital role in several biochemical reactions. Most of Fe is present in the haemoglobin (blood) and myoglobin (muscle tissues) pigments, cytochromes and other proteins, participating in the transport, storage and utilization of oxygen (Lall,1995). This element is also present in a number of enzymes (peroxidase, catalase, hydroxilases and flavine enzymes), therefore is an essential element of the daily diet (Belitz *et al*., 2004). According to IOM (2005), in average, the DRI for this element is 8 mg/day, however, in order to provide a sufficient supply of Fe to persons who require higher amounts (children, women before menopause and pregnant or nursing women), the Fe DRI can reached up to 27 mg/day. Iron deficiency causes anaemia. On the other hand, hemochromatosis and thalassemia involve an excessive storage of iron probably caused by an excessive intake of supplements. Symptoms of acute toxicity are vomiting, diarrhoea and intestinal problems (Curry & Liu, 2010).

Total content of Fe in seafood can vary greatly. Usually, Fe fish concentrations are lower than 10 mg/kg. Nevertheless, fish species with a high amount of dark muscle, as pelagic ones (sardine, mackerel, tuna, etc.), contain more Fe than others fish species, attaining 30 mg/kg, due to the high level of Fe in dark muscle (Lall, 1995). Species like crustaceans can also have amounts higher than 10 mg/kg (Lall, 1995).

Zinc (Zn)

Zinc is an essential element for humans. Its essential function is based on its role as an integral part of a number of metalloenzymes and as a catalyst for regulating the activity of specific zinc-dependent enzymes (Lall, 1995). According to IOM (2005), in average, the DRI for this element is 8 mg/day. A deficient diet in Zn can lead to the occurrence of diseases such as anorexia, stunting, parakeratosis, alopecia, hypogonadism, and delayed healing. On the other hand an excess of Zn can cause metal fume fever (fever, chills) (Fraústo da Silva, 1985). Zinc poisoning has been reported as a result of consumption of soured food kept in zinc-plated-metal containers (Belitz *et al*., 2004).

This element is present in fish and other seafood in mg/kg amounts and there have been no reports of concentrations in the edible parts of seafood that form a hazard to health (Oehlenschläger, 2002). In general, fish have low Zn contents when compared to molluscs and crustaceans. Fish muscle has an average of about 3-5 mg/kg of zinc, while molluscs and crustaceans can reach much higher values. Oysters for example can display average values of 850 mg/kg (Lall, 1995).

3.2.1.3 Ultra trace elements

Chromium (Cr)

Chromium is considered an essential nutrient for humans. It may have a role in activating enzymes and in maintaining the structural stability of proteins and nucleic acids, but the primary physiological role of Cr is to enhance insulin action (Lall, 1995; Vincent, 2004) by activating the enzyme phosphoglucomutase (Belitz *et al*., 2004). Trivalent Cr and its salts are usually the most stable form of Cr and the main form found in plants and animals (FDA, 1993a). The hexavalent Cr salts are less stable and more biologically reactive. This last form causes allergic reactions and has neurotoxic and carcinogenic effects (Racek, 2003). According to IOM (2005), in average, the DRI for this element is 35 µg/day. This value can be surpassed with a diet in seafood however, in agreement to Council for Responsible Nutrition (CRN) (Hathcock, 2004) Cr supplements at levels up to 1,000 µg per day are regarded as safe for adults. The symptoms of Cr deficiency caused by long-term total parenteral nutrition are severely impaired glucose tolerance, a loss of weight, and confusion. Because of the high excretion rates and the very low absorption rates of most forms of Cr, acute toxicity is uncommon.

The Cr content in fish was reported to be between 0.02 and 0.5 mg/kg (Oehlenschläger, 1997). However, levels as high as 5 or 12 mg/kg have been found in molluscs and crustacean edible tissues (Lall, 1995). Generally, the edible part of the muscle contains lower levels of Cr than liver and other organs.

Manganese (Mn)

One of the functions of Mn is as a cofactor activating a large number of enzymes such as arginase, amino peptidase, alkaline phosphatase, lecithinase or enolase (Belitz *et al*., 2004). Since the chemistry of Mn ion is similar to that of the Mg ion, many enzymes can be activated by either Mn or Mg however, certain enzymes, for example, glycosyl tansferases are highly specific for Mn activation (Lall, 1995). Manganese can also be an integral part of some metalloenzymes (for example pyruvate oxidase) (Fraústo da Silva, 1985). Moreover, Mn is also necessary in lipid and carbohydrate metabolism and brain function (Lall, 1995). According to IOM (2005), in average, the DRI for Mn is 1.8 mg/day. Only a small part of Mn is absorved by diet (IOM, 2001) and even in higher amounts, Mn is relatively non toxic (Belitz *et al.,* 2004). However, an excessive intake can lead to mental disorders (manganese madness) (Fraústo da Silva, 1985). Its deficiency can cause bone deformities, sterility and ataxia.

Seafood is not a good source of Mn and is considered a minor component (Oehlenschläger, 1997). Most muscle fish contain 0.1 to 0.4 mg/kg (Lall, 1995), nonetheless molluscs and crustaceans contain significantly higher levels than fish (Lall, 1995). Subsequently, seafood only contributes to the DRI with a minor percentage.

Nickel (Ni)

Nickel is an essential nutrient that is only required in very small amountsThis metal is present in some biologic molecules such as enzymes urease and CO dehydrogenase (Fraústo da Silva, 1985). Nickel also enhances insulin activity (Belitz *et al*., 2004). Its absorption into the body is affected by factors of consumption, the acidity of the gut, and various binding or competing

substances, including other minerals such as Ca, Fe, Mg and Zn (Curry & Liu, 2010). According to IOM (2005), in average, the DRI for this element is 0.5 mg/day. Nickel deficiency is rare due to the low level of requirement, and its relatively high availability in the diet, but experiments have shown that at a cellular level, Ni deprivation results in changes in the membrane properties and other structures. Deficiency has also been associated with low blood glucose levels, abnormal bone growth, altered metabolism of Ca and vitamin B12 and poor absorption of ferric iron (Curry & Liu, 2010). Nickel toxicity is usually not a problem unless several grams are ingested from non-dietary sources. The toxicity of Ni is classified into four categories: allergies, cancer, non-malignant respiratory disorders and iatrogenic poisoning (FDA, 1993b).

For the common population the major source of Ni is the diet. Nickel is widely distributed in foods. The concentration of Ni in seafood varies, depending of species and polluted or non-polluted catch area. In general, molluscs and crustaceans have higher values than fish species. Levels can range from 0.02 mg/kg to 1 mg/kg (Carvalho *et al.,* 2005; Sivaperumal *et al*., 2007).

Selenium (Se)

Selenium is recognized both as an essential trace element and a toxic agent. It is an integral component of the enzyme glutathione peroxidase (GSH) (selenoprotein) in human and animal tissues, which, together with vitamin E and the enzymes catalase and superoxide dismutase, acts as an antioxidant, thereby protecting cells against oxidative damage (Lall, 1995). It is also involved in thyroid metabolism through iodothyroninedeiodinase enzyme and is needed for the proper functioning of the immune system (Sirichakwal *et al.,* 2005). The presence of Se reduces the availability of metal ions (such as mercury and cadmium), blocking them in insoluble compounds, which is a process of great importance (Lall, 1995; Plessi *et al*., 2001; Feroci *et al.,* 2005). As a result, a deficiency of Se increases the toxicity caused by those metals. In addition, Se deficiency has been reported to cause an overload of Fe and unbalanced distributions of other elements in vivo, such as Mg, Ca, Cu and Zn (Feroci *et al*., 2005). On the other hand, excessive ingestion or inhalation of Se can be toxic to man and animals, although in humans its occurrence is rare. The symptoms include depression, nervousness, and dermatitis, garlic odour of the breath, gastrointestinal disturbances, and excessive tooth decay (Sivaperumal *et al*., 2007). According to IOM (2005), the average DRI for this element is 55 µg/day.

Seafood contains substantially higher levels of Se than red meats and poultry and is thus considered one of the major dietary sources of this element in addition to cereal and dried fruit (Lall, 1995). Among seafood, marine fish are the best source. The Se content is quite constant and varies little between species (Oehlenschläger, 1997). Reported values are about 0.2 – 1.0 mg/kg (Lall, 1995; Oehlenschläger, 1997; Carvalho *et al*., 2005; Sirichakwal *et al.,* 2005; Garg & Ramakrishna, 2006; Sivaperumal *et al.,* 2007). Dietary reference intake can be easily reached in a normal seafood diet.

3.2.2 Non-essential elements?

3.2.2.1 Bromine (Br)

Bromine is one of the most abundant and ubiquitous within the recognized trace elements in the biosphere. This element has not been conclusively shown to perform any essential function in plants, microrganisms and animals (Pavelka, 2004) since not enough information is available about the metabolism of bromide ion, the most common form of this element. However, inorganic bromide can exert both therapeutic as well as the toxic effects (Pavelka, 2004). It is proved that in thyroid gland, bromide replace iodide (Pavelka, 2004). In this way, seafood that are a source of bromine and iodine, and depending on their bromine/iodine ratio and whether someone is hypothyroid or hyperthyroid, it can have beneficial or unfavourable effect on thyroid functions when regularly consumed. There is no DRI established for Br, nevertheless, 1 – 3 mg per day is an estimated median daily intake of Br for food and water (Acu-cell Nutrition, 2010b).

Bromine data on seafood are sparse, however according to some authors the average Br concentration in fish is about 2 mg/kg, while in molluscs and crustaceans can reach much higher values (Varo, 1992; Barrento *et al.,* 2009).

3.2.2.2 Rubidium (Rb)

Rubidium, a rarely studied alkali metal, may be an essential ultra trace element for humans and other organisms (Campbell *et al.,* 2005). However, the circumstantial evidence supporting Rb essentiality is still limited. Rubidium deficiency in goats reportedly results in depressed food intake, growth, and life expectancy and increased spontaneous absorption (Anke & Angelow, 1995) These finding with goats are complemented by earlier reports that Rb can possibly acts as a nutritional substitute for K in some functions, especially in lower forms of life (Nielsen, 2000). Since this element is relatively non toxic and is not associated with any chronic disease, it has not received any media attention or supplement industry. The typical daily dietary intake of Rb of 1 to 5 mg probably ca be considered safe and, if essential, adequate (Nielsen, 2000).

Very little information exists in regard on the concentrations and distribution of Rb in freshwater and marine food webs (Campbell *et al*., 2005). A study performed by Carvalho *et al.* (2005) reported values around 2 mg/kg (dry weight) in some fish species and octopus eaten in Portugal.

3.2.2.3 Strontium (Sr)

There is some difficulty regarding the classification of this metal relatively to its essentiality. The human body absorbs Sr as if it is Ca and due to the chemical similarity, the stable forms of Sr might not pose a significant health threat. In fact, the levels found naturally may actually be beneficial. There is a long history of medical research regarding strontium's benefits, beginning in the 1950s (Wikipedia, 2010a). Studies indicate a lack of undesirable side-effects, and for that the evidence that Sr is essential to human health is complex. Nevertheless, recent studies show that adequate doses of specific essential nutrients, including Sr, prevent and fight against osteoporosis. This mineral is clinically effective in preventing fractures and bone loss and

increases bone mineral density. Medical literature suggests that a judicious use of low doses of Sr (for example some compounds of Sr, as strontium ranelate, gluconate, carbonate, lactate and chlorate) can be an effective and safe way to assist patients in restoring and maintaining bone health (Meunier *et al*., 2004; Genuis & Schwalfenberg, 2007). It also seems to have an effective role in dental caries being strontium chloride used as part of some toothpastes formulation (Acu-cell Nutrition, 2010c). There is no DRI established for Sr, nevertheless, 1 – 5 mg per day is an estimated median daily intake of Sr for food and water (Acu-cell Nutrition, 2010c).

Studies on contents of Sr in seafood are scarce. Generally, fish present lower amounts of Sr (1-5 mg/kg) than molluscs and crustaceans (Segar *et al*., 1971; Teeny *et al*., 1984; Vlieg *et al*., 1991; Garg & Ramakrishna, 2006) being the highest values found in crustaceans (Barrento *et al*., 2008).

3.2.3 Toxic elements

Despite of the indisputable nutritional value of seafood, these products can accumulate in their edible part some pollutants. Among these pollutants it can be detached the dioxins, polychlorinated biphenyl compounds (PCBs) and toxic elements like Hg, Cd, Pb and arsenic (As) (Fremy & Bordet, 2002). The danger of their presence in the marine environment implies not only the toxicity of proper environment but also a considerable level of these pollutants in the trophic chain, which may constitute a risk for the human health (Burger & Gochfeld, 2005).

The presence and concentration of toxic elements in the aquatic environment, including biota, specifically in seafood which are used for human diet is based both on natural and anthropogenic sources (Oehlenschläger, 2002). Natural levels of these elements are due to marine volcanism, geological anomalies and geothermal events while anthropogenic are principally related with intensive metallurgy. Fish and other seafood have always contained certain amounts of toxic elements as a consequence of habitat. In open seas, unaffected by pollution, seafood mostly carries just the natural burden of toxic elements content. In polluted areas, in waters which have no sufficient exchange with the world oceans, in estuaries, in rivers and especially in places which are close to sites of industrial activities, the concentrations of toxic elements exceed the natural load (Oehlenschläger, 2002). The main reasons of the toxicity of elements derive from the fact that many of these can replace metals in active sites of enzymes, bind to other radicals of enzymes and other biological molecules, replacing certain groups in biological molecules, complexing or forming precipitates with enzyme metals or other groups involved in metabolism, catalyzing the breakdown of essential metabolites, combined with membranes, altering their permeability, and replace elements with electrochemical functions (Fraústo da Silva, 1985; Goyer, 1997). Mercury, Cd, Pb and As are cumulative toxic that can be assimilated, stored and concentrated by living organisms through the food chain, causing sometimes serious physiological effects. The animals are usually more sensitive to contamination by toxic metals than plants, and humans are considered the most sensitive and the most important target species. Since 2001, there are legal limits for Cd, Pb and Hg,

proposed by EU, for fish muscle in general, for some particular fishes, for crustaceans and for molluscs (EU, 2006, 2008). Moreover, the Joint FAO/WHO Expert Committee for Food Additives (JECFA) has recommended the provisional tolerable weekly intakes (PTWI) (WHO, 1999; 2003a). **Table 3.6** summarizes these values for As, Cd, Pb and Hg and some usually contents in seafood.

Table 3.6 - Legal limits (LL), ranged values (RV) and PTWI for As, Cd, Pb and Hg in seafood.

	As		Cd		Hg		Pb	
Seafood	LL[a]	RV[b]	LL	RV[c]	LL	RV[c]	LL	RV[c]
Fish in general	-	0.05 - 450	0.05	< 0.01 - 0.05	0.50	< 0.05 - 0.50	0.30	< 0.1 - 0.2
Particular Fish[d]	-	-	0.10/0.30	< 0.01 - 0.12	1.0	< 0.05 - 2.4	-	< 0.1 - 0.2
Crustaceans (excluding brown meat)	-	< 0.1 - 270	0.50	< 0.01 - 1.1	0.50	< 0.05	0.50	< 0.1 - 0.1
Cephalopods (without viscera)	-	4.0 - 50	1.0	0.01 - 3.8	0.50	< 0.05 - 0.17	1.0	< 0.1 - 0.2
Bivalves molluscs	-	0.13 - 240	1.0	0.03 - 1.4	0.50	< 0.05 - 0.05	1.5	< 0.1 - 0.4
PTWI (µg/kg bw)		50		7[e]		5		25

[a] - Legal limits proposed by EU (2006, 2008) (mg/kg); [b] - Values in mg/kg, dw in Neff (2004); [c] - Values in mg/kg, ww, in Afonso *et al.* (2005); [d] - For example tuna and swordfish; [e] - Actually, European Food Safety Authority (EFSA, 2009) recommended a tolerable weekly intake (TWI) of 2.5 µg/kg bw, however this work was only considered the value of 7 ug/kg bw, recommended by WHO (2003a).

To evaluate the importance of studying the content of some toxic elements in the studied species, is made subsequently a brief approach to these elements.

3.2.3.1 Arsenic (As)

Arsenic is an important and ubiquitous non-metal element which has become one of the toxic trace elements under concern in recent decades (Karadjova *et al.*, 2007). The toxicity of As depends on its oxidation state and molecular form. For example As (III) is more toxic than As (V), while arsenobetaine and arsenocholine, arseno-organic compounds, are relatively non-toxic (Storeli & Marcotrigiano, 2000; ATSDR, 2007a). The high intakes of inorganic As, mainly due to chronic exposure to elevated levels in drinking water are associated with several toxic effects including increased risk of numerous types of cancer (IOM, 2001; Belitz *et al.*, 2004; ATSDR, 2007a). Studies performed in chickens, rats and goats were done to test As essentiality (Belitz *et al.*, 2004). Its deficiency in goats appears to cause growth alterations, reproduction problems, milk production and sudden dead (McDonald *et al.,* 2002). Its metabolic role is not yet understood. It appears to be involved in the metabolism of methionine. Choline can be replaced by arsenocholine in some of its functions (Belitz *et al.*, 2004). The general population may be

exposed to As in air, drinking water, and food, but human intake of As occurs mainly via food chain, and it is loosely related to the consumption of seafood (**Figure 3.2**).

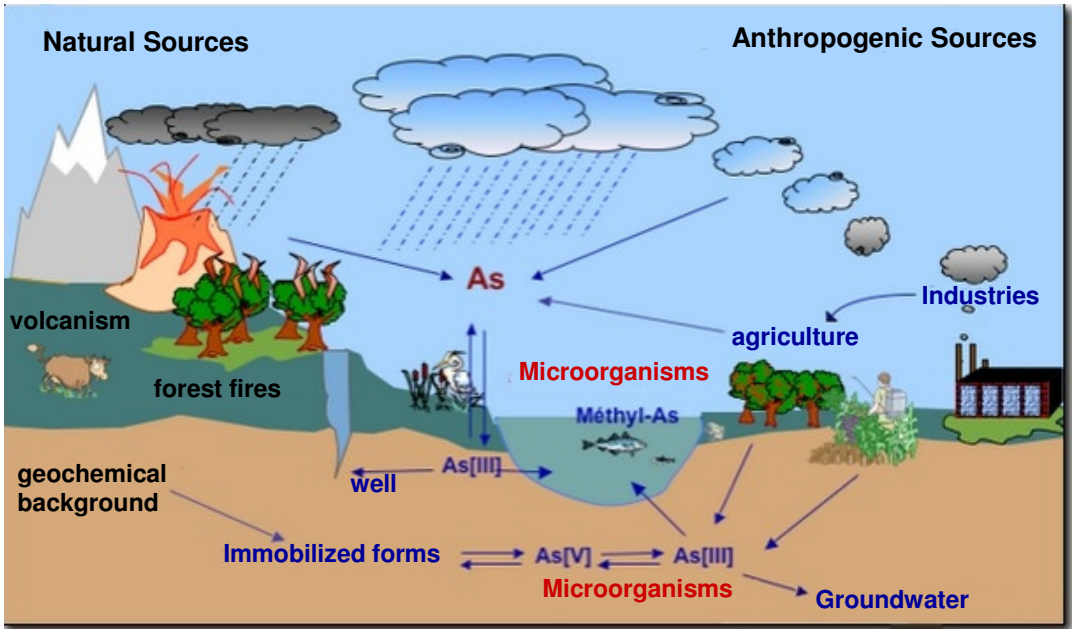


Figure 3.2 - Schematic representation of the cycle of arsenic in the environment. (Picture source site: GDR, 2009).

Marine organisms are known to accumulate total As in the range of 1 – 100 mg/kg from their environment and food sources. The majority of As is present as organoarsenic species, metabolised from inorganic As present in seawater or accumulated from food sources such as algae or other fish. Providentially, arsenobetaine is the major component (typically more than 90 % present As) in a variety of seafood products and it is considered as a non-toxic compound (Ackley, *et al.*, 1999; Muñoz *et al.*, 2000; Cava-Montesinos *et al.*, 2005). In EU no legal limits for the As levels in seafood have been established so far. Nevertheless, the Food and Drug Administration (FDA) suggests a limit of 76 mg/kg for crustaceans (FDA, 1993c) and the World Health Organization (WHO) recommends a tolerable daily intake of 0.05 mg As/kg body weight from food (WHO, 1983).

3.2.3.2 Cadmium (Cd)

Cadmium is an industrial and environmental pollutant that affects adversely a number of organs in humans. It is also a natural element in the earth´s crust. It is usually found as a mineral combined with other elements such as oxygen (cadmium oxide), chlorine (cadmium chloride), or sulphur (cadmium sulphate, cadmium sulphide) (ATSDR, 2008). Anthropogenic sources of Cd include mining operations, metallurgical industries, the corrosion of galvanized zinc, industrial production of batteries and the application of fertilizer and sewage sludge to farm land (Fraústo da Silva, 1985; Goyer & Clarkson, 2001; Castro-González & Méndez-Armenta, 2008; ATSDR, 2008). In foods of animal origin, and as happens in humans, Cd is found mainly in internal organs like the liver and kidney (Goyer & Clarkson, 2001; ATSDR, 2008; Castro-

González & Méndez-Armenta, 2008). This metal interacts with the metabolism of four essential elements: Zn, Fe, Ca and Cu (Goyer, 1997; Peraza *et al.*, 1998). Another important nutritional aspect of Cd is its interaction with the sulphydryl -rich protein, metallothioneins (MT).

Table 3.7 summarizes the major Cd interactions with some elements.

Table 3.7 - Major Cd interactions with micronutrients (Adapted from Peraza *et al.*, 1998).

Metal nutrient	Interaction and mechanism	Effects of nutrient on metal toxicity
Cd - Zn	Competes for GI* absorption; Cd interferes with Zn metabolism	Reverses Cd toxicity (i.e. decreases growth, increase lesions and testicular necrosis)
Cd - Fe	Decreases Fe absorption and metabolism (Cd possibly binds with ferritin and transferrin)	Supplementation corrects anaemia: increases hematocrit and increases haemoglobin levels
Cd - Ca	Decreases intestinal Ca transport; increases Cd deposits in bone tissue in a Ca deficient state	Sufficiency protects against bone deformities, osteomalacia, and osteoporosis (Itai-Itai disease)
Cd - Cu	Interferes with Cu metabolism, possibly by decreasing Cu absorption	Corrects Cd-induced decreased plasma ceruloplasmin concentrations
Cd - Se	Selenium shifts Cd binding to higher molecular weight proteins	MT can bind with essential nutrients

*GI - Gastrointestinal.

Cadmium-zinc interactions There are many similarities between Cd and Zn. Both metals are members of the group IIB in periodic table and both have a similar tendency to form tetrahedral complexes and therefore it seems that the two metals have similar interactions with the human body (Peraza *et al.*, 1998; Brzóska & Moniuszko-Jakoniuk, 2001). Cadmium has an inhibitory effect on the activity of Zn-containing enzymes such as carboxypeptidase and α-mannosidase. Zinc is also replaced by Cd in MT. Some of the symptoms of chronic Cd toxicity are similar to those of Zn deficiency. In both cases, growth failure, parakeratotic lesions and impaired glucose tolerance occurs.

Cadmium-iron interactions The development of anaemia is one of the symptoms associated with Cd intoxication. This is a result of the inhibitory effect of Cd on Fe metabolism and absorption. Cadmium decreases hematocrit and haemoglobin levels in exposed workers. Ferritin or transferrin could be involved in the Cd-Fe interaction observed during Cd intoxication (Peraza *et al.*, 1998).

Cadmium-calcium interactions Cadmium toxicity affects Ca metabolism, and individuals with severe Cd nephropathy may have renal calculi and excess excretion of Ca, probably related to increases urinary loss. Associated skeletal changes are probably related to Ca loss and include bone pain, osteomalacia, and/or osteoporosis. Bone changes are part of Itai-Itai disease, a syndrome recognizes in postmenopausal multiparous women living in the Funchu area of Japan prior to and during World War II. The syndrome consists of severe bone deformities and chronic renal disease (Peraza *et al.*, 1998; Goyer & Clarkson, 2001).

Cadmium-copper interactions The most obvious disturbance in Cu metabolism caused by Cd is the reduced plasma ceruloplasmin concentrations. Ceruloplasmin is the protein responsible for transporting Cu throughout the circulatory system. Another explanation for the Cd-Cu interaction is that the two metals compete for binding sites on MT. Copper displaces Cd from MT because of its higher affinity for the protein (Peraza *et al*., 1998).

Cadmium-metallothionein interactions The accumulation of Cd in the kidneys to some extent without apparent toxic effect is possible because of the formation of Cd-thionein or metallothionein, a metal-protein complex with a low molecular weight. Metallothionein is primarily a tissue protein and is ubiquitous in most organs, but it exists in the highest concentration in the liver, particularly following recent exposure to Cd, and in the kidneys, where it accumulates with age in proportion to Cd concentration. Cadmium bound to metallothionein within tissues is fortuitous result of the chemical similarity between Cd and the essential trace metals (Peraza *et al*., 1998; Goyer & Clarkson, 2001).

Cadmium-selenium interactions Nutritional studies have shown that Se offers some protection against acute Cd-induced toxicity. The protective role of Se probably come into play by the shifting of Cd binding from MT to higher molecular-weight proteins. This allows MT to be unencumbered and therefore able to bind essential nutrients such as Zn and Cu (Peraza *et al*., 1998).

Hypertension, neurological disorders and carcinogenicity Some epidemiologic studies suggest that Cd is an etiologic agent for essential hypertension, others suggested a relationship between abnormal behaviour and/or decreased intelligence in children and adults exposed to Cd and still other studies have shown a relationship between occupational (respiratory) exposure to Cd and lung cancer and possibly prostate cancer (Goyer & Clarkson, 2001; Castro-González & Méndez-Armenta, 2008).

Cadmium pathway to humans occurs mainly through two sources. One source is through inhalation of Cd particles during industrial or everyday activities, among which the inhaled Cd^{2+} from cigarette should be considered as highly hazardous because Cd is easily absorbed by the lungs (Goyer, 1997; Castro-González & Méndez-Armenta, 2008). Another is the oral route through water and food contaminated with Cd, particularly leafy vegetables, grains, cereals, fruits, organ meat, and seafood.

Cadmium content in fish muscle is generally low, ranging between 0.01 to 0.1 mg / kg wet weight (Francesconi, 2007), however it may be deposited in greater quantities in fish organs like kidneys and liver. These organs can be deeply contaminated and should preferably not be consumed (Oehlenschläger, 2002). However, the situation with marine invertebrates like molluscs and crustaceans is different. Molluscs, especially cephalopods, are active Cd accumulators (Bustamante *et al*., 1998). Cephalopods, like octopus, squid or cuttlefish can store large amounts of Cd in their intestines while their mantle and tentacle contain less amount (Oehlenschläger, 2002). If the elimination of all intestines is not done from cephalopods immediately after catch, Cd can migrate from the intestines into the muscle tissue. Probably, the Cd level in these muscle tissues will surpass the legal limits. Some bivalve molluscs can also

have high contents of Cd and the levels must be regularly monitored. The higher Cd content in molluscs is the justification for higher legal limits of Cd for this group of seafood.

3.2.3.3 Mercury (Hg)

Mercury is one of the most important pollutants both because of its effect on marine organisms and because it is potentially hazardous to humans (Storelli *et al.*, 2003). This element may occur naturally in the environment (mineral, deposits, volcanoes, forest fires, oceanic emission, and crust degassing) or be released by human activities such as agriculture industry (fungicides, seed preservatives), mining, mineral processing and combustion of fossil fuels, by pharmaceuticals, as pulp and paper preservatives, catalysts in organic syntheses, in thermometers and batteries, in amalgams and in chlorine and caustic soda production (Renzoni *et al.*, 1998; Oehlenschläger, 2002). The toxicology and the environmental behaviour of Hg are complex, since its toxicity, mobility, and bioaccumulation depend on its chemical form (Storelli *et al.,* 2003), elemental or metallic, inorganic or organic. **Figure 3.3** shows the main aspects of the biogeochemical cycle of Hg. Mercury in the atmosphere can be transported over great distances, so even areas without natural or anthropogenic sources of Hg, the contamination may be present, and thus it is a global pollution problem. In the atmosphere, almost all Hg is in elemental form (Hg^0). By reaction with ozone and OH radicals, the Hg^0 is oxidized to Hg^{2+}, being less volatile, it condenses and is deposited. In aquatic ecosystems, the Hg^{2+} can be reduced to Hg^0, returning to the atmosphere, or biotransformed into organic forms. Biotransformation is the methylation of Hg^{2+} by sulphate-reducing bacteria, which live in conditions of anoxia, usually in the sediment or the water column (EPA, 2001).

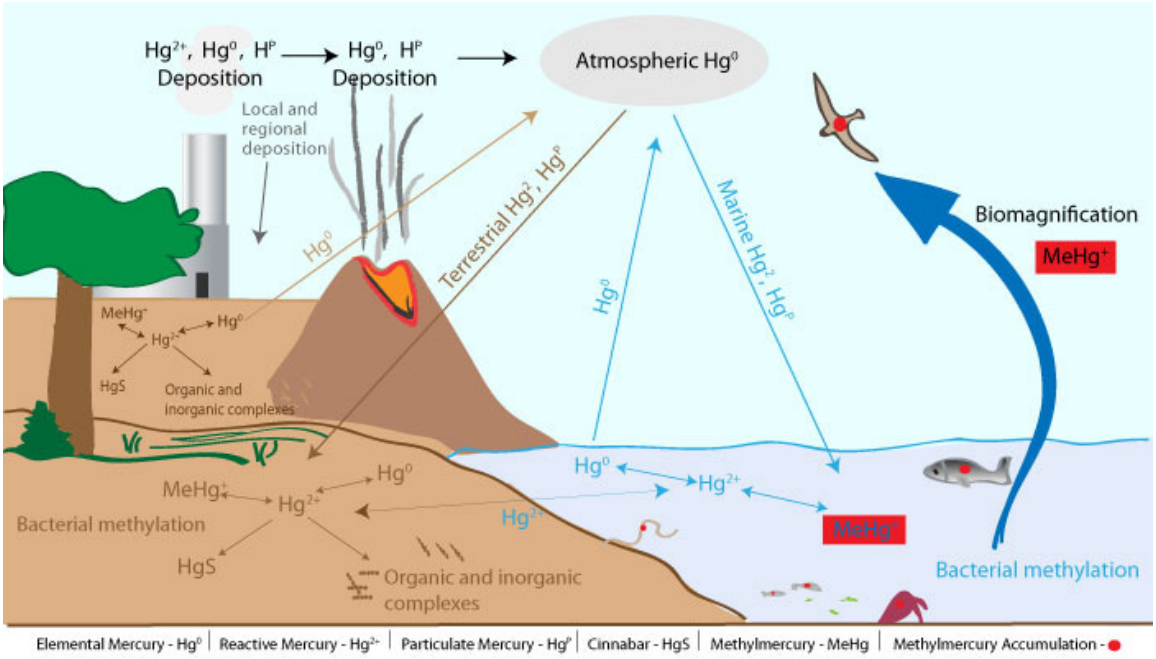


Figure 3.3 - Conceptual biogeochemical mercury cycle. (Picture source site: Annenberg Learner, 2010)

The organic compound that is formed is usually the monometylmercury (CH_3Hg^+ - MeHg) which, besides being the most toxic form, is quite stable and it is absorbed easily during digestion and accumulates in aquatic organisms. It also enters fish bodies directly through their skin and gills. Beyond MeHg methylation of Hg (II), it can also be produced dimethylmercury ($(CH_3)_2Hg$) that is quite volatile, and does not accumulate in aquatic organisms (Augelli *et al.*, 2007).

Elemental Hg is poorly absorbed from the gastrointestinal tract but is almost completely absorbed through respiratory tract, rapidly crossing the blood-brain barrier and causing neurotoxity. Its vapour accounts for most occupational mercury exposures. At the cellular level the dissolved vapour is oxidized to Hg^{2+}, which causes nephrotoxiity (Peraza *et al.*, 1998; Goyer & Clarkson, 2001, Hajeb *et al.*, 2009). Inorganic Hg is hydrophilic and thus is the most nephrotoxic form, affecting mainly the straight segment of the proximal tubule. The mechanism of toxicity of inorganic Hg is by impairing mitochondrial function. Mercury possibly promotes mitochondrial membrane leakage and increases mitochondrial oxygen consumption with subsequent increased production of hydrogen peroxide (Peraza *et al.*, 1998). The mercuric ion also has a high activity for ligands containing sulfhydryl groups, strongly inhibiting the activity of enzymes that contain those groups (Hajeb *et al.*, 2009). The mercuric chloride is the inorganic salt whose toxicity is well known. Its oral ingestion causes severe abdominal pain, bloody diarrhea and suppression of urine. Ulceration, haemorrhage and necrosis gastrointestinal tract is accompanied by a circulatory collapse (Goyer & Clarkson, 2001).

The MeHg organometallic species is a lipophilic compound easily absorbed. It crosses the blood brain barrier and placenta and reacts with their target organs, the tissues of the brain, being a neurotoxic agent that affects the development of the nervous system, resulting in psychological disturbance, impaired hearing, loss of sight, ataxia, loss of motor control and general debilitation (Storeli *et al.*, 2005). It also causes blockage of binding sites of enzymes, interferes in protein synthesis, hampers thymidine incorporation into DNA (Uría & Sanz-Medel, 1998). The MeHg suffers biotransformation to compounds of Hg (II) in tissues, by breaking the carbon-mercury (Goyer & Clarkson, 2001). Various studies suggest that disruption of the molecule MeHg causes the formation of free radicals, which affect the lipid membranes of neurons, leading to their damage. This situation should not be strange that the toxic effects of MeHg might be reduced by antioxidants such as selenium (Lall, 1995; Sivaperumal *et al.*, 2007). The MeHg also has a high affinity for sulphydryl groups and, therefore, the complexes with SH groups, in which the amino acid cysteine is particularly rich, serve ligand and provide transport of Hg in blood and central nervous system (Carvalho *et al.,* 2005; Hajeb *et al.*, 2009). One of the most notable cases of MeHg poisoning occurred in Minamata Bay (Japan) between 1932 and 1968, after discharges by an industrial unit in the bay, causing a high number of victims, and for that reason known as Minamata disease. Also in Iraq, between 1971 and 1972, one of the most serious cases was due to the consumption of bread made with seeds that had been treated with MeHg to prevent fungal growth. The peasants to get rid of contaminated seed, threw them in rivers and lakes, contaminating the water and aquatic organisms. This

resulted in the deaths of more than five thousand people and another 100 000 were disabled (Virtanen *et al*., 2007; Afonso *et al*., 2007).

Several nutrients have been found to affect the toxicity of Hg. Of these, Se has been the most widely studied. In general, Se has a protective effect, delaying the onset of mercury toxicity or reduces the severity of the effects of both inorganic forms of Hg and MeHg (Goyer, 1997). Selenium protection against inorganic Hg may involve a variety of mechanisms such as the direct stoichiometric complexing of Hg to reduce its availability. The reduction of Hg availability has not been proven because Hg levels are not decreased by Se. It may possible that Se interferes with the metabolism of inorganic Hg by reacting with the mercuric ion to form a compound that is less toxic than the mercuric ion. Selenium influences the oxidation rate of Hg^0 to mercuric ions that seems to be species dependent. The protection of Se against MeHg is not clear. Methylmercury is degraded to mercuric ion. The rate of this process is also species dependent. Selenium may protect against MeHg as described for inorganic Hg (Peraza *et al*., 1998). There may also be another protective mechanism involving Se. When MeHg is degraded to inorganic Hg, the methyl moiety can also be further degraded by homolyis to methyl free radicals. These molecules may initiate a chain reaction peroxidation of various lipid constituents. Selenium-dependent GSH protects the cells by catalyzing the reduction of hydrogen peroxide and other organic hydroperoxides to products of greater stability (Peraza *et al.,* 1998). Another nutrient that protect against MeHg toxicity is vitamin E. The mechanism of action of vitamin E is that of a scavenger of radicals that otherwise initiate MeHg breakdown. Vitamin E also can react with methyl radicals that might be formed in the breakdown. Nevertheless, the most effective action of vitamin E may be related to its location in the cell membranes and to its ability to stabilize membranes by interacting with unsaturated fatty acid chains (Peraza *et al*., 1998).

Methylmercury bioaccumulates in the aquatic food chain and is found in all species of fish and fish-eating animals. In humans, the major exposure to MeHg also occurs via food, with the major sources being fish and fish products. Almost 100 % of the Hg present in fish is MeHg. The highest concentrations are found in large and old predatory fish, such as sharks, swordfish, tuna and pike (Virtanen *et al*., 2007). The occurrence of Hg in seafood is a common subject for human health risk evaluation and is of particular concern to the food agencies. The combination of the metal concentrations obtained in the seafood products analyzed and the consumption information permits to estimate the dietary intake. To evaluate the health risk of this estimated dietary exposure it have to be compared with the PTWI that are recommended by JECFA: 5 µg Hg/kg body weight (WHO, 1999), no more than 1.6 µg MeHg/kg body weight (WHO, 2003a). In order to support in the accuracy of such recommendations, total Hg of marketed seafood is regulated. Maximum allowable levels differ between countries. Actually, EU established a level of 0.50 mg/kg wet weight for the majority of fishes and 1.0 mg/kg wet weight for those species that are recognized as naturally accumulating elevated levels of Hg (large, long-lived, carnivorous fishes) (EU, 2006, 2008).

With all this information is therefore important, the assessment of Hg levels in fishery products not only as a factor of toxicologically, but also for the assessment of potential impacts on public health (Muñoz *et al.,* 2005).

3.2.3.4 Lead (Pb)

Lead is a naturally-occurring bluish-gray metal that is rarely found in its elemental form, but occurs in the Earth´s crust primarily as the mineral galena (PbS), and to a lesser extent as anglesite ($PbSO_4$) and cerussite ($PbCO_3$). This metal is not a particularly abundant element, but its ore deposits are readily accessible and widely distributed throughout the world. Its properties, such as corrosion resistance, density, and low melting point, make it a familiar metal in pipes, solder, weights, and storage batteries (ATSDR, 2007b). The principal routes of exposure for general population is food and environmental sources. These sources include Pb based indoor paint in old dwellings, in dust, in contaminated drinking water, in air from combustion of Pb-containing industrial emissions, hand-to-mouth activities of young children living in polluted areas, Pb-glazed pottery, and, less commonly, Pb dust brought home by industrial workers on their shoes and clothes (Goyer & Clarkson, 2001). Because of health concerns, Pb from paints and ceramic products, caulking, and pipe solder has been dramatically reduced in recent years. The use of Pb as an additive to gasoline was also banned in most countries of the world (Goyer, 1997; ATSDR, 2007b). Actually, 95-98 % of all Pb present in the environment can be traced back to anthropogenic activities (Oehlenschläger, 2002). Lead toxicity affects several organs systems, including the nervous, haematopoietic, renal, endocrine, and skeletal, depending on the age of the subject and the size of the dose, but the effect of major concern is the impairment of cognitive and behavioural development in infants and young children (Goyer, 1997; Castro-González & Méndez-Armenta, 2008). Lead can bind covalently to organic ligand such as alkyl groups forming stable organometallic compounds as tetraethyl Pb (used as a gasoline additive). These forms can easily cross the cell membrane by diffusion, accumulating particularly in tissues rich in lipid structures. In humans, organic forms cross the blood brain barrier and affect the central nervous system (Goyer & Clarkson, 2001).The symptoms of Pb poisining are headache, irritability, abdominal pain and various symptoms related to the nervous system (Järup, 2003). Chronic Pb toxicity in humans often develop dullness, irritability, poor attention span, epigastria, constipation, vomiting, convulsions, coma and death. Children may be affected by encephalopathy with lethargy, mental dullness, vomiting, irritability, and anorexia; in severe cases, the prolonged exposition of Pb can decrease the cognitive function and increase behaviour disorders, specially aggression, psychosis, confusion and mental deficit (Järup, 2003; ATSDR, 2007b). Blood Pb level indicates a recent exposure, whereas bone Pb level, which forms 90-95 % of Pb burden in adults and 80-95 % of total Pb in children indicates a chronic exposure (Kakkar & Jaffery, 2005).

Nutritional deficiencies of essential elements can increase the hazard from Pb exposure by enhancing absorption and toxicity of dietary Pb. The essential elements with the most market

influence on Pb levels and toxic effects are Ca, Fe and Zn. **Table 3.8** summarizes the major Pb interactions with some elements.

Table 3.8 - Major Pb interactions with micronutrients (Adapted from Peraza *et al*., 1998).

Metal nutrient	Interaction and mechanism	Effects of nutrient on metal toxicity
Pb - Ca	Competes for binding sites on intestinal mucosal proteins	Sufficiency decreases GI* absorption of Pb and decreases concentration of Pb in critical organs
Pb - Fe	Competes for Fe transport systems (i.e. ferritin) of the intestine	Supplementation may decrease Pb absorption and toxicity (i.e. haematopoiesis depression)
Pb - Zn	Competes for GI uptake	Supplementation decreases Pb GI absorption, decreases Pb tissue accumulation, and thus decreases Pb toxicity (i.e. inhibitory effects on δ-ALAD**)

*GI - Gastrointestinal; **δ-ALAD - δ-aminolevulinic acid dehydratase.

Lead-calcium interactions There are several suggestions in the lead toxicity literature that the two metals are metabolically related (Goyer, 1997). These interactions occur at the cellular and molecular levels and are a result of the ability of Pb to mimic or displace Ca during specific physiologic processes. For example, Pb can block Ca efflux from cells by substituting for Ca in Ca^{2+}/Na^{+} adenosine triphosphate (ATP) pumps. Although it is not possible to mimic the animal studies in people, balance studies conducted by several authors (Goyer, 1997) shows an inverse relationship between dietary Ca and Pb absorption and retention. However, there is little information regarding the effect of excess Ca, on Pb absorption.

Lead-iron interactions Iron deficiency also enhances Pb absorption and promotes Pb toxicity. This is more evident in pregnant women and young children that are more susceptible to dietary Pb (Peraza *et al*., 1998). Lead inhibits two major enzymes of the heme biosynthetic pathway: δ-ALAD and ferrochelatase. Lead also interferes with mitochondrial energy metabolism, which is necessary to reduce ferric ion to ferrous ion before insertion of Fe into the porphyrin ring. When Fe deficiency is present ferrochelatase is more sensitive to these effects of Pb and results in depression of haematopoiesis. Therefore, iron supplementation may prevent this toxic effect of Pb on haematopoiesis (Peraza *et al*., 1998, Goyer & Clarkson, 2001).

Lead-zinc interactions These interactions are not so well defined as those between Pb and Ca or Pb and Fe. Their is a close inverse relationship between blood Pb and activity of Zn-containing heme enzymes, particularly δ-ALAD, which suggests that Pb replaces Zn in these enzymes (Goyer, 1997).

The Pb content in fish muscle from fish that is caught in the open sea is very low, amounting to 2 – 10 µg/kg. The uptake of Pb through the food chain is of little importance since the concentration of Pb in fish does not increase with trophic level and age but rises with increasing concentration in water (Oehlenschläger, 2002). Elevated Pb content in muscle tissue is reported only from areas with intensive industrial and agriculture activities and input of untreated

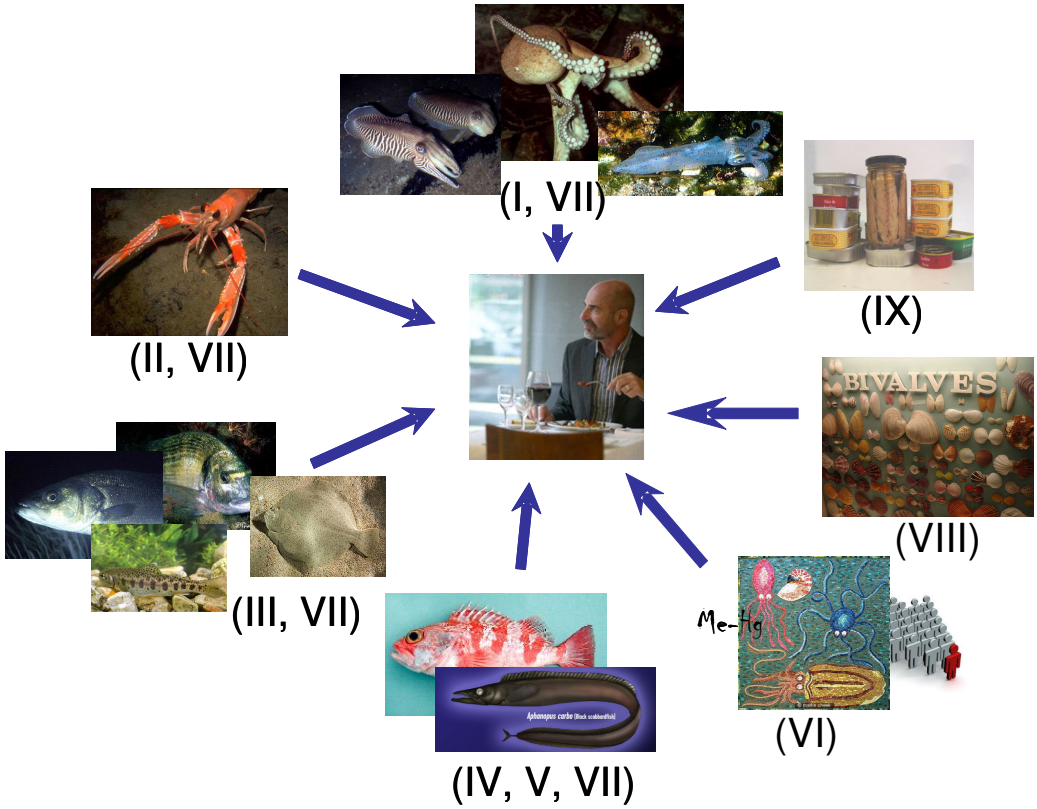
municipal and industrial waste waters (Oehlenschläger, 2002). In invertebrates as molluscs and crustaceans, Pb levels can be higher than in fish and can reach values of approximately 1 mg/kg. This is caused by an active accumulation of Pb in the digestive tract, hepatopancreas, of both molluscs and crustaceans. Like for Cd, the digestive tract of these kind of seafood has to be removed prior to consumption, preferably immediately after catch, to prevent a migration of Pb from intestine into muscle tissue.

3.3 Risk assessment of seafood consumption

The use of risk assessment is achieving progressive importance and recognition as a scientifically based approach to food safety and quality standards. According to Wal & Pascal (2000), the term "risk" can be defined as the probability of an adverse effect on a given organism, population or environment, as a result of exposure to a hazard. On the other hand, benefit is something that promotes or enhances well-being in the live organisms. To perform a risk assessment of a population that is exposed to certain food contaminants is necessary to understand and characterize this hazard, i.e., to determine their toxicological properties and establish the relationship between dose and adverse effects manifested (Nasreddine & Parent-Massin, 2002). The process of risk assessment can be divided into four stages: 1 - hazard identification, 2 - hazard characterization, 3 - exposure assessment, and 4 - risk characterization. The latter contains all the information obtained in the first three steps, performing qualitative and quantitative toxicological properties of an agent and its harmful effects on individuals exposed (Kroes *et al.*, 2002).

Today is generally accepted that seafood is important in a healthy and balanced human diet (WHO, 2003b) principally by the positive effects of omega-3 polyunsaturated fatty acids. However, seafood products are also a source of persistent chemical contaminants that accumulate in the marine food chain. Therefore, an increase in fish consumption to achieve an adequate intake of omega-3 polyunsaturated fatty acids may also increase the intake of these contaminants. Because of this phenomenon, a quantitative assessment of the intake of nutrients and contaminants via seafood consumption - and to ensuing health risk - have to be carried out in order to have more detailed information about the contribution of the various species to nutrient/contaminant intake (Sioen *et al.*, 2007). Actually, these nutritional-toxicological conflict related to seafood consumption has already been discussed at the Commission of the Codex on Contaminants in Food (Commission of the Codex Alimentarius) and a FAO/WHO expert consultation on the risks and benefits of seafood consumption was schedule in 2010, showing the pertinence of this subject (Sioen *et al.*, 2009). On the paper VI it is presented a mathematical-statistical framework for quantifying the probabilities of risk associated with seafood consumption. In addition, the estimation of the intake of MeHg is done through the cephalopod species as well as through their consumption in Portugal, and thus, the estimation of the portion of a given population at risk (probability of exceeding MeHg PTWI) is also performed.

4 DESIGN OF THE STUDY



Three of the studies included in this work were designed to characterize some seafood, not so well studied in Portugal, in what concerns proximate and elemental composition as well as to provide knowledge of their contribution to dietary reference intake (DRI) or tolerable upper intake level (UL) (I-III).

Elements, as Cd, Pb and Hg, considered non essential and harmful to humans were also quantified in the same seafood species and some deep water fish species (I-III, V). The relation of Se/Hg and correlations between elements and biological parameters were also studied (I-II, IV-V). The risk assessment, in terms of MeHg, in the cephalopods consumed in Portugal, was also analysed (VI).

In addition, three other papers deal with the As contents in some seafood consumed in Portugal (VII), toxic elements in bivalve molluscs (VIII) and the influence of canning in the concentration of toxic elements (IX).

5 MATERIAL AND METHODS

5.1 Biological material

All fishery products used in this study were purchased directly from outlets in the Lisbon area or in several commercial farms located in Portugal, obtained from cruise ships organized by the Instituto Nacional de Recursos Biológicos (INRB, IP / L-IPIMAR) under the project "Programa Nacional de Amostragem Biológica / Recolha de Dados" (PNAB/EU Regulation) and collected at various production areas localised along the Portuguese coastal zone and estuaries, without a periodicity established, between 2000 and 2007.

As sampling plays an important role in obtaining representative results, it was tried, whenever possible, to obey the Commission Directive 2001/22/EC of 8 March 2001 (EU, 2001) and more recently the Commission Directive 2005/4/EC of 19 January 2005 amending Directive 2001/22/EC (EU, 2005) and Commission Regulation (EC) No 333/2007 of 28 March 2007 (EU, 2007), laying down the sampling methods and the methods of analysis for the official control of the levels of Pb, Cd, Hg and 3-MCPD in foodstuffs.

Studied samples were several species of fish, including farmed ones, three cephalopods, several bivalve molluscs and one crustacean. Canned seafood was also studied. The common name, scientific name and the number of analysed samples are described in **Table 5.1**. The morphology, habitat and fishing mode are described in detail in **Annex A** of this thesis (Data collected from the databases, "FishBase", "CephBase" and also Sanchez (1992)). Seafood products studied were selected taking into account the higher consumption in our country and the fact that some of these species have been little studied in Portugal.

Table 5.1 - List of studied species.

Species	Product	Scientific name	Samples (number)
Bivalves*	11 species	-	554
Canned fish**	10 products	-	1682
Cephalopods	Cuttlefish	*Sepia officinalis*	10
	Octopus	*Loligo vulgaris*	10
	Squid	*Octopus vulg*aris	10
Crustacean	Norway lobster	*Nephrops norvegicus*	10
Deep-water fish	Blackbelly rosefish	*Helicolenus dactylopterus dactylopterus*	50
	Black scabbardfish	*Aphanopus carbo*	410
Farmed fish	Gilthead seabream	*Sparus aurata*	10
	European seabass	*Dicentrarchus labrax*	10
	Trout	*Oncorrinchus mykiss*	10
	Turbot	*Psetta maxima*	7

* See paper VIII; ** See paper IX.

All fishery products were transported from the place of purchase to the laboratory refrigerated or already frozen. Upon arrival at the laboratory, all specimens were weighed and measured according to its type and to the criteria described by Sanchez (1992). Within each species, it was analyzed the edible part of several specimens, individually whenever possible. Once homogenized, samples were placed in plastic bags, previously coded and frozen at -30 ° C until further analysis. Time is a factor that does not change the mineral composition, as they do not volatilize at room temperature or to usual temperatures in frozen storage (Nunes, personal presentation).

The laboratory equipment used in the analysis was washed and/or decontaminated (HNO_3 20%, v/v), taking into account the analysis to be undertaken and to avoid any contamination. The mill granulator (Retsch GM 200) used in the homogenization of the samples had a layer of titanium.

5.2 Methodologies

The techniques used at Unit "Valorização dos Produtos da Pesca e Aquicultura" (U-VPPA) of INRB, I.P./L-IPIMAR, are validated in accordance with the Guide RELACRE 2000 (RELACRE, 2000) and IPAC (Portuguese Institute for Accreditation) Guides including the Guidelines for Accreditation of Laboratories for Chemistry (2001, 2005). The chemical laboratory of the Unit U-VPPA, which hosted the techniques used is accredited by IPAC (2010) for determining moisture, mineral, fat, total Hg, Cd and Pb. The technique for determining the elements for EDFRX was validated by the laboratory of the Atomic Physics Centre (Experimental Atomic Physics Section) of Science College of Lisbon University (FCUL).

5.2.1 Proximate composition

The proximate composition - crude protein, fat, moisture and mineral contents - was determined according to AOAC (1998) methods.

5.2.1.1 Water

Dispersion of the sample, drying at a temperature of 105 ± 2 ºC and cooling until constant weight.

Weigh approximately 10 g of homogenized sample with the accuracy of ± 0.001 g (balance Mettler Toledo, AG 204) for a glass crystallizer previously weighed. Then dried at 105 ± 2 ºC overnight (oven Memmert, ULE 500). Next day withdrew from the oven the crystallizer to a desiccators and cool at least 30 minutes and weigh. Repeat this last operation until constant weight.

The water content of the product, expressed in grams per 100 g of the sample is given by:

$$100 - \left[\frac{m_3 - m_1}{m_2} \right] \times 100$$

where

m_1 - mass in grams, of the crystallizer;

m_2 - mass in grams, of the test sample;
m_3 - mass in grams, of all of the crystallizer and the test sample, after drying.

The result is calculated based on the determination of at least two tests on the same sample.

5.2.1.2 Crude protein

Sample digestion with sulphuric acid in the presence of a catalyst. Alkalinisation of the extract with excess sodium hydroxide. Steam distillation to release ammonia and receipt in a boric acid solution followed by titration with a hydrochloric acid solution. The total nitrogen content is calculated based on the quantity of ammonia produced. The crude protein content is calculated by multiplying the result by a conventional conversion factor of 6.25.

Weigh 0.5 to 1 g of homogenized sample with the accuracy of ± 0.001 g (balance Mettler Toledo, AG 204) to a filter paper for a Kjeldahl tube. Add a pellet catalyst (Kjeltab S/3,5) and 20 ml of concentrated sulphuric acid (Merck, 95-97 %, m/m). Place in the apparatus of digestion (Tecator, Digestion System 20 - 1015 Digester) and digest about 1 hour (310 ° C for 10 minutes and subsequently at 450 º C for 50 minutes). Cool the tubes and proceeded to the distillation of ammonia, according to the instructions of the distillation apparatus (Tecator, Kjeltec Auto - 1035 Analyser). The sample extract is alkalinized with excess sodium hydroxide solution (Merck, 40 % m/v), followed by steam distillation to release ammonia and receipt in a boric acid solution (Merck, 1 % m/v with indicator: mixture of methyl red and bromocresol green) and finally is titrate with a hydrochloric acid solution (Merck titrisol, 0.1 N). A blank test is perform, following the procedure described above, employing the same quantities of all reagents used, except the product to be analyzed.

The crude protein of the product, expressed in grams per 100 g of the sample is given by:

$$\left[\frac{14\times(V_a - V_b)\times N}{(m/1000)}\right]\times 100\times F$$

Where:
V_a - volume in millilitres of hydrochloric acid solution of known titre, spent in the sample titration;
V_b - volume in millilitres of hydrochloric acid solution of known titre, spent in the blank titration;
N - concentration, expressed as normality, of the hydrochloric acid solution;
m - mass in grams of test sample;
F - conversion factor of Kjeldahl nitrogen in protein (F = 6.25 for fishery products).

The result is calculated based on the determination of at least two tests on the same sample.

5.2.1.3 Fat

Extraction of fat in sample with ethylic ether. Removal of solvent by evaporation, drying and weighing.

Weigh 10 g of homogenized sample with the accuracy of ± 0.001 g (balance Mettler Toledo, AG 204). Add an equal amount of anhydrous sodium sulphate (Panreac) to the test sample and

transfer quantitatively to the extraction cartridge, drag all traces of sample with filter paper, which was also introduced into the cartridge. Cover it with filter paper, fat-free. Place the cartridge in a Soxhlet extractor. Place a volume of about 80 ml of ethylic ether (Panreac) into a round-bottom flask, previously dried in an oven for 30 minutes, cooled in desiccators and weighed, and put also solvent into extractor enough to cover the cartridge. Put the flask with the extractor in the heating battery (SBS, PC 6L) for 7 hours. After extraction, remove the flask and eliminate the solvent by using a hot plate (Schott-Geräte, CK 111) about 35 ° C. Dry the flask containing the extract in an oven (Memmert, ULE 500) for 30 minutes and, after cooling in desiccators, weigh the flask. Repeat these two last operations until constant weight.

The fat of the product, expressed in grams per 100 g of the sample is given by:

$$(m_3 - m_2) \times \frac{100}{m_1}$$

Where:

m_1 - mass in grams, of test sample;

m_2 - mass in grams, of round-bottom flask;

m_3 - mass in grams, of the round-bottom flask with the extract after drying.

The result was calculated based on the determination of at least two tests on the same sample.

5.2.1.4 Mineral

Drying the sample followed by carbonization and incineration at a temperature of 500 ± 25 ºC. Determination of the residue mass.

Weigh 5 g of homogenized sample with the accuracy of ± 0.001 g (balance Mettler Toledo, AG 204) to crucible, previously dried in an muffle furnace (Heraeus, MR 170E) for 30 minutes, cooled in desiccators and weighed previously. Put the crucibles for drying the sample in the oven (Cassel) to at least 100ºC overnight. Then, transfer the crucible to the muffle furnace, raising the temperature very slowly, until 500 ± 25 ºC. Let stand for 16 hours (overnight) for incineration. Remove the crucible from the muffle furnace, cool in desiccator and weigh. Perform incineration, cooling and weighing operations until two successive weightings do not differ by more than 1 mg.

The mineral content of the product, expressed in grams per 100 g of the sample is given by:

$$\frac{(m_3 - m_1)}{(m_2 - m_1)} \times 100$$

Where:

m_1 - mass in grams, of empty crucible;

m_2 - mass in grams, of crucible with test sample;

m_3 - mass in grams, of crucible with mineral residue.

The result is calculated based on the determination of at least two tests on the same sample.

5.2.2 α-Tocopherol

The content of α-tocopherol was determined by the method described by Piironen *et al*. (1984).

Extraction of fat from homogenized samples. Dissolution of the oil obtained in n-hexane followed by injection in High Pressure Liquid Chromatograph (HPLC).

The extraction of fat is based on the method described by Bligh & Dyer (1959). Solutions of methanol / chloroform (2:1) (99.8 %, m/m, Merck), saturated sodium chloride (99.5 % m/m, Merck) and Butylated hydroxytoluene (BHT - 99 % m/m, AnalytiCals) solution (50 g/l) with chloroform (99.8 %, m/m Merck) have to be cooled at 5 º C before use. Weigh about 12.5 grams (balance Mettler Toledo, AG 204) of the sample into a tube and added 37.5 ml of methanol / chloroform solution and stirred between two-seven minutes in the Ultra-Turrax (IKA, T25). After, add five ml of saturated sodium chloride solution and homogenized seven minutes in the Ultra-Turrax. Add 12.5 ml of chloroform with BHT and shake for 30 seconds to five minutes on the Ultra-Turrax. Then, add 12.5 ml of cold ultrapure water and homogenized 30 seconds to five minutes on the Ultra-Turrax. Put the sample in the ultrasonic bath (Sonorex, DK255P) for 10 minutes. Filter for a Kitasato, using a Buchner funnel. Transfer the filtrate to a separating funnel. Collect the organic phase (bottom) filtering with a filter paper over sodium sulphate anhydrous for pear-shaped flask previously weighed. Evaporate the chloroform in rotary evaporator (Büchi, RE 121) (bath at 40 ° C, 291 mbar.) Weigh the pear-shaped flasks. Collect a known amount of oil in a known volume of solution of BHT (50 mg/l) in n-hexane (≥ 98 %, m/m, Merck). Transfer the mixture (oil / BHT in n-hexane) to vials for injection. When necessary to concentrate the sample, evaporate the solvent with the help of an evaporator of samples (Reacti, Therm III) with a stream of nitrogen). Add 100-200 µl n-hexane and transferred to vials for injection with "insert".

For chromatographic analysis, inject 20 µl of the mixture into the HPLC system (Jasco, PU-980). Selected the wavelengths, excitation 292 nm and emission, 324 nm. Use as mobile phase a mixture of n-hexano/2-propanol (≥ 99.9 %, m/m, Merck) to 0.7% with a flow rate of 0.5 ml/minute.

Draw two calibration curves from the readings obtained for solutions 1, 2, 5, 8, 10, 15 and 20 and 8, 20, 40, 50, 100 and 200 µ of α-tocopherol (95 % m/m, Sigma) using as solvent n-hexane.

The α-tocopherol is identified by comparison with retention times of standards. The peaks are integrated using software Borwin in version 1.2. The calculation of α-tocopherol content, expressed in mg/100 g, is given by the relationship:

$$\frac{A}{10 \times m}$$

where:

m - mass in grams, of the test analysis;

A - α-tocopherol content expressed in g/ml.

The result is calculated based on the determination of at least two tests on the same sample.

5.2.3 Elemental composition

Techniques of Ultraviolet-Visible absorption spectrometry (UV-Vis), Atomic Absorption Spectrometry (AAS) and Energy Dispersive X-Ray Fluorescence (EDXRF) to analyse chemical elements were used. These techniques were chosen for being the most suitable for the elements studied in this work, and also because they are available in the laboratories where the experimental part of this thesis were done. Some operating conditions for all elements analysed by these techniques are shown in **Table 5.2** at the subchapter 5.2.3.6.

All reagents used had a high purity and ultrapure water was used (obtained by the system Milli-Q Plus Millipore).

5.2.3.1 Quantification of K, Na, Mg, Ca, Zn, Fe, Cu, Mn, Cr, Ni, Cd and Pb by flame atomic absorption spectrometry

The levels of K, Na, Mg, Ca, Zn, Fe, Cu, Mn, Cr, Ni, Cd and Pb were determined by flame atomic absorption spectrofotometry (FAAS), based on the methodology proposed by Jorhem (2000) and technical procedures in use in the Unit U-VPPA of INRB, IP / L-IPIMAR.

Incineration of the sample followed by dissolution in nitric acid. After dilution of the sample proceed to read the content of the element to be determined (K, Na, Mg, Ca, Zn, Fe, Cu, Mn, Cr, Ni, Cd and Pb) by flame atomic absorption.

First, it is necessary to obtain the ash of the sample. For that, weigh 5 g or 10 g of homogenized sample (5 g for K, Na, Mg, Ca, Zn, Fe, Cu and Mn and 10 g for Cr, NI, Cd and Pb) with the accuracy of ± 0.001 g (balance Mettler Toledo, AG 204) to quartz crucible. Put the crucible for drying the sample in the oven (Cassel) to at least 100°C overnight. Then, transfer the crucible to the muffle furnace (Heraeus, MR 170E), raising the temperature very slowly, until 500 ± 25 ºC. Let stand for 16 hours (overnight) for incineration. Remove the crucible from the muffle furnace and cool in desiccators. Then moisten the ash with nitric acid (65 % m/m, Merck) and evaporate carefully to dryness on a hot plate (Schott-Geräte, CK 111). The crucible is taken back to the oven at ± 400 °C for 20-30 minutes to obtain white ash.

In the case of K, Na, Mg, Ca, Zn, Fe, Cu and Mn add 6 ml of nitric acid 15% (v/v) hot to dissolve the ash and then transfer and filter (Macherey-Nagel 640 w, ∅ = 7 cm) to a 25 ml volumetric flask. Wash the crucible with 6 ml of the same acid and then with ultrapure water. Both washing solutions pass through the filter. Cool and volume with ultrapure water. In the case of Cr, Ni, Cd and Pb add 3 ml of 15% nitric acid to dissolve the hot ash and then transfer and filter to a 10 ml volumetric flask. The crucible was washed with 2 ml of the same acid and then with ultrapure water. Both washing solutions pass through the filter. Cool and volume with ultrapure water.

Read at the absorption wavelength corresponding to each element and record the maximum absorption signal obtained in the atomic absorption apparatus (Varian, Spectr AA 20).

For the blank, put equal volume of nitric acid to 15% without sample in the adequate volumetric flask and make up the final volume with ultrapure water.

To set up the calibration curve, prepare 100 ml of a standard concentration of 10 µg/ml from the standard solution (1000 mg/L, Merck) for each element, using nitric acid as solvent, 5% (v/v). From the solution of 10 µg/ml, also called the intermediate solution, prepare five standard solutions to use in the calibration curve (calibration solutions). Plot a calibration curve from the readings obtained for these solutions.

The calculation of the content of elements in mg/kg, wet weight is given by:

$$\frac{(A \times V)}{m}$$

Where:

A - reading in µg/ml;

m - mass in grams, of the test sample;

V - volume of the dissolution of the sample.

The result is given by the arithmetic mean of at least two parallel determinations.

5.2.3.2 Quantification of Cl, S, Br, Sr, Rb, Se and As by energy dispersive X-ray fluorescence

The content of various elements was determined by EDFRX, based on methodology described by Carvalho *et al.* (2005) and Custódio *et al.* (2005), in the laboratory of the Atomic Physics Centre of FCUL.

Use of a X radiation source to ionize internal levels of constituent atoms of the sample. In the reorganization of the atom and return to the ground state, these atoms can release the excess energy through the emission of a photon X, of energy equal to the difference in binding energy levels between which the transition occurred. This radiation is characteristic of the element. The detection and analysis of this spectrum allows the identification and quantification of constituents of the sample.

First, it is necessary to obtain the freeze-dried sample. For this, a portion of the frozen sample is distributed evenly in a Petri dish. It is placed on the freeze-dryer (Edwards, Modulyo Freeze-Dryer) for 48 hours (at a temperature of - 45 ºC and a pressure of about 10^{-1} atmospheres). After, homogenize the freeze-dried samples and again placed in plastic bags properly identified (species, number), vacuum packed and stored at - 20 ° C until further analysis.

Put the freeze-dried sample (a coffee spoon) in a cylinder and compress it into a press (Graseby, Speac) at a pressure of 10 Torr, forming a circular tablet of about 2 cm in diameter and 1mm thick. Glue the tablet to the Mylar film of the slide (50x50 mm). The choice of these materials is due to the fact that they have low atomic number and its characteristic radiation was not detected. Put the slide containing the sample tablet on a stand and link the equipment. This consists of a PW 1140 X-ray tube (100kV, 80mA; Phillips, Eindhoven) equipped with a changeable molybdenum secondary target and Si(Li) detector (Oxford, High Wycombe). The energy resolution is 135 eV at 5.9 keV and the acquisition system is a Nuclear PCA card (Oxford, High Wycombe). Expose each tablet to radiation during 1000 seconds. The spectrum is obtained by the Quantum MCA software.

From the spectra (energy versus intensity) determine the energy corresponding to each observed peak. Identify the existing elements in the sample by comparing these energies with the theoretical energies corresponding to different elements (Table of X-ray emission energies). After calculating the intensity of each radiation, the concentration of the detected elements is determined by the expression:

$$I_i = I_0 K_i c_i m C_i$$

Where:

I_i - X radiation intensity, characteristic of the element i;

I_0 - incident radiation intensity;

K_i - experimental calibration factor;

c_i - concentration of element i;

m - sample surface mass (g/cm^2);

C_i - damping factor.

The quantification is done by the software "XRF - Quantitative Calculation (fundamental parameters)" for Windows. The result of the content of each analyzed element, expressed in mg /kg wet weight, is calculated taking into account its content in mg/kg dry weight (given by the software) and moisture of each sample.

The result is given by the arithmetic mean of at least two parallel determinations.

5.2.3.3 Quantification of P by Ultraviolet-Visible absorption spectrometry

The content of P was determined by UV/Vis, based on methodology described in ISO 13730 (1996).

All equipment and utensils used in this technique should be free of residues of detergent containing phosphates. Materials have to decontaminate in a solution of 25% hydrochloric acid for 24 hours.

Drying the sample and the incineration of residue (500 ± 25 ºC). Acid hydrolysis of the ashes with nitric acid, filtration and dilution, followed by formation of a yellow compound after the addition of a mixture of ammonium monovanadate and ammonium heptamolybdate. Spectrophotometric measurement at 430 nm.

In this case it is necessary to have the ash of sample. For that, weigh 5 g of homogenized sample with the accuracy of ± 0.001 g (balance Mettler Toledo, AG 204) to crucible, previously decontaminated. Put the crucible for drying the sample in the oven (Cassel) at least 100ºC overnight. Then, transfer the crucible to the muffle furnace (Heraeus, MR 170E), raising the temperature very slowly, until 500 ± 25 ºC. Let stand for 16 hours (overnight) for incineration. Remove the crucible from the muffle furnace and cool in desiccators.

Reconstitute the ash obtained with 10 ml of HNO_3 (65 % 1 + 2 ultrapure water , v/v). Cover the crucible with a watch glass, put it on the hot plate (Schott-Geräte, CK 111), boil about 8 minutes. Cool and transfer to volumetric flask of 100 ml. Dilute with ultrapure water, mix and filter (Macherey-Nagel, GF-1, ∅ = 10 cm). Pipette a volume V of filtrate (not exceeding 20 ml)

for a volumetric flask of 100 ml, add 30 ml of colorimetric reagent (mix 1 volume of nitric acid solution with a volume of solution of ammonium monovanadate (2.5 g/L) and mix. Then add a volume of solution of ammonium heptamolybdate (50 g/L) and mix. The colorimetric reagent should be pale yellow and completely clear) make up the volume with ultrapure water and let stand at least 15 min at room temperature. Measure the absorbance of this solution at 430 nm (quartz cells of 1 cm optical path) in the spectrophotometer (UNICAM UV/Vis UV_2) and corrected with a blank.

For the blank, measure 2 ml of nitric acid solution and 30 ml of colorimetric reagent to a volumetric flask of 100 ml, dilute to volume with ultrapure water and mix.

To set up the calibration curve, measure 10, 20, 30, 40, 50 ml of standard phosphate solution (1000 mg/L, Merck) for volumetric flasks of 100 ml. Add 10 ml of nitric acid solution, dilute to volume with ultrapure water and mix. Next, measure 20 ml of each of these standard solutions of phosphate to 100 ml volumetric flasks, add 30 ml of colorimetric reagent (described above) make up the volume with ultrapure water and let stand at least 15 min at room temperature. These standard solutions are respectively the concentrations of 10, 20, 30, 40 and 50 µg of P_2O_5/ml. Measure the absorbance of these solutions in the spectrophotometer at 430 nm, corrected with the blank. Establish the calibration curve marking the values of absorbance measurements.

The calculation of phosphorus content, expressed as grams of phosphorus pentoxide per kilogram of wet weight sample is given by:

$$\frac{10 \text{ x } C}{m \text{ x } V}$$

where:

C = concentration of sample solution obtained from the calibration curve in µg/ml of P_2O_5;

m = mass of test portion in grams;

V = volume of filtrate used in the colorimetric determination in ml.

The result is given by the arithmetic mean of at least two parallel determinations.

5.2.3.4 Quantification of total mercury by cold vapour atomic absorption spectrometry

Total mercury (Hg_T) was determined by cold vapour atomic absorption spectroscopy (CVAAS) according to the method developed by Hatch & Ott (1968) and described by Joiris *et al.* (1991).

Digestion of the sample with sulphuric acid. Oxidation of Hg to mercuric ion with potassium permanganate in acid medium. Reduction of mercuric ion to elemental form with tin chloride. The vaporized Hg is put into circulation in the cell absorption spectrophotometer using a bubbler system. The readings are made at the absorption wavelength of 253.7 nm.

Weigh approximately 1 g of homogenized sample with accuracy of 0.001 g (balance Mettler Toledo, AG 204) to Erlenmeyer flasks of 150 ml. Add 20 ml of concentrated sulphuric acid (95-97 %, Merck) carefully and shaking slightly. Then place the Erlenmeyer flasks in an oven (Durocell, Model 55) set at 60 ± 2 º C for 6 hours. After cooling, place the Erlenmeyer flasks in a

tank with water, cooled with ice, and add about 25 ml (or more if necessary) of a solution of potassium permanganate (5 % m/v, Merck) to provide persistent purple colour. After a period 3-4 hours, transfer carefully to a 300 ml flask already containing 30 ml of distilled water. Add washings of Erlenmeyer flasks of 150 ml, until a total volume of 100 ml. After, add 5 ml of a solution of hydroxylamine hydrochloride (1.5 % m/v, Merck). After decolourization of the sample, add 2-3 ml of a solution of tin chloride (II) (20 % m/v in hydrochloric acid 20 % (v/v) Merck) and introduce the bubbler of the spectrometer (Bacharach, Coleman MAS 50D) into to the flask of 300 ml, quickly adjusting to the whole system and without leaks. Finally, there is the maximum absorption obtained by the equipment at 253.7 nm.

The blank is carried out on 100 ml of distilled water by adding a few drops of potassium permanganate, and proceeding in an identical manner as for a sample.

For the calibration curve, prepare intermediate Hg solutions of 10 µg/ml and 0.1 µg/ml in 0.5 M HNO_3, using the standard solution 1000 ug/ml (Merck). Draw two calibration curves obtained from the readings of 0.3, 0.4, 0.5, 0.8 and 1.0 ml and 1.0, 2.0, 3.0, 4.0 and 5, 0 ml, prepared from standard solution of 0.1 µg/ml, corresponding values of 0.03, 0.04, 0.05, 0.08 and 0.1 g/ml (low range) and 0.1, 0.2, 0.3, 0.4 and 0.5 g/ml (high range), respectively. The quantification is done by the software "MercuReport II", versão 1.00, XRF, 1994, Bacharach, Inc., USA, for Windows.

The calculation of the Hg_T content in mg/kg, wet weight is given by:

$$\frac{A}{m}$$

Where:

A - reading in µg;

m - mass in grams, of the test sample.

The result is given by the arithmetic mean of at least two parallel determinations.

5.2.3.5 Quantification of organic mercury by atomic absorption spectrometry

The determination of organic mercury (Hg_{Org}) was based on the method described by Scerbo & Barghigiani (1998).

Hydrolysis of the freeze-dried sample with hydrobromic acid followed by extraction of organic compounds with toluene. Removal of organic compounds of Hg using a solution of cysteine. Thermal and chemical decomposition of the sample (cysteine containing organic compounds of Hg) in the oven of the Hg analyser. Selective retention of Hg in an amalgam of gold then release after heating. Trawling of Hg vapour by oxygen through cell absorption spectrophotometer. Reading the absorption at a wavelength of 253.7 nm.

First, it is necessary to obtain the freeze-dried sample. For this, a portion of the frozen sample is distributed evenly in a Petri dish. It is placed on the freeze-dryer (Edwards, Modulyo Freeze-Dryer) for 48 hours (at a temperature of - 45 ºC and a pressure of about 10^{-1} atmospheres).

After, homogenize the freeze-dried samples and again placed in plastic bags properly identified (species, number), vacuum packed and stored at - 20 °C until further analysis.

Weigh about 200 mg of freeze-dried sample (balance Mettler Toledo, AG 204) to a centrifuge tube (FEP with screw cap of ETFE, Nalgene). Add 10 ml of hydrobromic acid (47 % m/m, Merck) and 20 ml of toluene (≥ 99 % m/m, Merck). Stir the mixture in a vortex (Heidolph, ReAX) for about 5 minutes and centrifuge (Sigma, 3K29) for 20 minutes at 3000 rpm. Remove 15 ml of organic phase and placed into another tube which was previously added 6 ml of cysteine solution (1 % L-cysteine hydrochloride, monohydrate in 12.5 % of sodium sulphate and 0.8 % of sodium acetate, all Merck). Add 15 ml of toluene to the tube containing the initial hydrobromic acid and repeat the process. Finally, shake (manually and vortex) the tube containing the solution of cysteine and toluene (30 ml) and centrifuge for 20 minutes at 3000 rpm. Transfer to another centrifuge tube 3 ml of cysteine for subsequent analysis. From this solution withdraw between 100 a 500 µl in a small boat. Put the small boat on Hg analyzer and read according to the instructions of the Hg analyser (LECO, AMA 254). The blank is carried out as described above except the step of weighing the sample that were not made. It is accepted the calibration curve introduced into the Hg analyzer software (0.10, 0.30, 1.00, 3.00, 10.00, 20.00, 30.00, 36.00 ng of Hg). To verify the calibration curve prepare one intermediate Hg solution of 10 µg/ml in 1% of HNO_3, using the standard solution 1000 mg/L (Merck). After, prepare two standard solutions of 0.1 µg/ml and 0.005 µg/ml to read in the mercury analyser.

The calculation of Hg_{Org} level, expressed as a percentage, is given by the relationship:

$$\left[\frac{\frac{6}{Va}\times(a-b)}{m}\times f\right]\times\left[\frac{100}{c}\right]$$

Where:

V_a - sample volume, in µl, analysed in the equipment;

a - concentration read on ng of the sample;

b - concentration read, on ng of the blank;

m - mass in grams, of the test analysis;

c - concentration read in mg/kg dry weight, of Hg_T ;

f - correction factor (f = 1.07)

The result is given by the arithmetic mean of at least two parallel determinations.

5.2.3.6 - Summary of operating conditions in the elemental composition analyses

In **Table 5.2** it can be observed some operating conditions in the elemental composition analyses.

Table 5.2 - Operating conditions in the elemental composition analyses.

Element	Technique	Apparatus	Conditions	Standard	Typical calibration curve	DL (mg/kg)	CRM* used
Br	EDXRF	Philips, PW 2184/00	-	-	-	0.8	-
Ca	FAAS	Varian, Spectr AA-20	λ = 422.7 nm; I = 10 mA, Slit = 0.5 nm	$Ca(N0_3)_2$,1000 mg/L, Merck	y = 0.2457x + 0.0073	0.08	LUTS-1 SMRD-2000
Cl	EDXRF	Philips, PW 2184/00	-	-	-	10	-
Cd	FAAS	Varian, Spectr AA-20	λ = 228.8 nm; I = 4 mA, Slit = 0.5 nm	$Cd(N0_3)_2$,,1000 mg/L, Merck	y = 0.1781x + 0.0003	0.01	TORT-2 DORM-2
Cr	FAAS	Varian, Spectr AA-20	λ = 357.9 nm; I = 4 mA, Slit = 0.2 nm	$Cr(N0_3)_3$,,1000 mg/L, Merck	y = 0.0805x + 0.0015	0.09	TORT-2 DORM-2
Cu	FAAS	Varian, Spectr AA-20	λ = 324.8 nm; I = 4 mA, Slit = 0.5 nm	$Cu(N0_3)_2$,1000 mg/L, Merck	y = 0.1239x + 0.0004	0.02	LUTS-1 DORM-2
Fe	FAAS	Varian, Spectr AA-20	λ = 248.3 nm; I = 5 mA, Slit = 0.2 nm	$Fe(N0_3)_3$, 1000 mg/L, Merck	y = 0.0813x + 0.0074	0.32	LUTS-1 SMRD-2000
Hg_T	CVAAS	Bacharach, MAS-50 D	λ = 253.7 nm	$Hg(N0_3)_2$ 1000 mg/L, Merck	$y = 3{,}348*10^{-4}x + 4.249*10^{-3}$	0.01	TORT-2 DORM-2
Hg_{Org}	AAS	LECO, AMA 254	λ = 248.3 nm; I = 5 mA,	$Hg(N0_3)_2$ 1000 mg/L, Merck	y = 31.87x + 0.0381		DORM-2 CRM-463
K	FAAS	Varian, Spectr AA-20	λ = 766.5 nm; I = 5 mA, Slit = 1 nm	$KN0_3$,1000 mg/L, Merck	y = 0.3888x - 0.0011	0.01	LUTS-1 SMRD-2000
Mg	FAAS	Varian, Spectr AA-20	λ = 285.2 nm; I = 4 mA, Slit = 0.5 nm	$Mg(N0_3)_2$, 1000 mg/L, Merck	y = 1.3149x + 0.0054	0.02	LUTS-1
Mn	FAAS	Varian, Spectr AA-20	λ = 279.5 nm; I = 5 mA, Slit = 0.2 nm	$Mn(N0_3)_2$, 1000 mg/L, Merck	y = 0.2036x + 0.0012	0.01	TORT-2 DORM-2
Na	FAAS	Varian, Spectr AA-20	λ = 589.0 nm; I = 5 mA, Slit = 0.5 nm	$NaN0_3$,1000 mg/L, Merck	y = 0.5863x + 0.0195	0.09	SMRD-2000
Ni	FAAS	Varian, Spectr AA-20	λ = 232.0 nm; I = 4 mA, Slit = 0.2 nm	$Ni(N0_3)_2$, 1000 mg/L, Merck	y = 0.0902x + 0.0004	0.02	LUTS-1 DORM-2
P	UV/Vis	Unicam, UV 5 220	λ = 430.0 nm	PO_4^{3-} 1000 mg/L, Merck	y = 0.0186x + 0.0008	0.01	SMRD-2000
Pb	FAAS	Varian, Spectr AA-20	λ = 217.0 nm; I = 10 mA, Slit = 1 nm	$Pb(N0_3)_2$, 1000 mg/L, Merck	y = 0.0368x + 0.0021	0.02	TORT-2 DORM-2
Rb	EDXRF	Philips, PW 2184/00	-	-	-	1.1	-

Continuation of **Table 5.2**

Element	Technique	Apparatus	Conditions	Standard	Typical calibration curve	DL (mg/kg)	CRM* used
S	EDXRF	Philips, PW 2184/00	-	-	-	10	-
Se	EDXRF	Philips, PW 2184/00	-	-	-	0.6	DORM-2 MA-A-2
Sr	EDXRF	Philips, PW 2184/00	-	-	-	0.5	TORT-2
Zn	FAAS	Varian, Spectr AA-20	λ = 213.9 nm; I = 5 mA, Slit = 1 nm	$Zn(N0_3)_2$, 1000 mg/L, Merck	y = 0.4484x + 0.01	0.06	TORT-2 DORM-2

* Certified Reference Material (results are shown in Papers).

5.2.4 - Handling of the data

5.2.4.1 Risk analysis

Toxic metals' risks to human health are assessed by comparing the weekly intake level with an appropriate safe exposure level, the PTWI for each toxic metal (Renwick *et al.* 2003). That is, assessing the probability of the intake level exceeding the respective PTWI value. There are two alternatives for estimating the risk; the plug-in (PI) and the tail estimation (TE) based estimators (Tressou *et al.* 2004). Both were used, the PI estimator for large probabilities and the TE estimator for the other situations (especially, for very low probabilities). The statistical methodology based on the extreme value theory (which has found application in various scientific fields, such as hydrology) was applied to the MeHg data of cephalopod species. This analysis is described in detail in the Paper VI and also in Cardoso *et al.* (2010b).

5.2.4.2 Statistical Analysis

The statistical treatment of the data was performed using the STATISTICA 6.1 software (Stat soft, Inc., Tulsa, OK74104, USA). The Kolmogorov-Smirnov or Lilliefors-test and the Levene´s test were used to check the normality and the homogeneity of variance of data, respectively (Zar, 1999). Data that corroborate these assumptions were analysed by Student t-test or single factor ANOVA analysis to evaluate the influence of some factors like sex or age groups on the concentrations of various elements, for each species. Comparisons were made using Kruskall-Wallis test when the conditions were nonparametric. In some cases data logarithmic transformation was applied to reduce the wide range of values. The Pearson correlation between elements of each species was determined. The significance level for statistical analyses was always tested at $\alpha = 0.05$ ($p < 0.05$). These analyses are described in detail in Papers I, II, III, IV, V and VII.

6 FINAL DISCUSSION

In this chapter it is intended to summarize the main results obtained on the selected species, which are versed in the nine papers incorporated in this thesis.

6.1 Cephalopods

Cephalopods are part of the traditional diet of coastal communities in southern European countries. In Portugal, they are important targeted species in fisheries, specially octopus, that has a high economic, social and culture value. The results obtained for proximate composition showed that they are a good source of protein (levels around 19%) and have an appreciable mineral content (about 2%). The fat level was very low (<1%) and consequently they can be considered lean species. These results are similar to those observed by other authors (Favier *et al.* 1995; Bandarra *et al.*, 2005). From paper I, it can be concluded that they are a good source of essential elements (mainly S, P, Zn and Cu) and, in general, their contents are in the same order of magnitude as those reported by other authors (**Table 6.1**). However, in the case of Zn, Fe and Cu, values may be different due to different sizes of the specimens and also due to different testing methodologies. Most part of the elements can be assumed to be incorporated by the diet because cephalopods are know to be carnivorous, active predators and have very high feeding rates (Villanueva & Bustamante, 2006). This may explain the high values of almost essential elements, particularly in the case of the octopus. Octopuses usually consume crustaceans (Smith, 2003), like crabs, that contain high levels of these elements. However, absorption also takes place from seawater. Until know, no data on the respective proportions of the elements incorporated from food and seawater has been published for cephalopods. Nevertheless, a similar behaviour to that of fish is expected to have place in cephalopods. Among the three cephalopods, squid shows the lowest concentrations of most essential elements. According to some authors (Guerra & Rocha, 1994; Coelho *et al.*, 1997) fish is always the main component of the squid diet, representing about 90 % of the prey found in their stomachs. This may explain the low values of essential elements in this species, since fish contains fewer minerals than crustaceans, preferred prey of the octopus. Unlike fish, K in cephalopods is low, and in the case of the octopus, is below the level of Na. In general, Na contents are considerably higher in shellfish than in finfish (Vlieg *et al.*, 1991). Thus, the ratio Na/K in octopus is high (2.6), which can be seen as an increased risk of developing cardiovascular disease and high blood pressure (Astorga España *et al.*, 2007).

Although the levels of essential elements are lower in squid, the three studied species can have a good contribution to the DRI of these elements, especially with regard to the Cu, S, P, Cl, Zn and Mg (**Table 6.2**), considering the DRI for adults.

Taking into consideration the limits set by EU (2006, 2008) for Hg, Pb and Cd, the mean levels detected of these toxic elements in cephalopods were always lower, although some specimens of octopus and cuttlefish (about 10 %) has shown high levels of Cd. This occurs very often in cephalopods, since these animals have the ability to concentrate Cd in the

Table 6.1 - Concentration of macro elements (K, Na, P, S, Mg and Ca) expressed as g/kg (wet weight), trace elements (Zn, Fe and Cu), ultra trace elements (Mn, Ni and Se) and toxic elements (As, Cd, Hg and Pb) expressed as mg/kg (wet weight) as reported in the literature for cephalopod species.

Cephalopods	Geographical area	K	Na	P	S	Mg	Ca	Zn	Fe	Cu	Mn	Ni	Se	As	Cd	Hg	Pb	Reference
Species:																		
Loligo vulgaris	Mediterranean Sea, Spain	2.6	3.9	2.2	4.7	0.62	0.36	12.4	15.2	10.1	0.49	< 0.1	-	3.1	0.06	0.04	0.12	Villanueva & Bustamante (2006)
Loligo vulgaris	Atlantic Ocean, Portugal	2.6	1.6	2.6	2.3	0.44	0.14	12.6	1.7	1.5	0.16	0.02	< 0.4	4.0	0.04	0.05	0.10	Present work
Octopus vulgaris	Atlantic Ocean, Spain	-	-	-	-	-	-	12.1	5.7	6.3	0.39	-	-	-	-	-	-	Soldevilla *et al.* (1987)
Octopus vulgaris	Atlantic Ocean, Portugal	-	-	-	-	-	-	10-60	9	0.8-40	0.3-0.5	-	0.2-0.4	8-26	Nd*-4	0.03-0.09	0.6-0.8	Seixas *et al.* (2005)
Octopus vulgaris	Atlantic Ocean	-	-	-	-	-	-	15.2	5.4	7.2	0.3	0.4	0.3	10.8	-	-	-	Napoleão *et al.* (2005)
	Portugal (3 areas)	-	-	-	-	-	-	13.6	4.6	5.0	0.4	0.3	0.3	11.2	-	-	-	
		-	-	-	-	-	-	12.0	4.8	5.2	0.3	0.3	0.3	6.6	-	-	-	
Octopus vulgaris	Mediterranean Sea, Spain	3.3	5.2	1.9	6.1	0.80	0.51	31.4	35.1	37.0	0.9	0.3	-	24.1	0.39	0.07	0.39	Villanueva & Bustamante (2006)
Octopus vulgaris	Atlantic Ocean, Portugal	2.5	-	-	-	-	0.14	14.2	21.8	2.0	-	0.05	0.22	-	0.02	0.10	0.01	Carvalho *et al.* (2005)
Octopus vulgaris	Atlantic Ocean, Portugal	2.2	5.7	1.5	2.6	0.94	0.21	17.7	4.2	3.8	0.31	0.02	< 0.4	25.9	0.38	0.13	0.02	Present work
Sepiotheutis bilineata	Pacific Ocean, New Zealand	2.5	2.2	2.1	2.4	0.41	0.13	9.2	3.6	1.5	0.14	-	-	3.99	-	-	-	Vlieg *et al.* (1991)
Sepia officinalis	Atlantic Ocean, France	-	-	-	-	-	-	14.1	3.2	2.1	0.11	0.04	-	-	0.02	-	0.04	Miramand & Bentley (1992)
Sepia officinalis	Mediterranean Sea, Spain	3.3	4.2	2.4	5.8	0.88	10.4	29.2	84.0	47.5	1.4	< 1.3	-	17.7	0.22	0.12	0.27	Villanueva & Bustamante (2006)
Sepia officinalis	Atlantic Ocean, Portugal	2.9	2.7	2.5	3.4	0.57	0.13	17.7	1.4	4.5	0.11	0.05	< 0.4	19.8	0.31	0.15	0.04	Present work

* Nd - Not detected.

Table 6.2 - Contribution (%) of essential elements in studied seafood (160 g portion) for DRI.

	Cephalopods (%)			Crustacean (%)	Farmed fish (%)			
DRI * (mg/day)	**Cuttlefish**	**Octopus**	**Squid**	**Norway lobster**	**European seabass**	**Trout**	**Turbot**	**Gilthead seabream**
K (4700)	10.9	7.6	8.9	11.9	17.7	17.6	11.1	15.9
Na (1500)	28.1	61.1	16.7	46.0	11.3	18.3	9.2	12.8
Cl (1800)	39.0	55.9	23.2	40.9	-	-	-	4.9
P (700)	56.7	34.6	58.5	51.9	56.9	59.2	40.2	61.9
S (800)	67.5	51.3	45.4	30.5	-	-	-	33.2
Mg (310)	29.3	48.4	22.5	30.7	19.1	19.1	12.4	17.5
Ca (1000)	2.2	3.4	2.2	15.3	4.6	4.5	1.8	3.7
Cu (0.9)	72.2	67.6	27.5	**179.4**	8.2	7.5	3.0	12.6
Fe (8)	2.6	8.4	3.3	39.2	10.0	8.9	5.2	9.6
Zn (8)	35.1	35.3	25.3	29.9	10.4	12.6	13.6	9.8
Cr (0.025)	-	-	-	73.6	**198.4**	**147.2**	**179.2**	**198.4**
Mn (1.8)	0.9	2.8	1.5	6.3	2.7	1.6	2.8	1.2
Ni (0.5)	1.6	0.6	0.6	4.0	1.3	0.6	0.6	1.3

* DRI - estimated for adults (IOM, 2005).

in mantle and arms, in addition to digestive gland (Bustamante *et al.*, 1998; Miramand & Bentley, 1992). The levels of these metals in squid samples, especially Cd, were always very low. This different behaviour is probably due to their diet and also to the different habitat. Mean values of As presented similar pattern: octopus showed the highest values and squid showed the lowest ones (VII), possibly due to the same facts. Taking into account the different PTWIs, the consumption of cephalopods does not pose a hazard to human diet, albeit moderation is recommended in the case of cuttlefish and octopus (**Table 6.3**) for which four or five meals per week should not be achieved taking into consideration the Hg_{Org} levels.

The results allow to draw a general recommendation for the consumption of these species **(Fig. 6.1**).

6.2 Crustacean

Crustaceans have been lauded for their health promoting characteristics. Norway lobster had a low fat and high protein and mineral contents, with a low energy value. These characteristics make this species interesting from a nutritional standpoint. Generally, other crustaceans, as blue crab and Atlantic spider crab, showed a similar proximate composition (Gökoðlu & Yerlikaya, 2003; Küçükgülmez *et al.*, 2006; Marques *et al.*, 2010). They are nutritional valuable sources of various minerals (Küçükgülmez *et al.*, 2006) as can be seen in **Table 6.4**. Related publications on these elements concentrations in similar crustacean species also reveal high values of essential elements, however there are some differences. Due to Ca metabolism in

Table 6.3 - Estimated weekly intake (µg/kg BW) of toxic elements in studied seafood (160 g portion per day, 60 kg BW) and number maximum of meals per week.

	EDI*				Number maximum of meals per week			
Seafood	**Cd**	**Hg_T**	**Hg_{Org}**	**Pb**	**Cd**	**Hg_T**	**Hg_{Org}**	**Pb**
Cephalopods:								
Cuttlefish	5.8	2.8	**2.5**	0.7	> 7	> 7	**4**	> 7
Octopus	**7.1**	2.4	**2.1**	0.4	**7**	> 7	**5**	> 7
Squid	0.7	0.9	0.8	1.9	> 7	> 7	> 7	> 7
Crustacean:								
Norway lobster	1.9	**7.5**	**5.8**	0.9	> 7	**4**	**1**	> 7
Farmed fish:								
Gilthead seabream	0.2	2.1	**1.7**	0.6	> 7	>7	**6**	> 7
Seabass	0.2	2.6	-	0.4	> 7	>7	-	> 7
Trout	0.2	1.1	-	0.9	> 7	>7	-	> 7
Turbot	0.2	1.1	-	0.9	> 7	>7	-	> 7
Deep-water fish:								
Blackbelly rosefish	0.2	**12.3**	**10.6**	0.4	> 7	**2**	**1**	> 7
Black scabbardfish (SZ)	0.4	**12.9**	**11.1**	0.7	> 7	**2**	**1**	> 7
Black scabbardfish (MA)	0.4	**27.1**	-	0.7	> 7	**1**	-	> 7
Black scabbardfish (AA)	0.4	**13.3**	-	0.9	> 7	**2**	-	> 7
Bivalves:								
Furrow shell	0.6	0.7	-	**26.1**	> 7	> 7	-	**6**
Mussel	2.4	0.4	-	3.7	> 7	> 7	-	> 7
Portuguese oyster	**6.9**	0.7	-	1.9	**7**	> 7	-	> 7
Canned seafood:								
Tuna	0.7	**5.2**	-	1.9	> 7	**6**	-	> 7
Sardine	0.4	0.6	-	3.7	> 7	> 7	-	> 7
Octopus	0.9	2.6	-	1.9	> 7	> 7	-	> 7
Squid	5.4	1.3	-	1.9	> 7	> 7	-	> 7
Mussel	3.0	0.6	-	5.6	> 7	> 7	-	> 7

*EDI - estimated weekly intakes; Bold numbers indicate higher EDI than the proposed PTWI.

crustaceans being much more active than other invertebrates (due to the moult) the levels of this metal are considerably high. The skeleton needs to shed regularly to allow increase in body size (Greenaway, 1985). High levels of Cu, Fe and Zn are also found in this species (however lower than the levels referred for other species). This is probably due to the fact that Norway lobster is a species that feeds on benthic sediments that are generally rich in trace elements. High levels of Cu are probably due to the requirement of this element by the respiratory pigment

Octopus
(*Octopus vulgaris*)

Cuttlefish
(*Sepia officinalis*)

Squid
(*Loligo vulgaris*)

Main Facts

Benefits

- Low fat
- Low energy value
- High levels of S, P, Mg, Zn and Cu
- Low levels of Pb

Hazards

- High levels of Cd and Hg

Advisable consumption
Up to 4 meals per week

Figure 6.1 - General recommendations for cephalopods consumption. (Picture source sites: Marine Life Information Network (a,b); Cefalópodos; As receitas da Amélie)

haemocyanin in crustaceans. According to the levels of essential elements found, this crustacean is a good contribution for the DRI of most essential elements (**Table 6.2**). However, the level of Cu greatly exceeds the DRI. Nevertheless, there is no problem in relation to consumption of this species because the estimated UL for Cu (IOM, 2005) is 10mg/day and the calculated value in this thesis is 1.7 mg/day (a meal of 160 g).

Regarding toxic elements (II), Norway lobster showed low mean values of Pb and Cd, inferior to EU limits (EU, 2006, 2008), being the estimated weekly intake, based on a 160 g portion per day and a person of 60 kg BW, also lower than the respectively PTWI (**Table 6.3**). Nevertheless, this species presented high levels of Hg (total and organic - about 78 % of total) and As (II, VII). These contents of Hg and As can be attributed to its habitat. In fact, this species usually lives in muddy sediments that always contain high levels of these elements and furthermore this species is considered a scavenger (Barghigiani *et al.*, 2000). In this case, EDI of Hg_T and Hg_{Org} exceeded the PTWI.

Table 6.4 - Concentration of macro elements (Cl, K, Na, P, S, Mg and Ca) expressed as g/kg (wet weight), trace elements (Zn, Fe and Cu), ultra trace elements (Mn, Ni, Cr and Se) and toxic elements (As, Cd, Hg and Pb) expressed as mg/kg (wet weight) as reported in the literature for crustacean species.

Crustacean	Geographical area	Cl	K	Na	P	S	Mg	Ca	Zn	Fe	Cu	Mn	Ni	Cr	Se	As	Cd	Hg	Pb	Reference
Species:																				
Homarus americanus	Atlantic Ocean, USA and Canada	4.4	2.6	3.2	-	1.7	0.25	0.47	29	2.3	10	0.61	-	-	0.85	11	0.02	0.10	0.10	Barrento *et al.* (2008)
Homarus gammarus	Atlantic Ocean, Scotland	4.0	2.6	2.0	-	1.7	0.22	0.46	26	1.8	9.6	0.40	-	-	0.56	17	0.02	0.15	0.10	Barrento *et al.* (2008)
Jasus edwardsii	Pacific Ocean, New Zealand	-	4.4	2.4	3.1	2.2	0.40	0.23	19	9.2	9.0	-	-	-	-	3.7	-	-	-	Vlieg *et al.* (1991)
Maja brachydactyla	Atlantic Ocean, Scotland	4.9	2.3	4.0	-	2.4	0.61	0.79	62.8	10.7	11.9	0.6	Trace	-	0.9	24	< 0.02	0.10	< 0.01	Marques *et al.* (2010)
Nephrops norvegicus	Ligurian Sea, Italy	-	-	-	-	-	-	-	13	-	6	1.4	-	-	-	-	0.14	0.68	0.82	Capelli *et al.* (1983)
Nephrops norvegicus	Aegean Sea, Greece	-	8.0	-	-	-	0.19	0.25	22	48	4	1.0	1.0	1.0	-	-	0.1	-	1.0	Karakoltsidis *et al.* (1995)
Nephrops norvegicus	Atlantic Ocean, Portugal	4.7	3.4	4.4	2.3	1.4	0.6	1.0	17.4	20.0	10.6	0.75	0.16	0.13	< 0.4	32.2	0.10	0.40	0.05	Present work
Panulirus inflatus	Gulf of California, Mexico	-	-	-	-	-	-	-	31.1	9.6	13.8	0.4	0.4	0.11	-	-	0.08	-	-	Páez-Osuna *et al.* (1995)
Portunus pelagicus	Kuwait Bay, Kuwait	-	-	-	-	-	-	-	60	-	31	0.39	-	0.12	0.04	-	-	-	0.48	Al-Mohanna & Subrahmanyam (2001)
Scylla serrata	Mahanadi estuary, India	-	2.3	-	-	-	-	0.72	74	41	31	3.0	-	-	0.11	-	-	-	0.05	Mohapatra *et al.* (2009)

Having regard to the percentage value obtained for the Hg_{Org}, consumption do not have to exceed a meal of Norway lobster per week. In spite of high levels of As, several authors (Ackley, et al., 1999; Muñoz *et al.*, 2000; Cava-Montesinos *et al.*, 2005) and also FDA (1993c) reported that most of the As compounds found in seafood are methylated, and therefore are relatively low in toxicity and do not present a concern to the consumer. In **Figure 6.2** it can be observed a draw of general recommendations for the consumption of this crustacean.

Norway lobster
(*Nephrops norvegicus*)

Main Facts

Benefits

- Low fat
- High protein
- High levels of P, Mg, Ca, Zn, Fe and Cu
- Low levels of Cd and Pb

Hazards

- High levels of Hg

Advisable consumption
Up to 1 meal per week

Figure 6.2 - General recommendations for Norway lobster consumption. (Picture sourece sites: Marine Life Information Network (c); Cozinha da Zela)

6.3 Farmed fish

Actually, farmed fish offers an alternative solution to the increasing market demand for fish and fish protein. The results obtained in this thesis confirmed this statement. They have a protein content of about 20 %, which is considered a good value for fishery products (Belitz, 2004). Fat levels where different among the four studied species ranging between 4 and 12 % (trout and European seabass, respectively). These dissimilar values are attributed to the different farm systems and feeds composition. They have a similar mineral content to those obtained in other studies performed in the same species (**Table 6.5**). The profile of main

Table 6.5 - Concentration of macro elements (K, Na, P, Mg and Ca) expressed as g/kg (wet weight), trace elements (Zn, Fe and Cu), ultra trace elements (Mn, Ni, Cr and Se) and toxic elements (As, Cd, Hg and Pb) expressed as mg/kg (wet weight) as reported in the literature for farmed fish species.

Farmed Fish	Country	K	Na	P	Mg	Ca	Zn	Fe	Cu	Mn	Ni	Cr	Se	As	Cd	Hg	Pb	Reference
Species:																		
Dicentrarchus labrax	Greece	-	-	-	-	-	9.0	10.24	0.77	1.45	0.98	0.03	-	-	0.05	-	0.2	Alasalvar *et al.* (2002)
Dicentrarchus labrax	Italy	-	-	-	-	-	3.35	-	1.17	-	-	-	0.19-0.51	-	0.09	-	0.18	Dugo *et al.* (2006)
		-	-	-	-	-	2.63	-	0.88	-	-	-	0.23-0.35	-	Nd*	-	0.12	
Dicentrarchus labrax	Turkey	4.6	0.8	3.7	0.32	0.64	2.8	24.7	-	0.547	-	-	0.282	-	-	-	-	Erkan & Özden (2007)
Dicentrarchus labrax	Spain	-	-	-	-	-	1.68	1.73	0.27	0.06	-	-	-	-	-	-	-	Fuentes *et al.* (2010)
	Greece	-	-	-	-	-	2.34	1.10	0.29	0.08	-	-	-	-	-	-	-	
Dicentrarchus labrax	Portugal	5.2	1.1	2.5	0.37	0.29	5.2	5.0	0.46	0.30	0.04	0.31	-	-	< 0.01	0.14	0.02	Present work
Oncorhynchus mykiss	Turkey	3.1	0.5	3.4	0.41	0.63	9.68	2.10	0.33	0.78	-	-	-	-	-	-	-	Gökoğlu *et al.* (2004)
Oncorhynchus mykiss	Portugal	5.2	1.7	2.6	0.37	0.28	6.3	4.5	0.42	0.18	0.02	0.23	-	-	0.01	0.06	0.05	Present work
Psetta maxima	Spain	-	-	-	0.25	0.99	8.25	1.15	0.27	0.31	-	-	0.33	-	-	-	-	Aubourg *et al.* (2007)
Psetta maxima	Portugal	3.2	0.9	1.8	0.24	0.11	6.8	2.6	0.17	0.32	0.02	0.28	-	-	0.01	0.06	0.05	Present work
Sparus aurata	Turkey	3.9	0.3	3.6	0,22	0.19	1.08	225	-	6.44	-	-	0.236	-	-	-	-	Erkan & Özden (2007)
Sparus aurata	Portugal	4.7	1.2	2.7	0.34	0.23	4.9	4.8	0.71	0.14	0.04	0.31	< 0.4	4.0	0.01	0.11	0.03	Present work

* Nd - not detected.

elements was analogous for all the species (III): K>P>Na>Mg>Ca, showing a low Na/K (≈ 0.3). The profile of trace and ultrace elements was somewhat different, mainly in what regards Cu, Cr and Mn. Turbot was the species that revealed the lowest mineral content. The apparent reason for this difference seems to be the diet. Some species contributed to more than 50 % for P, 19% for Mg and 17 % for K DRIs (European seabass and trout) (**Table 6.2**). Chromium percentages of farmed fish are above the DRI (more than 100 %). However, according to the Council for responsible Nutrition (CRN) (Hathcock, 2004), Cr supplements at levels up to 1,000 µg per day are regarded as safe for adults. Such limit is above the results found in this study (maximum: 0.05 mg Cr in a portion of 160 g). Considering the contaminant elements (As, Cd, Hg and Pb), farmed fish showed low values (III, VII). Thus, estimated weekly intakes were consistently lower than the PTWI set by WHO/FAO (WHO, 1999, 2003a), except for Hg_{Org} (only analyzed in samples of gilthead seabream). However, there seems to be no problem because it is possible to get six meals without reaching the PTWI, which is unlikely to accomplish. A general draw of consumption recommendation can be seen in **Figure 6.3**.

Gilthead seabream (*Sparus aurata*)

European seabass (*Dicentrarchus labrax*)

Rainbow trout (*Oncorhynchus mykiss*)

Turbot (*Psetta maxima*)

Main Facts

Benefits

- High protein
- Low Na/K
- High levels of K, P and Mg
- Low levels of Cd and Pb

Hazards

- Possible high levels of Hg_{Org}

Advisable consumption

Up to 6 meals per week

Figure 6.3 - General recommendations for farmed fish consumption. (Picture source sites: Acquario Marino Mediterraneo; El maestro Pescador; ZipcodZoo; Wikipedia Commons; Leonardo Gama)

6.4 Deep-water fish

Deep-water fish species are generally predators with a long life span that tend to make them accumulate more toxic metals, especially Hg, than other marine species. Blackbelly rosefish and black scabbardfish studied in this thesis (IV-V) showed high levels of Hg_T. Comparing the observed values with those of other authors (**Table 6.6**) it is verified that, in general, the levels of Hg_T are similar for black scabbardfish although the geographic area is different. With regard to blackbelly rosefish, values obtained in this study were higher than those recorded in studies conducted by Monteiro *et al.* (1991) and Andersen & Depledge (1997). This finding may be due to the fact that the specimens studied are larger than those in the two other studies. Organic mercury levels were about 86 % of the Hg_T for both species. Similar percentages for other deep-water fish species were obtained by others (Andersen & Depledge, 1997; Storelli *et al.,* 2003, 2005; Magalhães *et al*., 2007). Significant positive correlations between Hg_T and length were found, which is in agreement with the results presented by Monteiro *et al.* (1991), Andersen & Depledge (1997) and Bebiano *et al.* (2007). It is also noted that the levels of Hg_T observed for black scabbardfish caught off the Azores and Madeira were significantly higher than those caught off the mainland. This is mainly due to the fact, of Azores and Madeira being volcanic regions and for that a potential source of Hg. Given the high levels of Hg, the molar ratio of Se:Hg was determined and the values were superior to one. Such value indicated that the absorption of Hg can be counteract by Se. Similar ratios were reported (Andersen & Depledge, 1997; Plessi *et al*., 2001; Cabañero *et al*., 2004). According the limits fixed by EU (2006, 2008) for Hg_T (1.0 mg/kg for black scabbardfish and 0.50 mg/kg for blackbelly rosefish), it was found that some specimens showed concentrations above the limits (30 % and 70 %, respectively). Taking into consideration these results, estimated weekly intakes of Hg_T and Hg_{Org} exceeded the PTWI (**Table 6.3**) and the consumption of the two species should not exceed one meal per week. On the other hand, mean levels of Cd and Pb in muscle sampleswere lower than the limits proposed by EU (2006, 2008) (data not showed for blackbelly rosefish). This pattern was also verified by other authors (**Table 6.6**). Total arsenic for both species were considered low when compared with other species (VII) and are in accordance with Mormede & Davies (2001). Thus, these latter three elements do not pose any problem to the consumption of these two species (**Table 6.3**). A draw of general recommendations for consumption of both species, is given in **Figure 6.4**.

6.5 Bivalves

Bivalves, as the other marine organisms, are a good source of nutrients. The consumption of some species provides a source of protein for human consumption (Astorga España *et al*., 2007). But, on the other hand, they are filter or deposit feeders; therefore, they can absorb toxic elements not only from food and water, but also from inorganic particulate materials they ingest (Özden *et al*., 2009). For that reason, many researchers have reported the potentiality of using bivalves as bioindicators or biomarkers for monitoring toxic metals contaminations in aquatic

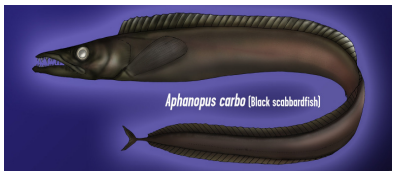
Table 6.6 - Concentration of toxic elements (As, Cd, Hg and Pb) expressed as mg/kg (wet weight) as reported in the literature for deep-water fish species.

Deep-water fish	Geographical area	As	Cd	Hg	Pb	Reference
Species:						
Aphanopus carbo	Atlantic Ocean, Portugal					
	Azores	-	0.03	0.89	0.04	Afonso *et al.* (2007)
	Madeira	-	0.01	0.90	0.02	
Aphanopus carbo	Atlantic Ocean, Portugal Madeira,	-	-	0.30-2.34	-	Bebiano *et al.* (2007)
Aphanopus carbo	Atlantic ocean, Scotland	-	0.004	-	0.009	Mormede & Davies (2001)
Aphanopus carbo	Atlantic Ocean, Portugal Madeira	-	-	0.4-1.9	-	Renzoni *et al.* (1998)
Aphanopus carbo	Atlantic Ocean, Portugal					
	Azores	-	< DL*	0.71	0.05	Present work
	Madeira	-	< DL	1.45	0.04	
	Mainland	2.9	0.02	0.69	0.04	
Lophius piscatorius	Atlantic Ocean, Scotland	8.6	< 0.002	-	< 0.002	Mormede & Davies (2001)
Helicolenus dactylopterus	Atlantic Ocean, Portugal Azores	-	-	0.260	-	Andersen & Depledge (1997)
Helicolenus dactylopterus	Atlantic Ocean, Portugal		0.01	0.55	Nd**	Carvalho *et al.* (2005)
Helicolenus dactylopterus	Atlantic Ocean, Portugal Azores	-	-	0.29	-	Monteiro *et al.* (1991)
Helicolenus dactylopterus	Atlantic Ocean, Portugal	4.4	0.01	0.66	0.02	Present work
Hoplostethus atlanticus	Atlantic Ocean, Scotland	-	0.010	0.42	0.010	Cronin *et al.* (1998)

* DL - Detection Limit; ** Nd - Not detected.

system (Liang *et al.*, 2004; Özden *et al.*, 2009). The results obtained in this thesis (VIII) illustrated the contamination level of production areas localised along the Portuguese coastal zone and estuaries. Total Hg levels were always very low (below 0.1 mg/kg) being the highest values observed in bivalves from the Tagus estuary. However, the concentrations found are well below the limit proposed by the EU, 0.5 mg/kg (2006, 2008). Similar values were found in other papers (**Table 6.7**). Furthermore, bivalves do not normally contain high concentrations of Hg because they are low on the trophic chain and have a short life cycle (Gutiérrez *et al.*, 2006).The results also leads to the conclusion that the areas studied are not be sources of anthropogenic Hg. The same can not be said with regard to Pb and Cd. Some samples of

Blackbelly rosefish
(*Nephrops norvegicus*)



Black scabbardfish
(*Aphanopus carbo*)

Main Facts

Benefits

- High protein
- Low fat
- High levels of K, Mg and Se
- Low levels of Cd and Pb

Hazards

- High levels of Hg

Advisable consumption
Up to 1 meal per week

Figure 6.4 - General recommendations for deep-water fish consumption. (Picture source sites: Fishbase; Cozinha sem tabus)

furrow shell of the Tagus estuary and some oysters of the Sado estuary showed very high levels of Pb and Cd, respectively (above the limits set for these species). Like otherestuaries, Tagus and Sado ones, are most intensively used and one of the most vulnerable coastal areas. They receive a multitude of inputs from point and diffusive sources (Blasco *et al.*, 1999, França *et al.*, 2005). Thus, these results indicate some pollution in these two estuaries, which is reflected in the bivalves living inside these areas. Furthermore, the furrow shell is considered a species that is a burrowing and a deposit-feeding bivalve, which may explain the high levels of Pb. Oysters are suspension feeders and can uptake metals from the re-suspended sediments, thus the high levels of Cd indicate that the sediments may contain high contents of this metal and, probably, the phytoplankton and zooplankton in the Sado estuary are also contaminated. The levels for both elements, reported by other authors for the same species, show great variability (**Table 6.7**). These dissimilarities indicate a different degrees of pollution in the different coastal and estuarine areas of the World. However, this accumulation of metals is also

Table 6.7 - Concentration of toxic elements (Cd, Hg and Pb) expressed as mg/kg (wet weight) as reported in the literature for bivalve mollusc species.

Bivalves	Geographical area	Cd	Hg	Pb	Reference
Species:					
Cerastoderma edule	Orwell and Stour Estuaries, UK	0.06-0.18	0.04-0.17	0.09-1.8	Wright & Mason (1999)
Cerastoderma edule	Wadden Sea, Germany	0.06	-	0.15	Jung *et al.* (2006)
Cerastoderma edule	Sado Estuary, Portugal	0.03	0.02	0.10	Present work
Crassostrea angulata	Guadalquivir Estuary, Spain	1.3	-	0.37	Blasco *et al.* (1999)
Crassostrea angulata	Sado Estuary, Portugal	0.37	0.04	0.1	Present work
Crassostrea gigas	Japan Sea, Russia	0.8-4.9	-	0.7-6.5	Shulkin *et al.* (2003)
Crassostrea gigas	Atlantic Ocean, Portugal	0.22	0.04	0.10	Present work
Donax trunculus	Marmara Sea, Turkey	0.05	0.09	0.57	Özden *et al.* (2009)
Donax trunculus	Atlantic Ocean, Spain	0.04	0.02	0.68	Usero *et al.* (2005)
Donax trunculus	Sado Estuary, Portugal	0.04	0.03	0.10	Present work
Mytilus chilensis	Maule Region, Chile	0.05-0.76	-	0.12-3.0	Tapia *et al.* (2010)
Mytilus chilensis	Strait of Magellan, Chile	0.17	-	-	Astorga España *et al.* (2007)
Mytilus edulis	Gulf of Maine, USA/Canada	0.29	0.08	0.47	Chase *et al.* (2001)
Mytilus edulis	Bohai Sea, China	0.34-1.96	-	0.16-0.60	Liang *et al.* (2004)
Mytilus edulis	Orwell and Stour Estuaries, UK	0.18-0.52	0.04-0.12	0.15-1.5	Wright & Mason (1999)
Mytilus edulis	Barents Sea, Russia	0.36	-	0.29	Zauke *et al.* (2003)
Mytilus edulis	Tagus Estuary, Portugal	0.13	0.02	0.20	Present work
Ruditapes decussatus	Atlantic Ocean, Spain	0.06-0.42	0.01-0.07	0.06-0.48	Usero *et al.* (1997)
Ruditapes decussatus	Ria Formosa, Portugal	0.02	0.02	0.10	Present work
Scrobicularia plana	Guadalquivir Estuary, Spain	0.24	-	2.28	Blasco *et al.* (1999)
Scrobicularia plana	Bilbao Estuary, Spain	5-18	-	16-36	Ruiz & Saiz-Salinas (2000)
Scrobicularia plana	Tagus Estuary, Portugal	0.16-0.71	-	2.4-13	França *et al.* (2005)
Scrobicularia plana	Tagus Estuary, Portugal	0.03	0.04	1.4	Present work

dependent of other factors such as the organism size and reproductive state (Usero *et al.*, 1997).

From the food safety point of view, it is important to control the contribution of bivalve molluscs to the weekly Cd and Pb intakes (**Table 6.3**). The results obtained showed that only furrow shell and Portuguese oyster surpassed the PTWI indicated by FAO/WHO and, for that the consumption of these two species have to be moderated. Nevertheless, it is very uncommon in Portugal that a person have six or seven meals per week of these bivalve species. General recommendations for consumption of these species can be graphically observed in **Figure 6.5**.

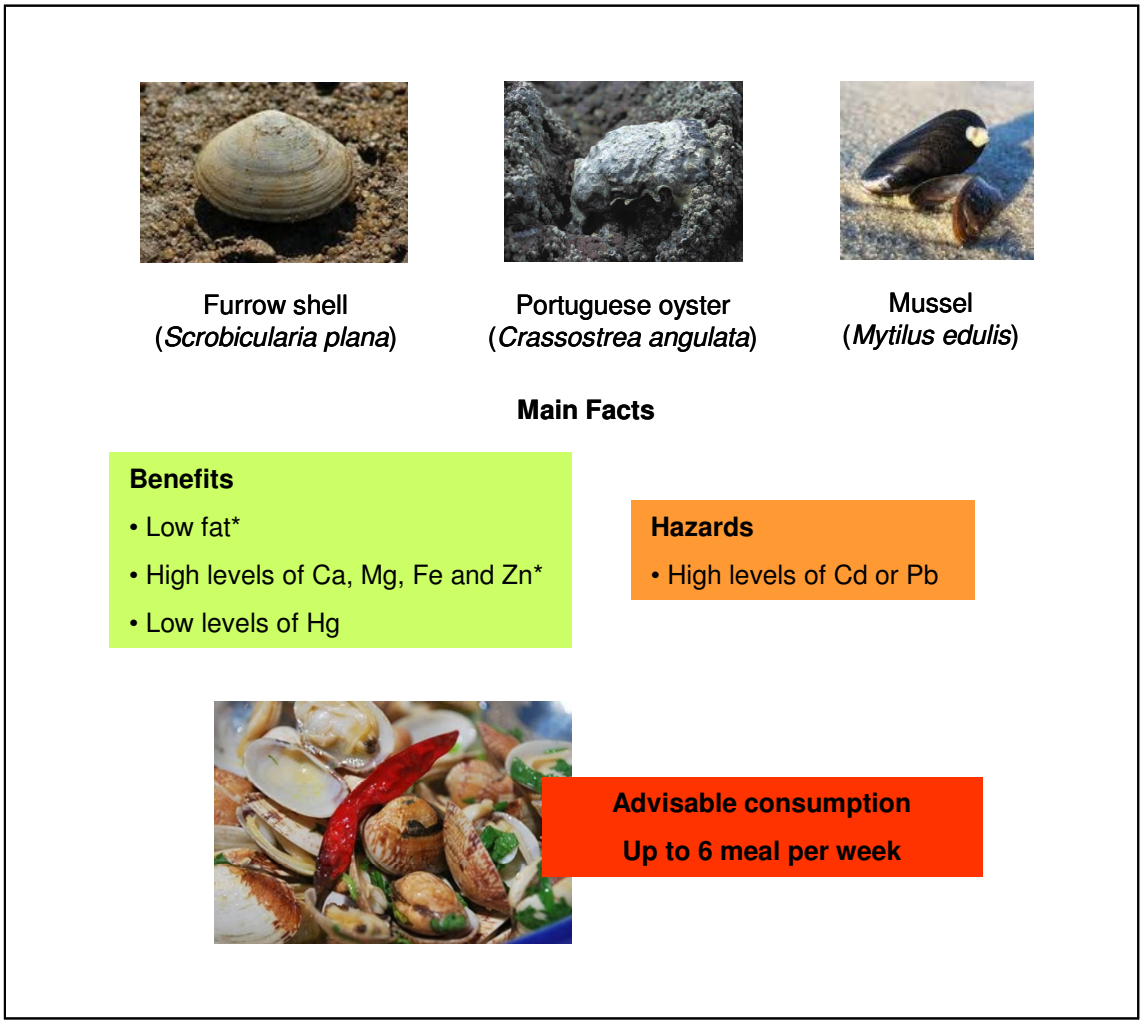


Figure 6.5 - General recommendations for bivalve molluscs consumption. *IPIMAR, 2008. (Picture source sites: Wikipedia (b); Marine Life Information Network (d); Pbase; Crónicas do rochedo)

6.6 Canned seafood

Canned seafood still record a reasonable demand in several countries of the world including Portugal. Their consumption is somewhat privileged because of its convenient and affordable use for most working families. These products, generally reflect the profile of raw-material, and therefore may also contain toxic elements. However, it is noteworthy that the processing steps can alter the concentration of toxic metals in fish prior to consumption (Rasmussen *et al.*, 2007;

Ganjavi *et al.*, 2010). In general, Portuguese canned seafood studied in this thesis, did not show mean high levels of the three toxic metals (IX). Lead was only detected in about 20 % of analysed samples and the maximum concentration found did not reach the proposed limits for EU (2006, 2008). These results were in good agreement with several authors (**Table 6.8**).

Table 6.8 - Concentration of toxic elements (Cd, Hg and Pb) expressed as mg/kg (wet weight) as reported in the literature for canned seafood.

Canned Seafood	Purchase country	Cd	Hg	Pb	Reference
Common name:					
Chub mackerel	Portugal	0.04	0.05	< 0.10	Present work
Horse mackerel	Turkey	0.25	-	0.16	Tuzen & Soylak (2007)
Mackerel	USA	-	0.04	-	Ikem & Egiebor (2005)
Mackerel	Slovenia	-	0.04	-	Miklavčič *et al.* (2011)
Mussel	Spain	0.005	-	0.007	Gutiérrez *et al.* (2004)
Mussel	Spain	-	0.03	-	Gutiérrez *et al.* (2006)
Mussel	Portugal	0.16	0.03	0.20-0.30	Present work
Sardine	Brazil	0.19-0.38	-	0.77-2.15	Tarley *et al.* (2001)
Sardine	USA	-	0.11	0.002	Ikem & Egiebor (2005)
Sardine	Turkey	0.19	-	0.09	Tuzen & Soylak (2007)
Sardine	USA	0.04	-	0.08	Shiber (2010)
Sardine	Slovenia	-	0.09	-	Miklavčič *et al.* (2011)
Sardine	Portugal	0.02	0.03	< 0.10-0.20	Present work
Squid	Spain	0.38-0.98	-	-	Cisneros-García *et al.* (1995)
Squid	USA	0.86-2.07	-	-	Galitsopoulou *et al.* (2009)
Squid	Portugal	0.29	0.07	< 0.10	Present work
Tuna	Libya	0.09-0.32	0.20-0.66	0.18-0.40	Voegborlo *et al.* (1999)
Tuna (white)	USA	-	0.407	-	Burger & Gochfeld (2004)
(light)		-	0.118	-	
Tuna	Iran	0.02	0.13	0.04	Khansari *et al.* (2005)
Tuna	USA	0.002	0.28	0.001	Ikem & Egiebor (2005)

Continuation of **Table 6.8**

Canned Seafood	Purchase country	Cd	Hg	Pb	Reference
Tuna	USA	-	0.10-0.33	-	Rasmussen & Morrissey (2007)
Tuna	Turkey	0.08	-	0.10	Tuzen & Soylak (2007)
Tuna	Italy	0.01-0.14	0.04-1.79	0.02-0.16	Storelli *et al.* (2010)
Tuna (Yellowfin)	Iran	0.03	-	0.15	Ganjavi *et al.* (2010)
Tuna (Skipjack)	Iran	0.02	-	0.07	Ganjavi *et al.* (2010)
Tuna	Slovenia	-	0.24	-	Miklavčič *et al.* (2011)
Tuna	Portugal	0.04	0.28	< 0.10-0.10	Present work

Previous works showed high levels of Pb, likely by the use of Pb soldered side seam in cans. Actually, this is not the used procedure and consequently the levels have decreased. Canned molluscs, especially of squid, showed the highest levels of Cd. Papers published on levels of toxic metals in canned shellfish are scarce. However, there are few that also mention the high values of Cd, principally in canned squid (**Table 6.8**). These high values are attributed to the presence of visceral tissue in this kind of canned seafood (Galitsopoulou *et al.*, 2009). Mercury levels were always very low and did not exceed the limits of 0.5 and 1.0 mg kg^{-1}, according to the species (EU, 2006, 2008). The exception occurred in some samples (around 0.5 %) of canned tuna that reached the proposed limit. These high figures are mainly due to the feeding behaviour of tuna, since it is a very voracious and predatory species (Khansari *et al.*, 2005; Storelli *et al.*, 2010). Probably, these Hg contents are also increased by the canning process (Rasmussen *et al.*, 2007) due to water release. There are several studies on Hg in canned tuna (**Table 6.8**). Overall, the Hg values vary somewhat, but are similar to those reported in this thesis. Differences are possibly due to different species of tuna, of different sizes and different capture sites, more or less polluted. According to the **Table 6.3**, only canned tuna slightly exceeded the estimated weekly intake for Hg_T, however the maximum number of meals allowed, six, seems unlikely in the Portuguese diet. Some consumption recommendations are generally described in **Figure 6.6**.

6.7 Risk assessment of seafood consumption

The simultaneous assessment of risks and benefits in seafood has been subject of various studies (Cohen *et al.*, 2005; Domingo *et al.*, 2007; van der Voet *et al.*, 2007; Gladyshev *et al.*, 2009; Sioen *et al.*, 2009). In this thesis, the hazard MeHg was chosen, since it is the component whose effects are most likely to contribute substantially to health impacts associated with seafood consumption. The results for the cephalopods group (VI) showed that squid presented the lowest risk, being octopus´ risk the higest as a result of higher MeHg content and greater

(eg. tuna, sardine, octopus, squid, mussel)

Main Facts

Benefits

- High protein*
- High levels of omega-3 fatty acids*
- High levels of K, P and Ca*
- Low levels of Pb

Hazards

- High levels of Hg or Cd

Advisable consuption

Up to 6 meal per week

Figure 6.6 - General recommendations for canned seafood consumption. *Bandarra *et al.*, 2004. (Canned seafood photo by author; Picture source site: Cozinha sem tabus)

consumption. Thus, whereas for squid no consumption restriction seems necessary, for cuttlefish and octopus an ideal maximal consumption of two meals per week must be advised. Other studies about risk assessment of methyl-Hg intake trough seafood were already done (Tressou *et al.*, 2004; Cardoso *et al.*, 2010a, 2010b). Regarding black scabbardfish, it was found (Cardoso *et al.*, 2010a) that people who consume two or three meals per week, have a high probability of being overly exposed to MeHg, about 1:55. This result confirms the data shown previously (Paper IV) that the black scabbardfish should be eaten sparingly. Another study (Cardoso *et al.*, 2010b), conceptualized to compare the risks and benefits associated to the consumption of the five most consumed seafood species in Portugal (cod, hake, sardine, horse mackerel and tuna), indicated that tuna and hake yielded the maximum probability of exceeding the MeHg PTWI, but somehow they are still considered negligible. The results also show that probabilities of surpassing the MeHg PTWI were lower than those of exceeding the EPA + DHA recommended daily intake. However, high seafood consumptions *per se*, do not

indicate much on the relation between risks and benefits to which a given population is subject. This information needs to be supplemented with a thorough knowledge about the species which are consumed, the consumption frequency and the amounts, included in the diet of a population.

7 MAIN CONCLUSIONS - FUTURE OUTLOOK

In this thesis it was studied the centesimal and elemental composition (essential and toxic elements) of several fishery and aquaculture products. It was also evaluated the MeHg risks associated with cephalopods consumption in Portugal. The results show that the studied species can give a relevant contribution to the DRI in an equilibrated diet and their consumption is considered healthy. However, some molluscs and deep-fish species have to be parsimoniously consumed due to the levels of toxic elements. It can also be state that MeHg risk assessment for Portuguese consumers reveals no danger concerning the observed squid consumption levels but octopus and cuttlefish consumption present a relatively high MeHg risk. Nevertheless, known the various assumptions used in this study, it can not be considered a public health problem.

These results can be used to complete databases and to establish and revise limits to be included in standards or legislation. However, this is a job that requires constant updating due, not only to the variety of species that already exist, but also to the introduction of new species in the market, either wild or farmed. Thus, it would be desirable to study the small pelagic fish (eg chub mackerel and horse mackerel) and other deep-water fish species given the relatively high volume of catches in Portugal, and the high number of new imported species that start to be very important in the Portuguese market, mainly due to the low price (eg Nile perch - *Lates niloticus* or panga - *Pangasius hypophthalmus*).

The risk analysis performed in this work is preliminary and need further support. For this, it will be necessary to gather consumption data, through surveys of consumption of selected fishery products. Relatively to contaminants, speciation analysis for As compounds is required to determine the toxic and non-toxic fractions in seafood since the toxicity of As is highly dependent on its chemical form, with inorganic As being the most toxic (Lin *et al.*, 2008). It will also be crucial to quantify other chemical contaminants as organic polychlorinated dibenzodioxins (PCDD), dibenzofurans (PCDF) and PCBs in the fishery and aquaculture products in Portugal because such compounds persist and bioaccumulate through the food chain (Karouna-Renier *et al.*, 2007). Moreover, although there are some models to assess the risk / benefit, eg QALIBRA Tool (QALIBRA, 2010), it would be interesting to look for new models.

Another area of future research will be the assessment of contaminant compounds bioaccessibility from seafood to human consumers. In current human health risk assessment, the maximum acceptable concentrations of contaminants in food are mostly based on the total concentrations. However, the total concentration of contaminants may not always reflect the available amount (He *et al.*, 2010). Bioaccessibility determination is thus required to improve the risk assessment of contaminants. These studies should be made not only in raw fishery products but also in cooked ones, since it is the latter form that they are consumed by humans.

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BIOLOGY OF SOME STUDIED SEAFOOD PRODUCTS

Common octopus (*Octopus vulgaris*)

Class: Cephalopoda

Order: Octopoda

Family: Octopodidae

Species: *Octopus vulgaris* (Cuvier, 1797)

Picture source site: Marine Life Information Network (a)
(Photographer: David Nicholson)

Portuguese name: Polvo comum

Diagnosis: mantle oval with wide open and devoid of fins. Tentacles with eight arms with two series of suckers, length about 4-5 times higher than the body. Inner shell reduced to small rods. Gray-brown, variable. Reaches 1 m total length.

Habitat: benthic, demersal and neritic (0-450 m). Circalittoral and can be found in the tidal zone.

Diet: crustaceans, molluscs, fish and other shellfish. Can occurred cannibalism.

Reproduction: temperate and subtropical zones usually with two times of posture: Spring (May-June) and Autumn (September).

Distribution: temperate seas, tropical and subtropical Atlantic, Indian and Pacific Oceans. It are not known the limits of their distribution. Seas of the Mainland, Azores and Madeira.

Type of fishing: bottom trawling, traps (buckets and pots), hand lines and by diving.

Economic importance: highly commercial cephalopod.

Common cuttlefish (*Sepia officinalis*)

Class: Cephalopoda

Order: Sepiida

Family: Sepiidae

Species: *Sepia officinalis* (Linnaeus, 1758)

Picture source site: Marine Life Information Network (b)
(Photographer: David Nicholson)

Portuguese name: Choco comum

Diagnosis: mantle oval, flattened dorso-ventrally, fins narrow, following almost the entire mantle and forming two lobes free at the back, eight short arms and two tentacles very long. Reach a maximum of 35 cm (mantle).

Habitat: between the surface and about 200 m depth, especially up funds of sand or silt.

Diet: small molluscs , crabs, shrimps and small fish. Can occur cannibalism.

Reproduction: breeds throughout the year, with peaks in areas with temperatures between 13 and 15 º C in the Mediterranean west between April and July. In Senegal and banks of the Sahara is between January and April.

Distribution: Atlantic Ocean, from the Baltic Sea to the South-Africa. Mediterranean Sea. Seas of the Mainland, the Azores and Madeira.

Type of fishing: bottom trawls, gillnets and traps.

Economic importance: commercial cephalopod.

Common European squid (*Loligo vulgaris*)

Class: Cephalopoda

Order: Tenthida

Family: Loliginidae

Species: *Loligo vulgaris* (Lammarck, 1798)

Picture source site: Cefalópodos

(Photographer: Unknown)

Portuguese name: Lula comum

Diagnosis: mantle elongate with posterior end in acute angle, more or less rounded fins occupy about two thirds the length of the mantle, eight short arms and two tentacles with two rows of suckers. Colour greyish-transparent. Reaches a maximum length of 40 cm (mantle).

Habitat: lives in water of about 20-250 m depth.

Diet: crustaceans, cephalopods and fish.

Reproduction: breeds throughout the year, with the most intensive spawning during summer-winter.

Distribution: Atlantic Ocean, from the North Sea to the Gulf of Guinea. Seas of the Mainland, the Azores and Madeira.

Type of fishing: bottom and pelagic trawls, hand lines, gill nets.

Economic importance: commercial cephalopod.

Norway lobster (*Nephrops norvegicus*)

Class: Malacostraca

Order: Decapoda

Family: Nephropidae

Species: (Linnaeus, 1758)

Picture source site: Marine Life Information Network (c)
(Photographer: Sue Scott)

Portuguese name: Lagostim

Diagnosis: first pair of thoracic legs with strong claws and elongated, bearing spines arranged longitudinally. Carapace covered with spines and hairs, especially on the front. Abdominal segments with transverse grooves furry and stopped in the middle. Two pairs of antennae, the second pair being much larger and thinner than the first. Pedunculated and mobile black eyes. General colour pink orange, red bands. Reaches a maximum length of 25 cm.

Habitat: lives in funds vessels 200-500 m, where digs galleries, but may be between approximately 50-800 m.

Diet: nocturnal species that feeds on detritus, crustaceans and worms.

Reproduction: stance in July.

Distribution: Atlantic Ocean, from Norway and Iceland to Morocco. Mediterranean Sea. Seas of the Mainland.

Type of fishing: bottom trawl, gill nets and traps.

Economic importance: highly commercial crustacean.

Gilthead seabream (*Sparus aurata*)

Class: Actinopterygii

Order: Perciformes

Family: Sparidae

Species: Sparus aurata (Linnaeus, 1758)

Picture source site: Acquario Marino Mediterraneo
(Photographer: Gianni Neto)

Portuguese name: Dourada

Diagnosis: black spot on top of the opercle , patch golden yellow crescent between the eyes, surrounded by two dark areas. Colour greyish dorsally, silvery on the belly. Longitudinal black band on the dorsal. It can reach up to 70 cm long.

Habitat: demersal (usually in rocky funds and / or sandy), can occur in brackish waters of coastal lagoons and estuaries, rarely exceeding 30 m depth, although flattered could reach 150 m.

Diet: mainly carnivorous, accessorily herbivorous. Feed on shellfish, including mussels and oysters.

Reproduction: hermaphrodite, in which males can become females.

Distribution: Atlantic Ocean, from the British Isles to Senegal. Mediterranean Sea. Black Sea. Seas of the Mainland.

Type of fishing: pelagic trawl and gill nets. Hook in angling. One of the most important fishes in saline and hyper saline aquaculture.

Economic importance: fisheries and aquaculture commercial fish, (used in gamefish).

European sea bass (*Dicentrarchus labrax*)

Class: Actinopterygii

Order: Perciformes

Family: Moronidae

Species: *Dicentrarchus labrax* (Linnaeus, 1758)

Picture source site: El maestro pescador
(Photographer: Robert Patzner)

Portuguese name: Robalo

Diagnosis: body rather elongate. It has two dorsal fins well separated and similarly, the length of the base of the second dorsal and anal fins, approximately equal, opercle with 2 flat spines. Colour silvery grey to bluish on the back, silvery on the sides, belly sometimes tinged with yellow. Young with some dark spots on upper part of the body. Vomerine teeth only anteriorly, in a crescentic band. It can reach up to 100 cm long.

Habitat: demersal, live along the coast, enters estuaries and rivers.

Diet: feed chiefly on shrimps and molluscs, but can also feeds on fish.

Reproduction: only one breeding season per year, which takes place in winter in the Mediterranean population (December to March), and up to June in Atlantic populations.

Distribution: Atlantic Ocean, from Norway to Morocco. Mediterranean Sea. Black Sea. Seas of Mainland.

Type of fishing: hook equipment, gill nets, seines on board, both pelagic and bottom trawls. One of the most important fishes in saline aquaculture.

Economic importance: fisheries and aquaculture commercial fish, (used in gamefish).

Rainbow trout (*Oncorhynchus mykiss*)

Class: Actinopterygii

Order: Salmoniformes

Family: Salmonidae

Species: Oncorhynchus mykiss (Walbaum, 1792)

Picture source site: ZipcodZoo

(Photographer: Massimo Lorenzoni)

Portuguese name: Truta arco-íris

Diagnosis: back bluish green iridescent with reflections and a pink band along the flanks. Belly whitish. Small black spots scattered throughout the body at par in adipose and caudal fins. It can reach 70 cm long.

Habitat: lakes and calm rivers. In Portugal, appears mainly in the northern rivers. Tolerates a wide temperature range (up to 24 º C).

Diet: feeds mainly on invertebrates, small fish and terrestrial insects that fall into the water.

Reproduction: breeds in late winter, early spring. Its maintenance in Portugal is due to successive repopulation.

Distribution: original species of western North America. Introduced in Europe in the late nineteenth century. In Portugal introduced to intensive fish farming.

Type of fishing: hook equipment. One of the most important fishes in fresh water aquaculture.

Economic importance: aquaculture commercial fish, (used in gamefish).

Turbot (*Psetta maxima*)

Class: Actinopterygii

Order: Pleuronectiformes

Family: Sctophthalmidae

Species: Psetta maxima (*Psetta maxima,* Linnaeus, 1758)

Picture source site: Wikimedia Commons
(Photographer: Luc Viatour)

Portuguese name: Pregado

Diagnosis: diamond-shaped body, with its ocular face covered with bone tubercles. It has eyes located on the left and separated from each other by a space larger than its diameter. The dorsal and anal fins not linger on the caudal peduncle of the blind side. Reaches a maximum of 75 cm long.

Habitat: adults live on sandy, rocky or mixed bottoms.

Diet: feeds mainly on other bottom-living fish (sand-eels, gobies, etc.), and also, to a lesser extent, on larger crustaceans and bivalves.

Reproduction: spawning season between April and August.

Distribution: Atlantic Ocean, from Norway to Morocco. Mediterranean Sea. Black Sea. Seas of Mainland.

Type of fishing: especially bottom trawling, angling equipment and gillnets. One of the most important fishes in saline aquaculture.

Economic importance: highly esteemed food fish in Europe, aquaculture commercial fish.

Blackbelly rosefish

(*Helicolenus dactylopterus dactylopterus*)

Picture source site: Fishbase

(Photographer: Pedro Miguel Niny Cambraia Duarte)

Class: Actinopterygii

Order: Scorpaeniformes

Family: Scorpaenidae

Species: *Helicolenus dactylopterus dactylopterus* (Delaroche, 1809)

Portuguese name: Cantarilho

Diagnosis: large heads and prickly. Inside of mouth black. Black spot located near the edge of the opercle. Reddish colour with white horizontal lists. It can reaches 44 cm long.

Habitat: bathydemersal; depth range 50 - 1100 m. Found in soft bottom areas of the continental shelf and upper slope.

Diet: feeds on both benthic and pelagic organisms (crustaceans, fish, cephalopods, and echinoderms).

Reproduction: November to December in southern waters and February to March in Mediterranean waters.

Distribution: Atlantic Ocean, from Norway to Morocco, including the Azores, Madeira and the Canary Islands and also in the Mediterranean except Black Sea.

Type of fishing: bottom trawl.

Economic importance: commercial fish.

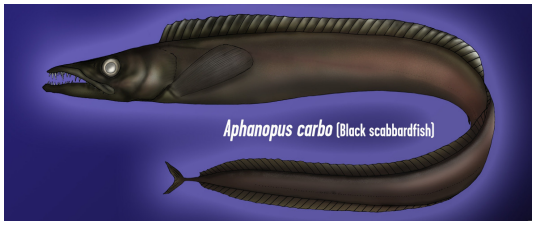
Black scabbardfish (*Aphanopus carbo*)

Class: Actinopterygii

Order: Perciformes

Family: Trichiuridae

Species: *Aphanopus carbo* (Lowe, 1839)



Picture source fish: Fishbase

(Picture by Fishpics)

Portuguese name: Peixe-espada-preto

Diagnosis: body elongated and flattened laterally. Dorsal fin with cut-out to separate the thorny part of the soft one. Strong spine below the anus. Black or very dark brown with metallic reflections. It can reaches 1.5 m long.

Habitat: bathypelagic (depth range 180 - 1600 m).

Diet: crustaceans, cephalopods and fish.

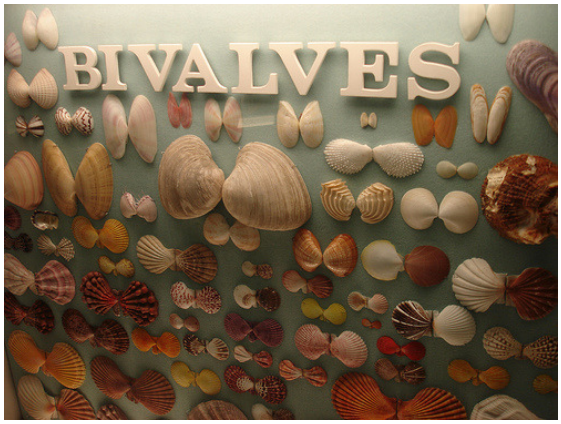
Reproduction: posture throughout the year.

Distribution: North Atlantic from the Strait of Denmark, Iceland and Norway to Madeira and Northwest Africa.

Type of fishing: deep water longline.

Economic importance: commercial fish.

Bivalves



Picture source site: Flickriver
(Photographer: Joethe Lion)

Class: Bivalvia

Most important orders: Mytiloida; Ostreoida; Veneroida

Most important families: Pectinidae; Macrtridae; Veneridae; Cardiidae; Donacidae; Scrobiculariidae; Mytilidae; Solenidae

Some species: Cerastoderma edule; Spisula solida; Ruditapes decussatus; Scrobicularia plana; Mytilus edulis; Solen marginatus; Crassostrea angulata

Portuguese names: Moluscos bivalves (berbigão; amêijoa-branca; amêijoa-boa; lambujinha; mexilhão; longueirão; ostra-portuguesa)

Diagnosis: with two calcified valves that are articulated through a dorsal position device, usually equipped with teeth, called the hinge. This ligament serves to open the valves, which, in contrast, are closed by contraction of one, two or three adductor muscles that are connected inside the valves. Maximum size of 3.5 to 17 cm, according to species.

Habitat: live in muddy or sandy bottoms in shallow coastal zone, coastal lagoons, lagoons and estuaries. Some attach themselves to rocks or submerged objects.

Diet: organic matter in the water column, especially plankton

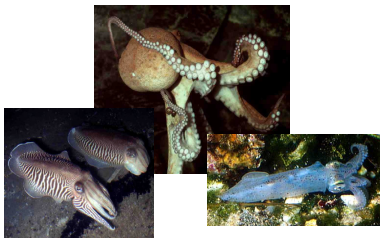
Reproduction: may be hermaphroditic or have separate sexes.

Distribution: Atlantic Ocean, from Norway to Morocco. Mediterranean Sea. Seas of Mainland and Madeira.

Type of fishing: bottom trawls, dredges and directly by hand.

Economic importance: highly esteemed seafood in Portugal, commercial bivalves.

Paper I



Food Chemistry 113 (2009) 1146–1153

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Elemental composition of cephalopods from Portuguese continental waters

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ARTICLE INFO

Article history:
Received 13 February 2008
Received in revised form 16 June 2008
Accepted 1 September 2008

Keywords:
Cephalopods
Octopus
Squid
Cuttlefish
Essential elements
Contaminant metals

ABSTRACT

Essential and contaminant elements concentrations were determined in the muscle tissue of octopus (*Octopus vulgaris*), squid (*Loligo vulgaris*) and cuttlefish (*Sepia officinalis*), caught off the Portuguese coast in 2004–2005. As expected, the largest concentrations found correspond to Cl, S, K, Na, P and Mg (average values between 629 mg $(100\ g)^{-1}$, for Cl, and 435 mg kg^{-1}, for Mg, in octopus and squid, respectively). Above average concentrations of Zn, Cu, Fe and Sr were also found. The highest total Hg concentration was found in cuttlefish (0.36 mg kg^{-1}); however, this value did not exceed the recommended limit proposed by EU (0.5 mg kg^{-1}). Lead levels observed in all samples were always significantly lower than the EU limit (1.0 mg kg^{-1}). Regarding Cd, the 1.0 mg kg^{-1} limit was only exceeded in two octopus samples. It may be concluded that the cephalopods studied do not constitute cause for concern, in terms of toxic elements, and could be safely used for daily intake of essential elements. Nevertheless, the squid contribution for elemental DI is minor in comparison to the other two species.

1. Introduction

Fish and shellfish are considered some of the most interesting dietary products, and their nutritional benefits are well-known. Seafood is rich in protein, with a balanced amino acid composition and a high proportion of polyunsaturated fatty acids (Belitz, Grosch, & Schieberle, 2004; Oehlenschläger, 1997). These species also contain most of the 90 natural elements (Causeret, 1962; Lall, 1995). The largest concentrations correspond to carbon, hydrogen, nitrogen, oxygen and sulphur (structural elements) followed by chlorine, potassium, phosphorus, sodium, magnesium and calcium (Lall, 1995; Oehlenschläger, 1997). Other elements are present at lower levels, being described as trace or ultratrace elements. These elements are classed as essential, when their biological roles are well-known, such as occurs with iron, copper, zinc, iodine, manganese, selenium or fluorine; non-essential, when their physiological functions have not been clearly demonstrated, such as occurs with nickel, vanadium and arsenic; and toxic, such as mercury, lead and cadmium (Lall, 1995).

Cephalopods are an excellent source of some essential elements (Oehlenschläger, 1997); however, given the morphological and biological characteristics associated to their habitat, some contaminant metals may accumulate in their tissues (Bustamante, Caurant, Fowler, & Miramand, 1998; Bustamante, Grigioni, Boucher-Rodoni, Caurant, & Miramand, 2000; Soldevilla, 1987). Among seafood species, cephalopods represent one of the most important groups captured in Europe (Fernández-Rueda & García-Flórez, 2007). In Portugal, cephalopods represented, in 2005, only approximately 8% of wholesale market sales; however, the corresponding values in terms of auction transaction were approximately three times higher than those registered for fish species (Fonseca, Campos, & Garcia, 2002).

Although, numerous studies on the elemental composition of cephalopod species exist (Napoleão, Pinheiro, & Sousa Reis, 2005; Raimundo, Caetano, & Vale, 2004; Seixas, Bustamante, & Pierce, 2005a, 2005b; Villanueva & Bustamante, 2006), their objectives were mostly related to environmental contamination and its use in biological monitoring. Most of these studies focus on amounts present in several organs such as digestive glands, branchial hearts and gills (Bustamante, Lahaye, Durnez, Churlaud, & Caurant, 2006; Miramand & Bentley, 1992; Miramand, Bustamante, Bentley, & Kouéta, 2006).

The primary aim of this work was to quantify the levels of a high number of essential and toxic elements in the muscle tissue of three cephalopods species much enjoyed by Portuguese consumers, using various analytical techniques. From a public health perspective, this study can provide consumers with better knowledge of nutritional characteristics and contamination problems associated to these species. Additionally, possible relationships

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0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.09.003

between concentrations and specimen age were also researched, for all species.

2. Materials and methods

2.1. Sample collection and preparation

Octopus (*Octopus vulgaris*), squid (*Loligo vulgaris*) and cuttlefish (*Sepia officinalis*), were purchased at the Peniche auction, in central Portugal (Atlantic North-eastern European coast), from May to September in 2004–2005. Collected specimens were immediately stored in plastic bags, which were placed in ice and taken to the laboratory. Upon arrival, mantle length, total weight, sex and maturity stages were recorded (Table 1). Edible parts (mantle and arms) were removed and homogenised in a food blender. Homogenised samples were vacuum-sealed in individual plastic bags, coded for easy identification, and stored at −21 °C until required for analysis. Specimen age was estimated by growth models (Bettencourt, 2000; Moreno, Azevedo, Pereira, & Pierce, 2007; Raya, 2001); the corresponding results are presented in Table 1.

2.2. Analytical methods

Determination of K, Na, Mg, Ca, Zn, Fe, Cu, Mn, Ni, Cd and Pb levels was performed by flame atomic absorption spectrometry, according to the procedures described by Jorhem (2000). All analyses were carried out at least in duplicate; an external calibration method was used for quantitative analysis. Edible portion samples (5–10 g wet weight) were dry-ashed at 500 °C under a gradual temperature increase. Ash was dissolved in concentrated nitric acid and the solution obtained was evaporated to dryness. The final residue was redissolved with 12 or 5 ml nitric acid 15% (v/v) and transferred to 25 or 10 ml volumetric flasks (10 ml for Pb, Cd and Ni, 25 ml for other elements); final volumes were adjusted with ultrapure water. Quantification of these elements was performed using a Spectr AA-20 spectrophotometer with deuterium background correction (Varian). Detection limits (DL, mg kg^{-1}, wet weight) were 0.01 (K), 0.09 (Na), 0.02 (Mg), 0.08 (Ca), 0.06 (Zn), 0.32 (Fe), 0.02 (Cu), 0.01 (Mn), 0.02 (Ni), 0.01 (Cd) and 0.02 (Pb).

Total Hg was determined by cold vapour atomic absorption spectrometry (CVAAS) according to the procedure developed by Hatch and Ott (1968) and described by Joiris, Holsbeek, Bouquegneau, and Bossicart (1991). For each specimen, 1 g of homogenised edible portion sample was digested with concentrated sulphuric acid. Sample mercury (Hg^0 and Hg_2^{2+}) was subsequently oxidised to Hg^{2+}, using potassium permanganate. Following Hg^{2+} reduction to Hg^0 with stannous chloride, volatile Hg^0 was bubbled into the Bacharach Coleman MAS-50D Mercury Analyser closed system (λ = 253.7 nm). Samples were analysed twice. Concentrations were calculated by interpolation using a linear calibration curve obtained by measuring the absorbance of standard solutions. The detection limit was 0.01 mg kg^{-1}, wet weight.

Phosphorus was determined spectrophotometrically according to ISO Standard 13730 (1996). Samples (5 g wet weight) were dry-ashed at 500 °C, followed by acid digestion and colorimetric measurement of a yellow compound resulting from the reaction between phosphorus and an ammonium vanadate and ammonium molybdate mixture, at 430 nm. The detection limit was 0.01 mg kg^{-1}, wet weight.

In order to analyse Cl, S, Br, Sr, Rb and Se, homogenised samples were freeze-dried for 48 h, at −45 °C and low pressure (approximately 10^{-1} atm). Samples were powdered and immediately vacuum-sealed, in individual coded plastic bags, which were subsequently stored at −21 °C, until further analysis. Concentrations of these six elements were determined using an EDXRF spectrometer, according to Carvalho, Santiago, and Nunes (2005). Powdered samples were pressed into 2.0 cm diameter pellets. Pellets were glued onto Mylar film, on a sample holder, and directly placed in the path of an X-ray beam, for quantification. Two pellets were prepared for each tissue sample. The detection limits (mg kg^{-1}, dry weight) for these elements were 10 (Cl), 10 (S), 0.8 (Br), 0.5 (Sr), 1.1 (Rb) and 0.6 (Se).

Blanks were always tested in the same conditions as the samples. All laboratory ware was cleaned with HNO_3 (10%) or HCl (20%) for 24–48 h and rinsed with ultrapure water (18.2 MΩ cm), to avoid contamination. Chemical reagents were pro analysis or superior. Commercial standard solutions (Merck, 1000 mg l^{-1}) were used for some elements.

Analytical data for elements are reported in mg $(100\,g)^{-1}$ or mg kg^{-1}, depending on element, on a wet weight basis. Mean moisture content in each species was 78% in cuttlefish and squid and 83% in octopus. These values were used for the conversion of results to dry weight basis for comparison with other studies.

Five certified reference materials were tested in the same conditions as the samples, in order to assess analytical method accuracy: LUTS-1 (non defatted lobster hepatopancreas), TORT-2 (Lobster hepatopancreas), DORM-2 (Dogfish muscle) from National Research Council of Canada, SMRD-2000 (Canned matrix meat) from Swedish Meats R&D and Scan Foods/National Food Administration, Sweden and MA-A-2 (Fish flesh) from International Atomic Energy Agency. Results obtained in this study were in good agreement with certified values (Table 2).

2.3. Statistical analysis

All data were analysed using the STATISTICA 6.1 software (Stat soft, Inc., Tulsa, OK74104, USA). Pearson's correlation between elements was performed. The Student *t*-test was used to evaluate the influence of sex on the concentration of various elements, for each species. Single factor ANOVA analysis was used to confirm the existence of significant differences between age groups, within the same species. Due to the absence of normality and variance homogeneity (Lilliefors-test and Levene-test, respectively), the Kruskal–Wallis non-parametric test was used to evaluate differences in element concentrations between studied cephalopods (Zar, 1999). Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. General

Data relative to 16 elements in common octopus, squid and common cuttlefish are presented in Table 3. Mean and standard deviation histograms are shown in Figs. 1 and 2, in order to com-

Table 1
Characteristics of studied cephalopods

Common name (specie)	*N*	Mantle length range (mm)	Weight (g)	Sex	Sex maturation state	Estimated age month (number in each class)
Common octopus (*O. vulgaris*)	10	115–245	427–4900	8 ♂,2 ♀	II, III	3 (2); 5 (2); 6 (3); 7 (3)
Squid (*L. vulgaris*)	10	130–420	65–1244	5 ♂,5 ♀	II, IV, V	7–8 (3); 9–10 (4); 11–13 (3)
Common cuttlefish (*S. officinalis*)	12	165–315	538–3100	6 ♂,6 ♀	III	6–8 (5); 9–12 (3); 13–15 (2); 24 (2)

Table 2
Results of analysis ($n = 4$) for some certified reference materials (mean ± sd, in mg kg^{-1})

		K	Na	P	Mg	Ca	Zn	Fe	Cu	Sr	Mn	Se	Ni	Hg	Pb	Cd
LUTS-1	Certified	948 ± 72	–	–	89.5 ± 4.1	203 ± 33	–	11.6 ± 0.9	15.9 ± 1.2	–	1.20 ± 0.13	–	0.200 ± 0.034	–	–	–
	Obtained	955 ± 23			90.8 ± 2.2	197 ± 16		12.1 ± 0.4	15.4 ± 0.2		1.28 ± 0.03		0.195 ± 0.009			
SMRD-2000	Certified	1859 ± 85	8533 ± 281	1075 ± 47	–	70.3 ± 8.3	–	6.33 ± 1.66	–	–	–	–	–	–	–	–
	Obtained	1938 ± 38	8346 ± 280	1108 ± 11		66.1 ± 7.4		4.94 ± 0.09								
TORT-2	Certified	–	–	–	–	–	180 ± 6	–	–	45.2 ± 1.9	13.6 ± 1.2	–	2.50 ± 0.19	0.27 ± 0.06	0.35 ± 0.13	26.7 ± 0.6
	Obtained						175 ± 1			43.4 ± 3.2	13.1 ± 0.1		2.37 ± 0.08	0.28 ± 0.00	0.35 ± 0.06	26.8 ± 0.1
DORM-2	Certified	–	–	–	–	–	–	142 ± 10	–	–	3.66 ± 0.34	–	–	4.64 ± 0.26	–	–
	Obtained							141 ± 4			3.62 ± 0.18			4.48 ± 0.12		
MA-A-2	Certified	–	–	–	–	–	–	–	–	–	–	1.7 ± 0.3	–	–	–	–
	Obtained											1.5 ± 04				

pare electrolyte, structural and nutrient element concentrations in the three cephalopod species. Total concentrations of Hg, Pb and Cd, metals considered toxic and regulated by the EU Commission (2006), are summarised in Table 4. Elements with related physiological roles were grouped together. Similar patterns were observed for all elements for the three cephalopod species; in general terms, the highest concentrations were found in octopus and the lowest in squid. The main elements were S, Cl, K, Na, P, Mg and Ca, followed by Br, Zn, Fe, Cu, Sr, Rb, Mn and Ni.

3.2. Electrolytes

The most abundant elements were the electrolytes K, Cl and Na, as illustrated in Fig. 1. Potassium contributes to the intracellular ion balance, as a monovalent cation; the two other elements constitute the main extracellular anions and cations, respectively, assuming an important role in acid–base balance (Lall, 1995). The chloride concentration was approximately 630 mg (100 g)$^{-1}$ in octopus; this value was significantly lower in squid ($p = 0.000$), of approximately 270 mg (100 g)$^{-1}$. Chloride data in seafood is scarce. In a study of fish species performed by Oehlenschläger (1997), lower values than those found in the present work were described. Nevertheless, in fish from Indian coastal areas, Cl concentrations are the highest, probably due to pollutant organochlorine compounds (Garg & Ramakrishna, 2006). Average Na levels displayed an identical pattern to Cl. As usually stated for fish, Na concentration in these cephalopod species is also equal to the molar amount of Cl. In general, Na contents are considerably higher in shellfish than in finfish (Vlieg, Lee, & Grace, 1991). The highest concentration of this element was found in octopus (572 mg (100 g)$^{-1}$), followed by cuttlefish (266 mg (100 g)$^{-1}$) and squid (157 mg (100 g)$^{-1}$). Similar contents were found in other studies involving molluscs (Lall, 1995; Segar, Collins, & Riley, 1971; Villanueva & Bustamante, 2006; Vlieg et al., 1991). Octopus was the cephalopod species showing the lowest K concentration, as opposed to other electrolytes (Fig. 1). Average K concentrations were between 223 mg (100 g)$^{-1}$, in octopus, and 289 mg (100 g)$^{-1}$, in cuttlefish. The average K concentration in octopus was significantly lower than in cuttlefish ($p = 0.01$); values for squid were not significantly different from the average values observed for the other two species. The range found in this study agrees with those obtained by several authors for cephalopods (Carvalho et al., 2005; Karakoltsidis, Zotos, & Constantinides, 1995; Lall, 1995; Villanueva & Bustamante, 2006).

3.3. Structural elements

Phosphorus and sulphur were the main structural elements found in seafood; in general terms, equal amounts of these two elements were found in marine species (Oehlenschläger, 1997). In this study, concentrations ranged between 107 and 285 mg (100 g)$^{-1}$ and 197 and 444 mg (100 g)$^{-1}$, respectively (Table 3). Squid showed the highest average value for P (260 mg (100 g)$^{-1}$), clearly above the average 200 mg (100 g)$^{-1}$ value found in the majority of seafood. Significant minimum P concentrations were observed in octopus samples, when compared to squid ($p = 0.000$) and cuttlefish ($p = 0.001$). Identical amounts were described by Lall (1995), in octopus, and wild juvenile cephalopods (Villanueva & Bustamante, 2006). Other authors reported similar concentrations for bivalve and fish species (Oehlenschläger, 1997; Segar et al., 1971; Teeny, Gauglitz, Hall, & Houle, 1984). Significantly larger concentrations of S were found in cuttlefish, when compared to squid ($p = 0.001$) and octopus ($p = 0.029$); the average value was 338 mg (100 g)$^{-1}$. The lowest average concentration was found in squid (229 mg (100 g)$^{-1}$). Vlieg et al. (1991) reported identical results for some squid species. The highest S concentrations in

Table 3
Elemental contents (wet basis) in edible part of the three studied cephalopods (mean ± standard deviation, median and range)

Elements	Common octopus (n = 10)		Squid (n = 10)		Common cuttlefish (n = 10)	
	Mean ± sd (median)	Range	Mean ± sd (median)	Range	Mean ± sd (median)	Range
Br (mg kg^{-1})	34.0 ± 3.7^{a} (34.1)	26.8–40.2	13.3 ± 1.4^{b} (13.7)	11.3–15.3	21.5 ± 2.9^{c} (21.4)	17.6–24.9
Ca (mg kg^{-1})	213 ± 108^{a} (177)	76–405	136 ± 43^{a} (128)	89–211	134 ± 26^{a} (135)	89–179
Cl (mg (100 g)$^{-1}$)	629 ± 97^{a} (652)	460–786	267 ± 36^{b} (266)	202–328	439 ± 75^{a} (436)	326–527
Cu (mg kg^{-1})	3.8 ± 1.6^{a} (3.4)	2.6–8.1	1.5 ± 0.2^{b} (1.5)	1.3–1.8	4.5 ± 2.5^{a} (4.3)	1.7–10.3
Fe (mg kg^{-1})	4.2 ± 1.7^{a} (3.6)	1.9–6.8	1.7 ± 1.2^{b} (1.2)	0.7–4.7	1.4 ± 0.7^{b} (1.2)	0.6–2.5
K (mg (100 g)$^{-1}$)	223 ± 38^{a} (227)	154–295	261 ± 55^{ab} (242)	193–343	289 ± 46^{b} (306)	210–359
Mg (mg kg^{-1})	938 ± 262^{a} (823)	651–1473	435 ± 108^{b} (433)	283–610	567 ± 99^{b} (506)	467–705
Mn (mg kg^{-1})	0.31 ± 0.07^{a} (0.30)	0.22–0.44	0.16 ± 0.03^{b} (0.17)	0.11–0.22	0.11 ± 0.04^{b} (0.09)	0.06–0.20
Na (mg (100 g)$^{-1}$)	572 ± 143^{a} (519)	399–793	157 ± 33^{b} (158)	90–194	266 ± 60^{b} (255)	186–352
Ni (mg kg^{-1})	0.02 ± 0.01^{a} (0.02)	<0.02–0.04	0.02 ± 0.01^{a} (0.02)	<0.02–0.04	0.05 ± 0.02^{b} (0.03)	0.02–0.09
P (mg (100 g)$^{-1}$)	147 ± 39^{a} (140)	107–195	260 ± 20^{b} (262)	230–285	249 ± 23^{b} (256)	208–280
Rb (mg kg^{-1})	0.44 ± 0.16^{a} (0.41)	0.22–0.73	0.68 ± 0.12^{b} (0.66)	0.51–0.95	0.77 ± 0.12^{b} (0.78)	0.54–0.91
S (mg (100 g)$^{-1}$)	257 ± 54^{a} (243)	207–383	229 ± 24^{a} (226)	197–270	338 ± 55^{b} (340)	235–444
Se* (mg kg^{-1})	<QL*	–	<QL	–	<QL	–
Sr (mg kg^{-1})	3.8 ± 0.5^{a} (3.8)	3.0–4.7	1.8 ± 0.2^{b} (1.7)	1.6–2.3	2.3 ± 0.3^{b} (2.3)	1.9–2.7
Zn (mg kg^{-1})	17.7 ± 2.2^{a} (16.9)	15.6–23.0	12.6 ± 1.3^{b} (12.3)	10.8–15.2	17.7 ± 2.3^{a} (17.2)	14.0–22.5

Mean ± sd with equal superscript letters for same element, indicates no statistical differences within species (p > 0.05).
* Selenium range always between DL and QL (quantification limit), error percentage too large to quantify concentration.

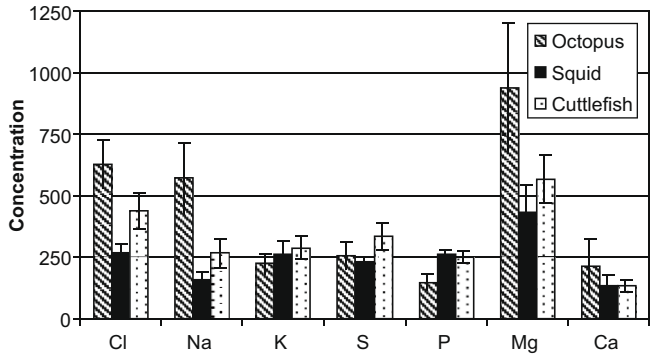

Fig. 1. Mean + sd comparison of macro nutrient elements in cephalopods (Cl, Na, K, S, P in mg (100 g)$^{-1}$, Mg and Ca in mg kg^{-1}).

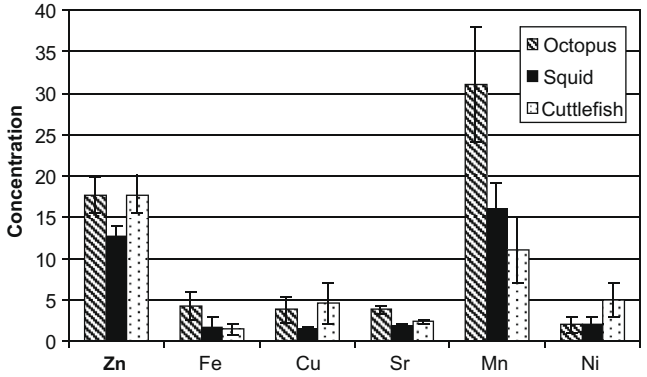

Fig. 2. Mean + sd comparison of micro nutrient elements in cephalopods (Zn, Fe, Cu and Sr in mg kg^{-1}, Mn and Ni in μg (100 g)$^{-1}$).

juveniles of these three species were reported by Villanueva and Bustamante (2006).

3.4. Nutrient elements

Regarding macro elements, the lowest levels observed corresponded to Mg and Ca. Fishery products are considered poor sources of Mg and Ca (Lall, 1995). Nevertheless, Mg concentrations are always higher than Ca concentrations (Oehlenschläger, 1997). This fact was also observed this study (Fig. 1). The magnesium:calcium ratio (mg/mg) in cephalopods was approximately 3 or 4. The average Mg concentration observed in octopus (938 mg kg^{-1}) was significantly higher than in the other two cephalopod species (p < 0.05), ranging between 651 and 1473 mg kg^{-1}. Squid samples showed the lowest levels, ranging between 283 and 610 mg kg^{-1} (Table 3). No significant differences were observed within species in what concerns average Ca levels, although the highest concentration (405 mg kg^{-1}) was found in octopus and the lowest in squid and cuttlefish (89 mg kg^{-1}). These levels are within the ranges observed by other researchers for some cephalopods, bivalve and gastropoda species (Carvalho et al., 2005; Oehlenschläger, 1997; Segar et al., 1971; Vlieg et al., 1991), although lower concentrations were observed in wild juvenile cephalopods in a study performed by Villanueva and Bustamante (2006). A previous study (Karakoltsidis et al., 1995) also reported some differences, namely in squid.

Iron, Cu, Zn and Mn are important elements for health. These elements play the role of functional elements in various metalloenzymes, which have particular catalytic function in living organisms. As frequently observed, concentrations of these elements in cephalopods are high, when compared to other seafood species (Bustamante et al., 2000; Causeret, 1962; Lall, 1995; Raimundo

Table 4
Cadmium, lead and total mercury (mg kg^{-1} wet basis) contents in edible part of the three studied cephalopods (mean ± standard deviation, median and range)

Toxic elements	Commom octopus (N = 10)		Squid (N = 10)		Common cuttlefish (N = 12)	
	Mean ± sd (median)	Range	Mean ± sd (median)	Range	Mean ± sd (median)	Range
Cd	0.38 ± 0.39^{a} (0.33)	0.03–1.3	0.04 ± 0.03^{b} (0.04)	0.01–0.10	0.31 ± 0.28^{a} (0.23)	0.03–1.0
Pb	0.02 ± 0.01^{a} (0.02)	<0.02–0.04	0.10 ± 0.03^{b} (0.09)	0.07–0.14	0.04 ± 0.01^{a} (0.04)	0.03–0.05
Total Hg	0.13 ± 0.06^{a} (0.12)	0.07–0.21	0.05 ± 0.02^{b} (0.05)	0.02–0.08	0.15 ± 0.10^{a} (0.11)	0.08–0.37

Mean ± sd with equal superscript letters for same element, indicates no statistical differences within species (p > 0.05).

et al., 2004). Of these four elements, zinc concentrations were the most relevant, corroborating the hypothesis that zinc is always present in seafood, but concentrations found in molluscs are generally higher (Celik & Oehlenschläger, 2004; Lall, 1995). In the present study, average levels reached 17.7 mg kg^{-1}, in octopus and cuttlefish samples (Fig. 2 and Table 3), while squid showed the lowest significant average value, of 12.6 mg kg^{-1} ($p = 0.000$). Similar values had also been found in previous studies (Miramand & Bentley, 1992; Napoleão et al., 2005; Seixas et al., 2005b; Soldevilla, 1987; Vlieg et al., 1991). The highest average Cu concentrations, approximately 4.0 mg kg^{-1}, were found in octopus and cuttlefish samples (no significant differences were observed), with some samples reaching 10 mg kg^{-1}. The maximum value found for squid samples was 1.8 mg kg^{-1} (Table 3). Soldevilla (1987), Seixas et al. (2005b) and Napoleão et al. (2005) found identical amounts for the same octopus species. Nevertheless, some results found in literature for similar species do not agree with the former (Carvalho et al., 2005; Miramand & Bentley, 1992; Miramand et al., 2006; Segar et al., 1971; Villanueva & Bustamante, 2006). In a different study, high concentrations were found in squid; however the species is not specified (Lall, 1995). Iron values found in octopus and squid samples were identical to Cu values (Fig. 2). The highest significant average Fe concentration, approximately 4.0 mg kg^{-1}, was found in octopus ($p = 0.00$); the concentration of this element was only approximately 1.5 mg kg^{-1} in squid and cuttlefish. Some authors observed higher levels (Karakoltsidis et al., 1995; Lall, 1995; Miramand et al., 2006; Seixas et al., 2005b; Villanueva & Bustamante, 2006) in identical species caught in other areas; however, similar concentrations were found in octopus specimens from the Portuguese coast (Napoleão et al., 2005), the Saharan Fishing Bank (Soldevilla, 1987) and the French Atlantic coast (Miramand & Bentley, 1992). Vlieg et al. (1991) also reported higher values than those observed in this study for arrow squid (*Nototodarus* sp.) and broad squid (*Sepiotheutis bilineata*). These different Fe concentrations may be explained by the different maturity stages of the specimens in question, sampling period and different habitats (Carvalho et al., 2005). Published data indicate that Mn amounts are generally low and identical in most seafood species (Astorga España, Rodríguez Rodríguez, & Díaz Romero, 2007; Bustamante et al., 2000; Oehlenschläger, 1997), although higher concentrations are found in some lamellibranchia and gastropoda (Segar et al., 1971). Manganese concentrations in this study were low, ranging between 0.06 and 0.44 mg kg^{-1}, in cuttlefish and octopus, respectively. The Mn profile was similar to that displayed for Fe (Fig. 2). The highest average concentration was found in octopus (0.31 mg kg^{-1}), followed by squid (0.16 mg kg^{-1}) and cuttlefish (0.11 mg kg^{-1}) (Table 3). These values correspond to the usual concentrations found in cephalopods (Miramand & Bentley, 1992; Napoleão et al., 2005; Seixas et al., 2005b; Soldevilla, 1987). Levels were higher in cuttlefish juveniles, reaching approximately 1.4 mg kg^{-1} (Villanueva & Bustamante, 2006). Significant differences in average Fe, Cu, Zn and Mn concentrations were always found between squid and octopus ($p = 0.003$, $p = 0.002$, $p = 0.000$ and $p = 0.01$, respectively).

The concentrations of the last four elements found in this study suggest that cephalopods may contribute significantly to the daily intake (DI) needs, especially in what concerns Cu and Zn.

Little is known about the function of Sr and Rb in organisms. Moreover, the number of studies on the concentrations of these two elements in fishery products is small; nevertheless, Varo (1992) found that Sr levels in seafood could be 20 times higher than in meat. The results of this study indicate that cephalopods may constitute a good source of Sr. Concentrations found were between 1.6 mg kg^{-1} (squid) and 4.7 mg kg^{-1} (octopus), The Sr profile was identical to that observed for some elements (Figs. 1 and 2); the average concentration in octopus (3.8 mg kg^{-1}) was significantly higher than those found in cuttlefish (2.3 mg kg^{-1}) and squid (1.8 mg kg^{-1}). Vlieg et al. (1991) found identical concentrations in squid species. Lamellibranchia and gastropoda species (Segar et al., 1971) also showed similar levels. Concentrations measured by Villanueva and Bustamante (2006) in the same species were higher, particularly in cuttlefish, possibly because this study focused on juveniles. Lowest concentrations were observed in studies performed in fish species (Garg & Ramakrishna, 2006; Teeny et al., 1984). Rubidium concentrations ranged between 0.22 and 0.95 mg kg^{-1}, respectively, in octopus and squid. These concentrations were lower than Sr concentrations and agree with the existing literature (Carvalho et al., 2005; Villanueva & Bustamante, 2006). In a study of fish species from different regions of India, performed by Garg and Ramakrishna (2006), a large dispersion of Rb concentrations was observed. No significant differences were found between squid and cuttlefish, for both elements. A similar concentration pattern was observed for Rb and K in the three species (octopus < squid < cuttlefish), which can be explained by the electrochemical affinity of these elements.

The last essential trace element analysed was Ni. A few years ago, the physiological functions of this element were not clear (Lall, 1995). Nowadays it is thought that Ni plays a role in hormone, lipid and cell membrane metabolism, as well as activating enzymes associated with the glucose breakdown and use (Acu-Cell, 2007). The nickel concentration profile was different from profiles observed for other microelements (Fig. 2). The average levels observed in octopus and squid, of 0.02 mg kg^{-1} (no significant differences were found), were lower than the average level observed in cuttlefish, of 0.05 mg kg^{-1}. These concentrations are considered low, possibly because the specimens collected originate from an unpolluted ocean area. These results agree with those published by other authors (Carvalho et al., 2005; Miramand & Bentley, 1992; Varo, 1992). Nevertheless, Napoleão et al. (2005) and Villanueva and Bustamante (2006) observed higher concentrations of Ni in the same octopus species.

3.5. Non-metals

Selenium and bromine are non-metals, classed as essential or toxic, depending on their concentration (Lall, 1995). Bromine data in seafood is relatively scarce; however, this element is known to have anti-seizure properties, as potassium bromide or sodium bromide, appearing to be effective trace elements regarding the prevention of hyperthyroid conditions (Acu-Cell, 2007). In this study, Br concentrations found in octopus ranged between 26.8 and 40.2 mg kg^{-1}, whereas average Br values found in squid and cuttlefish samples were lower than those found in octopus (13.3 and 21.5 mg kg^{-1}, respectively). Significant differences were observed between the three species ($p = 0.000$ for squid/octopus and $p = 0.03$ for squid/cuttlefish and octopus/cuttlefish). Garg and Ramakrishna (2006) found similar average Br concentrations, between 43 and 93 mg kg^{-1} (dry weight basis), in fish from different regions of India, having considered this element as a pollutant.

Selenium in low concentrations, in addition to its role as an essential micronutrient for normal growth and reproduction, has a protective effect against toxic elements in organisms (Barghigiani, Pellegrini, ÚUlivo, & De Ranieri, 1991; Feroci, Badiello, & Fini, 2005). Although Se was found in all samples analysed, the concentrations observed ranged between the detection limit (DL) and the quantification limit (QL). Concentrations found in squid and cuttlefish samples were close to the QL (1.0 mg kg^{-1}, dry weight; approximately 0.2 mg kg^{-1}, wet weight) whereas concentrations found in octopus were closed to the DL (0.6 mg kg^{-1}, dry weight; approximately 0.1 mg kg^{-1}, wet weight). Similar values were found in studies performed in molluscs (Astorga España et al., 2007; Napoleão et al., 2005; Plessi, Bertelli, & Monzani, 2001; Seixas et al., 2005b). Seafood species usually showed values

between 0.10 and 0.60 mg kg^{-1}, wet weight basis (Carvalho et al., 2005; Lall, 1995; Oehlenschläger, 1997; Plessi et al., 2001; Varo, 1992).

3.6. Toxic elements

Non-essential functions are known for Hg, Cd and Pb, which are considered harmful (Ruiter, 1995). Concentrations in marine organisms reflect environmental pollution (Belitz et al., 2004; Carvalho et al., 2005; Ruiter, 1995); bioaccumulation and biomagnification are observed in some organisms. Lead is considered a chronic or accumulative poison (Seixas et al., 2005b). In the species studied, Pb concentrations were the lowest, among toxic elements. The profile observed for this element is different from the profiles observed for the remaining two. Of all samples analysed, squid reached the highest concentration, 0.14 mg kg^{-1}, representing almost one tenth of the limit proposed by the EU for cephalopods (1.0 mg kg^{-1}), whereas average concentrations were low in cuttlefish and octopus samples, of 0.04 and 0.02 mg kg^{-1}, respectively. These values agree with the results published by some authors (Martí-Cid, Bocio, Llobet, & Domingo, 2007; Miramand & Bentley, 1992) despite being lower than results published by others (Raimundo et al., 2004; Seixas et al., 2005; Villanueva & Bustamante, 2006).

Mercury is one of the most toxic elements, with seafood representing one of its major sources in the human food chain (Plessi et al., 2001). Total Hg levels detected in cephalopods were always lower than the limit set by the EU (0.5 mg kg^{-1}), in all samples studied. Average Hg concentrations were similar in octopus and cuttlefish, of approximately 0.14 mg kg^{-1}; however, maximum concentrations were found in cuttlefish (0.37 mg kg^{-1}) whereas the lowest levels, of approximately 0.02 mg kg^{-1}, were found in squid. These values agree with results reported by other authors, for the same species (Bustamante et al., 2006; Pierce, Stowasser, Hastie, & Bustamante, 2007; Villanueva & Bustamante, 2006). In general terms, it may be said that total Hg concentrations in cephalopods are low (Plessi et al., 2001; Raimundo et al., 2004; Seixas et al., 2005a, 2005b; Villanueva & Bustamante, 2006) and do not represent a risk for human consumption.

Molluscs very often accumulate cadmium in digestive glands (Bustamante et al., 1998); however, minor amounts are also found in the mantle and arms (Miramand & Bentley, 1992). In most organisms, this element may compete with Fe, Cu and Zn, which turns its presence into a serious hazard (Carvalho et al., 2005). Regarding the cephalopods studied, modest average amounts were found in squid (0.04 mg kg^{-1}), when compared to octopus and cuttlefish, in which average concentrations of 0.38 and 0.31 mg kg^{-1} were found, respectively (Table 4). In squid, data obtained agrees with data obtained by other authors (Martí-Cid et al., 2007; Pierce et al., 2007; Villanueva & Bustamante, 2006). The limit proposed by the EU for cadmium (1.0 mg kg^{-1}) was reached in one cuttlefish sample and exceeded in two octopus samples, despite this fact the average levels did not exceed the indicative value. Bustamante et al. (1998), Raimundo et al. (2004) and Miramand et al. (2006) found identical concentrations for similar species. Nevertheless, lower concentrations were reported in cuttlefish, in different studies (Martí-Cid et al., 2007; Miramand & Bentley, 1992; Villanueva & Bustamante, 2006). In octopus, some authors also observed higher levels (Seixas et al., 2005b; Soldevilla, 1987), possibly due to the different environments.

In general terms, mean and median concentrations of these toxic elements are identical (Table 4), which indicates that the cephalopod population in the study habitat may be well-known. Statistical analysis performed for these three toxic metals did not reveal significant differences between octopus and cuttlefish. This fact shows that squid must have a different behaviour from the other two cephalopod species, probably due to its size and diet.

3.7. Influence of age, sex and relationships between element levels

Comparison between age groups was performed for each species. Differences in Hg concentrations were only found in squid. No significant differences were found between age groups in octopus and cuttlefish. Several studies were performed in seafood to test the significance of differences or find correlations between age or size and Hg concentrations; however, the results obtained were inconsistent (Pierce et al., 2007; Raimundo et al., 2004; Seixas et al.,2005a, 2005b). In a similar way to what occurred in the present study, Pierce et al. (2007) found that body size was a significant factor in *Loligo forbesi*, whereas other authors (Raimundo et al., 2004; Seixas et al., 2005b) did not find any significant differences in octopus. Significant differences in element concentrations with sex were only found for Mn and only in squid and cuttlefish ($p = 0.001$ and $p = 0.03$, respectively). Mn concentrations were higher in females of these two species, probably due to reproductive requirements.

Numerous positive and negative correlations were observed among elements for all cephalopod species (data not show). Some of the most significant ($p < 0.005$ and absolute correlation coefficient $r \geqslant 0.8000$) positive correlations found were between K/Mg/Zn, Mn/Fe, Zn/Cu, Fe/Cd, and Cu/Cd. In octopus some negative correlations were also found, such as Sr/K and Sr/P. Seixas et al. (2005b) found similar relationships with Cd. These correlations may be explained by the Cd interaction with the metabolism of these essential metals (Peraza, Ayala-Fierro, Barber, Casarez, & Rael, 1998).

3.8. Daily and provisional tolerable weekly element intakes

Several suggestions have been made regarding daily intake (DI) values relative to elements (Belitz et al., 2004; Lall, 1995; Oehlenschläger, 1997). The values proposed by Belitz et al. (2004) and Acu-cell (2007) are listed in Table 5. Considering the concentrations found in the present work, DI percentages for each element are also shown in Table 5. Analysis of the results included in this table shows that cephalopods may contribute significantly to the DI of S, P, Mg, Zn and Cu, representing 10–38% of the DI. Daily intakes of Ni and Sr are also important. Additionally, Ca, Fe and Mn percentages are small, ranging between 0.3% and 3%. The Na percentage (based on minimum intake for good health) is higher than the K percentage, especially for octopus, which indicates a high Na/K ratio. This fact is important, since some studies suggest an increased risk of developing high blood pressure and cardiovascular disease (Astorga España et al., 2007). The contribution of octopus and cuttlefish is more relevant than that of squid, in general terms. This dif-

Table 5
Daily intake and contribution of each element in octopus, squid and cuttlefish

Elements	Daily intake (DI)[a] (mg)	Percent of DI in a 100 g portion of cephalopods (octopus–squid–cuttlefish)
Ca	1000	2–1–1
Cl	3500	18–8–12
Cu	1–1.5	30–12–36
Fe	15	3–1–1
K	2000–5900	6–7–7
Mg	300–400	27–12–16
Mn	2–5	1–0.5–0.3
Na	<2300	25–7–12
Ni	0.025–0.030	7–7–18
P	800–1200	15–26–25
S	800–1000	28–25–38
Sr[b]	1–5	15–7–9
Zn	10–15	14–10–14

[a] Belitz et al. (2004).
[b] Acu-Cell (2007).

ference could be explained by a different diet. According to the Joint Food and Agriculture Organisation/World Health Organisation (FAO/WHO) (WHO, 1999, 2003) provisional tolerable weekly intakes (PTWI) for Hg, Cd and Pb are 5 μg kg^{-1} body weight, 7 μg kg^{-1} body weight and 25 μg kg^{-1} body weight, respectively. With basis on a weekly average consumption of fishery products in Portugal of 1120 g (160 g/day) (FAO, 2007), an average human body weight of 60 kg and the average Hg, Cd and Pb values found in this study (Table 4), estimated weekly intakes of Hg and Pb are much lower than established PTWIs, for all three species. Nevertheless, the PTWI of 7 μg kg^{-1} body weight for cadmium is almost reached or slightly exceeded when cuttlefish (6.7 μg kg^{-1} body weight) and octopus (9.7 μg kg^{-1} body weight) are consumed. However, it would be very unlikely for a person to consume the aforementioned amounts of these species per week; therefore, the values obtained are overestimated.

4. Conclusion

The elemental profile is quite similar for the three species studied, the major elements being Cl, Na, K, S, P, Mg and Ca, followed by Br, Zn, Fe, Cu, Sr, Rb, Se, Mn and Ni. This indicates that these elements may have the same physiological importance in the three cephalopod species. Results obtained in this study also suggest that cephalopods may constitute a good source of some essential elements, such as P, Mg, Zn and Cu. In general, comparison of element concentrations in octopus, squid and cuttlefish shows that the lowest concentrations of most elements are found in squid; therefore, its contribution for DIs is lower than that of the other two species. Regarding Hg and Pb intakes, consumption does not guide to any concerns, although it should be moderate when considering Cd intake, especially regarding octopus.

Acknowledgements

The authors would like to thank PNAB/EU Data Collection Regulation, particularly Dr. João Pereira, for providing cephalopod specimens. We would also like to thank Amparo Gonçalves for her help with statistical analysis. We are grateful to Pedro da Conceição, Fernanda Queirós and Maria do Céu Reis for their participation in sample preparation. This work was sponsored by the QCA III PO MARE Project (22-05-01-FDR-00008 – "Vigilância, Segurança e Qualidade Alimentar").

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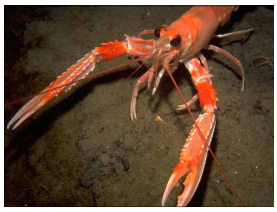
Paper II



Research Article

Received: 4 May 2009 Revised: 24 July 2009 Accepted: 28 July 2009 Published online in Wiley Interscience: 24 September 2009

(www.interscience.wiley.com) DOI 10.1002/jsfa.3754

Chemical characterisation of *Nephrops norvegicus* from Portuguese coast

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Abstract

BACKGROUND: Among seafood products, crustaceans are an important group owing to their nutritional benefits and high market value. The aim of this study was to evaluate the proximate composition as well as essential and non-essential elements in the edible muscle of *Nephrops norvegicus* (Norway lobster).

RESULTS: The studied crustacean showed a high protein level of about 210 g kg^{-1} and a mineral level of about 24 g kg^{-1}. The pattern for the elements studied was Cl > Na > K > P > S > Ca > Mg > Br > Fe > Sr ≈ Zn > Cu > Mn > Rb > Se > Ni > Cr. Regarding non-essential elements, the average total Hg content was 0.40 mg kg^{-1}, but two samples exceeded the legal limit set by the European Union (EU) for Hg in crustaceans (0.50 mg kg^{-1}). The levels of Pb and Cd found in all samples were below the EU limits (0.50 mg kg^{-1} for both metals).

CONCLUSION: *Nephrops norvegicus* is a good source of protein and contains most minerals at levels sufficient to satisfy the dietary reference intake. The amounts of Pb and Cd are not a concern. Hg levels indicate some contamination; however, taking into account the type of consumption of this species, it can be concluded that this does not represent a risk in terms of the human diet.

Keywords: *Nephrops norvegicus*; essential elements; non-essential elements; FAAS; EDXRF

INTRODUCTION

The importance of seafood products in a balanced diet is well known. They are protein-rich with a good amino acid composition and a high proportion of polyunsaturated fatty acids.[1,2] These products contain most of the 90 elements that appear in nature.[3,4] C, H, N, O and S (structural elements) followed by macroelements such as Cl, K, P, Na, Mg and Ca constitute the largest amounts found.[1,4] Other elements such as Fe, Cu, Zn, I, Mn, Se, Ni and Cr occur in lower concentrations and are considered trace or ultratrace elements. Depending on their physiological functions, elements are classified as either essential when their functions are well confirmed or non-essential when their roles have not been well demonstrated.[4] Some elements such as Br and Sn remain to be classified. Hg, Pb and Cd are non-essential elements found in marine fish products. Since 2001 there have been legal limits set by the European Union (EU)[5,6] on Hg, Pb and Cd levels in several types of food, including seafood.

Among seafood products, crustaceans are important not only owing to their nutritional properties but also because of their high market value.[7] Shellfish are widely consumed in Southern Europe; for example, the annual consumption per capita in Portugal reached 11.5 kg in 2006.[8] *Nephrops norvegicus* (Norway lobster) production in 2007 was 226.5 t out of 970.9 t of total crustacean catches.[9] In spite of this, the biochemical composition of some deep-sea crustacean species has not been studied to the same extent as that of others.[10] Several studies have been done in an attempt to determine trace metal accumulation in crustaceans instead of studying element benefits.[11–20]

The aim of this study was to characterise the edible muscle of *N. norvegicus* in terms of proximate and elemental composition and therefore to contribute to a better knowledge of nutritional benefits and hazards of this species.

EXPERIMENTAL

Sample collection and preparation

Nephrops norvegicus specimens were caught off the southwestern Portuguese coast, Algarve (Portimão, 36° 47.6′ N, 8° 9.7′ W, depth 377 m; Sagres, 36° 55.4′ N, 9° 7.3′ W, depth 589 m) by the R/V *Noruega* from Instituto Nacional dos Recursos Biológicos in 2004. Collected specimens were immediately frozen on board, stored in plastic bags, transported to the laboratory and kept at −21 °C

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Table 1. Biological characteristics of studied *Nephrops norvegicus*

Class	Carapace length range (mm)	Total weight range (g)	*n*
I	28–30	13–17	2 pools (10 ♂, 10 ♀)
II	34–36	24–29	2 pools (10 ♂, 10 ♀)
III	40–42	40–48	2 pools (10 ♂, 10 ♀)
IV	46–48	61–73	2 pools (10 ♂, 10 ♀)
V	55–57	104–128	2 pools (10 ♂, 10 ♀)

until preparation. After thawing at room temperature, carapace length, total weight and sex were recorded (Table 1). The male and female are distinguished by a difference in shape of the first pair of pleopodes: they are rigid and amended in the copulation organ in males, while they are much thinner and flexible in females. Ten composite samples (ten specimens in each) of edible muscle, five for males and five for females, two pools for each carapace length class (28–30, 34–36, 40–42, 46–48 and 55–57 mm), were obtained and homogenised in a food blender. Homogenised samples were vacuum sealed in individual plastic bags, coded for easy identification and stored at −21 °C until required for analysis.

Sample analysis

The proximate composition – protein (Kjeldahl method), fat (Soxhlet extraction with ethyl ether), moisture (sample dried overnight at 105 °C) and ash (sample incinerated at 500 °C to constant weight) – was determined according to AOAC methods.[21]

Quantification of K, Na, Mg, Ca, Zn, Fe, Cu, Mn, Ni, Cr, Cd and Pb levels was performed by flame atomic absorption spectrometry (FAAS) according to Jorhem.[22] The method is based on sample (10–15 g) incineration and dissolution in HNO_3. All analyses were carried out at least in duplicate using a Spectr AA-20 spectrometer (Varian Australia, Mulgrave, Australia) with deuterium background correction. Quantification was made by the external calibration method. Detection limits (DLs, mg kg^{-1} wet weight) were calculated from the residual standard deviation (RSD) of the response and the slope (S) of the calibration curve for each element: DL = 3.3 × RSD/S. The values obtained were 0.01 (K), 0.09 (Na), 0.02 (Mg), 0.08 (Ca), 0.06 (Zn), 0.32 (Fe), 0.02 (Cu), 0.01 (Mn), 0.02 (Ni), 0.09 (Cr), 0.01 (Cd) and 0.02 (Pb) mg kg^{-1} wet weight.

Total Hg was determined by cold vapour atomic absorption according to the method developed by Hatch and Ott[23] and described by Joiris *et al*.[24] using a Coleman MAS-50D mercury analyser system (Bacharach, Inc., Pittsburgh, PA, USA). In this method, homogenised samples (1 g) are first digested with concentrated H_2SO_4, then Hg is oxidised to Hg^{2+} with 50 g L^{-1} potassium permanganate. Subsequently, Hg is reduced to Hg^0 with 200 g L^{-1} stannous chloride, and the volatile Hg^0 is bubbled into the closed system of the mercury analyser. All samples were analysed in duplicate. Concentrations were calculated from linear calibration obtained by measurement of the absorbance of standard solutions. The DL (calculated as above) was 0.01 mg kg^{-1} wet weight.

P was determined spectrophotometrically according to ISO 13 730[25] using a UNICAM UV 5 220 spectrophotometer (ATI UNICAM, Cambridge, UK) In this method, samples (5 g) are incinerated and dissolved in 200 g L^{-1} HCl. Colorimetric measurements are performed on the yellow compound obtained from the reaction of P with a mixture of ammonium vanadate and ammonium molybdate. Quantification was made by the external calibration method. The DL (calculated as above) was 0.01 mg kg^{-1} wet weight.

All laboratory ware was cleaned with 100 g L^{-1} HNO_3 or 200 g L^{-1} HCl for 24–48 h and rinsed with ultrapure water (18.2 MΩ cm). All chemicals were of reagent grade. Commercial standard solutions (1 g L^{-1}) for K, Na, Mg, Ca, Zn, Fe, Cu, Mn, Ni, Cr, Cd, Pb, Hg and P (Merck, Darmstadt, Germany) were used.

To analyse Cl, S, Br, Sr, Rb and Se, samples were freeze-dried (48 h at −40 °C and ∼0.1 atm), pulverised, immediately vacuum sealed in individual coded plastic bags and stored at −21 °C until further analysis. Determination of these six elements was carried out according to Carvalho *et al*.[26] using an energy-dispersive X-ray fluorescence (EDXRF) spectrometer consisting of a PW 1140 X-ray tube (100 kV, 80 mA; Philips, Eindhoven, The Netherlands) equipped with a changeable Mo secondary target and Si(Li) detector (Oxford, High Wycombe, UK). The energy resolution was 135 eV at 5.9 keV and the acquisition system was a Nucleus PCA card (Oxford, High Wycombe, UK). Two pellets were prepared for each sample. DLs (mg kg^{-1} dry weight) for these elements were determined by the signal-to-noise approach, where the equipment compares the signal of each element with blank samples and establishes the minimum concentration at which the element is reliably detected. The values obtained were 10 (Cl), 10 (S), 0.8 (Br), 0.5 (Sr), 1.1 (Rb) and 0.6 (Se) mg kg^{-1} dry weight.

Analytical data for elements are reported as g kg^{-1} or mg kg^{-1} on a wet weight basis.

Five certified reference materials were tested under the same conditions as the samples in order to assess the accuracy of the analytical method: LUTS-1 (National Research Council of Canada, Ottawa, Canada) non-defatted lobster hepatopancreas (used only in the FAAS technique for essential elements); TORT-2 (National Research Council of Canada, Ottawa, Canada) lobster hepatopancreas; DORM-2, dogfish muscle (National Research Council of Canada, Ottawa, Canada); SMRD-2000, canned matrix meat (Swedish Meats R&D and Scan Foods/National Food Administration, Kävlinge, Sweden); MA-A-2, fish flesh (International Atomic Energy Agency, Vienne, Austria) (used only in EDXRF technique). The results obtained in this study were in good agreement with the certified values (Table 2).

Considering the provisional tolerable weekly intake (PTWI, expressed in mg kg^{-1}) of non-essential metals, a portion of seafood and a person's body weight (BW, expressed in kg), one can estimate the maximum number of meals (X) that can be consumed:

$$X = (\text{PTWI} \times \text{BW})/(C \times D)$$

where C is the mean concentration of metals in seafood (expressed in mg kg^{-1}) and D is the portion of seafood (expressed in g) per meal.

Statistical analysis

All data analysis was carried out using Statistica 6.1 (StatSoft, Inc., Tulsa, OK, USA). Normality and variance homogeneity were confirmed by the Lilliefors test and the Levene test respectively.[27] The Pearson correlation between elements was determined. The Student t test was used to verify the influence of sex on various elements. Significant differences were considered at $P < 0.05$.

RESULTS AND DISCUSSION

The proximate chemical composition of *N. norvegicus* edible muscle showed the normal pattern for crustaceans.[2,28,29] Thus the protein content, about 210 g kg^{-1} (206 ± 7 g kg^{-1}), was similar to that found by Rosa and Nunes[10] in the same lobster species and by Gökoðolu and Yerlikaya[30] in *Portunus pelagicus* (swim crab).

Table 2. Results of analysis ($n = 4$) for some certified reference materials (mean ± standard deviation, mg kg^{-1} dry weight)

Material		K	Na	P	Mg	Ca	Zn	Fe	Cu	Sr	Mn	Se	Ni	Cr	Hg	Pb	Cd
LUTS-1[a]	Certified	948 ± 72	–	–	89.5 ± 4.1	203 ± 33	12.4 ± 0.8	11.6 ± 0.9	15.9 ± 1.2	–	1.20 ± 0.13	–	0.200 ± 0.034	<DL[b]	–	–	–
	Obtained	955 ± 23			90.8 ± 2.2	197 ± 16	13.7 ± 0.7	12.1 ± 0.4	15.4 ± 0.2		1.28 ± 0.03		0.195 ± 0.009	0.079 ± 0.012			
SMRD-2000	Certified	1859 ± 85	8533 ± 281	1075 ± 47	–	70.3 ± 8.3	–	6.33 ± 1.66	–	–	–	–	–	–	–	–	–
	Obtained	1938 ± 38	8346 ± 280	1108 ± 11		66.1 ± 7.4		4.94 ± 0.09									
TORT-2	Certified	–	–	–	–	–	180 ± 6	105 ± 13	106 ± 10	45.2 ± 1.9	13.6 ± 1.2	5.63 ± 0.67	2.50 ± 0.19	0.77 ± 0.15	0.27 ± 0.06	0.35 ± 0.13	26.7 ± 0.6
	Obtained						175 ± 1	109 ± 3	93 ± 5	43.4 ± 3.2	13.1 ± 0.1	5.34 ± 0.43	2.37 ± 0.08	0.65 ± 0.05	0.28 ± 0.00	0.35 ± 0.06	26.8 ± 0.1
DORM-2	Certified	–	–	–	–	–	25.6 ± 2.3	142 ± 10	2.34 ± 0.16	–	3.66 ± 0.34	1.40 ± 0.09	19.4 ± 3.1	34.7 ± 5.5	4.64 ± 0.26	0.065 ± 0.007	0.043 ± 0.012
	Obtained						23.7 ± 2.6	141 ± 4	2.50±0.55		3.62 ± 0.18	1.40 ± 0.10	16.4 ± 0.2	30.6 ± 2.4	4.48 ± 0.12	0.066 ± 0.004	0.040 ± 0.003
MA-A-2	Certified	–	–	–	–	–	–	–	–	–	–	1.7 ± 0.3	–	–	–	–	–
	Obtained											1.5 ± 0.4					

[a] Values in mg kg^{-1} as bottled.
[b] Detection limit.

The mean mineral content ranged from 23.3 to 26.1 g kg^{-1}, in agreement with values recorded in *P. pelagicus* and *Callinectes sapidus* (blue crab)[30,31] but higher than those found in fish.[1] The fat content was always lower than 10 g kg^{-1} and the moisture content ranged between 750 and 800 g kg^{-1}. These values are also in accordance with those published in the literature.[10,31,32]

Tables 3 and 4 present the concentrations (mean, standard deviation (SD) and range) of macroelements and trace elements respectively found in *N. norvegicus* edible muscle. The profile for mineral content was Cl > Na > K > P > S > Ca > Mg > Br > Fe > Sr ≈ Zn > Cu > Mn > Rb > Se > Ni > Cr. There were no significant differences between males and females in the levels of any of these elements ($P > 0.05$).

The major elements present in *N. norvegicus* muscle were K, Cl and Na. Of these, K contributes to the intracellular ion balance as a monovalent cation, while Cl and Na are the main extracellular anion and cation respectively, assuming an important role in the acid–base balance.[4] The mean levels of K, Cl and Na were about 3.4, 4.7 and 4.4 g kg^{-1} respectively. The mean content found for Cl is much higher than that reported by Causeret[3] and Oehlenschläger[1] in fish species (average ∼1 g kg^{-1}). A higher level of K was observed by Karakoltsidis *et al*.[32] in the same lobster species (8.0 g kg^{-1}). This discrepancy could be due to the different geographical catching areas or even to the season. In contrast, some authors observed lower levels of Na and K in blue crab (3.2 and 3.0 g kg^{-1} respectively), European lobster (3.2 and 2.6 g kg^{-1} respectively) and American lobster (2.0 and 2.6 g kg^{-1} respectively).[30,33] Nevertheless, the values found for these two elements in the present study are comparable to those reported in other shellfish species.[4,26]

P and S were the second most abundant elements in *N. norvegicus* muscle. In living organisms, 80–85% of P is present in bones, the remainder being found in extracellular fluids, intracellular structures and cell membranes.[4] P is directly involved in energy-producing cellular reactions.[4] S is present in amino acids, polypeptides, proteins and enzymes. The mean contents of these two elements were 2.3 and 1.4 g kg^{-1} respectively. Values of S content are scarce in the literature; however, a study by Vlieg *et al*.[34] showed concentrations of S between 1.3 and 2.2 g kg^{-1} in three species of crustaceans, while Barrento *et al*.[33] reported levels around 1.7 g kg^{-1} in European and American lobsters. Levels of P are identical to those indicated by other authors in fish[1,35] and some shellfish.[34,36,37] Crustaceans such as blue and swim crabs showed a slightly lower content.[30,31] The similar results found for other crustaceans of different habitat, depth and genus indicate that different crustacean species use and metabolise these two elements in an identical manner.

Two cations that are essential nutrients for life are Ca and Mg. Ca participates in a number of regulatory functions, while Mg is essential for energy-requiring biological functions.[4] Seafood is considered a poor source of these elements, and fish species generally contain more Mg than Ca.[1] However, *N. norvegicus* muscle showed higher concentrations of these elements, with the mean content of Ca being greater than that of Mg (1.0 and 0.6 g kg^{-1} respectively). It is commonly accepted that crustaceans show this pattern owing to a biomineralisation process. In fact, crustaceans have a particularly active Ca metabolism and have the ability to form cyclically not only an external structure but also Ca storage forms.[38] Other authors have also indicated higher Ca than Mg levels in such species.[4,28,33,39] Thus, compared with other seafood, *N. norvegicus* can be a good source of Ca in human nutrition.

Data on Br in seafood are scarce, but it is known that this non-metallic element in the form of potassium bromide or sodium bromide has anti-seizure properties and seems to be an effective trace mineral in regard to hyperthyroid conditions.[40] According to Varo,[36] the average Br concentration in fish is about 2 mg kg^{-1}. In the present study, *N. norvegicus* showed higher amounts ranging from 26.8 to 44.3 mg kg^{-1}. Barrento *et al*.[20,33] and Mohapatra *et al*.[41] also found levels around 20 mg kg^{-1} or even higher in some crustaceans. These high concentrations of Br in crustaceans are probably due to species biology, habitat, food source and environment.

Shellfish usually show high levels of Fe, Zn and Cu and in this sense could make a good contribution to the daily requirement of these elements, which play an important role in human life. As constituents of metalloenzymes, Fe, Zn and Cu give rise to particular catalytic functions: Fe is essential in the transport, storage and utilisation of oxygen; Zn controls many processes of carbohydrate, lipid and protein metabolism and nucleoprotein synthesis; Cu is a cofactor for enzymes involved in glucose metabolism and haemoglobin synthesis. In crustaceans, Cu is required by the respiratory pigment haemocyanin. Many studies have been performed to determine levels of these three elements, some of them in crustaceans caught in several areas.[14,15,32,33,42,43] In the investigated species, high contents of Fe, Zn and Cu were reported. The mean concentrations of Fe, Zn and Cu found in *N. norvegicus* in the present study were 20.0, 17.4 and 10.6 mg kg^{-1}, with the largest quantity being detected for Fe (35.4 mg kg^{-1}). A different result was obtained by Capelli *et al*.[44] for *N. norvegicus* caught in the Ligurian Sea, where Zn and Cu contents were lower (13 and 6.2 mg kg^{-1} respectively). However, some studies performed on lobsters, shrimps and crabs indicated similar concentrations of Fe, Zn and Cu to those detected in the present study.[17,19,45,46] Data presented for the three elements in *N. norvegicus* edible muscle are in close agreement for this type of crustacean, which is one of the seafood species that can be a good source for human daily intake of Fe, Zn and Cu.

Few data are available on Sr and Rb in seafood, and the functions of these elements in organisms are unknown. The present study indicated a global mean Sr concentration of 17.8 mg kg^{-1} in *N. norvegicus* edible muscle. Levels of Rb (0.23–0.67 mg kg^{-1}) were lower than those of Sr, in agreement with the literature.[26,47,48] Agusa *et al*.,[49] on the other hand, reported higher Rb than Sr levels in some fish species from several locations in Cambodia, Indonesia and Thailand. In a study by Seixas and Pierce,[50] *Octopus vulgaris* showed Rb levels between 3 and 5 mg kg^{-1} dry weight, somewhat higher than those found in *N. norvegicus*. Species of Lamellibranchiata and Gastropoda showed similar levels of Sr to those observed in *N. norvegicus* in the present study.[37] Another study reported values around 35 mg kg^{-1} in other lobsters.[33] Some fish species contained less Sr,[26] while others from Manila Bay, especially demersal fish, accumulated higher levels,[47] probably owing to high levels of water contamination. A study by Varo[36] revealed lower levels of Sr and Rb in canned shrimp (11 and 0.2 mg kg^{-1} respectively).

The transition metal Mg is important as a cofactor activating a high number of enzymes to form metal–enzyme complexes and also as a constituent of certain metalloenzymes.[4] For example, the enzymatic function of Mn in lipid and carbohydrate metabolism and brain function is well known. The mean Mn concentration found in *N. norvegicus* was 0.75 mg kg^{-1}. This is considerably higher than the values usually found in fish and is in accordance with the report by Lall[4] that molluscs and crustaceans contain

Table 3. Macroelements (g kg^{-1} wet weight) in edible muscle of *Nephrops norvegicus* (mean ± standard deviation (SD) of males and females, global mean ± SD and range)

Value	Cl	Na	**K**	P	S	Ca	Mg
Mean ± SD (5 ♂)	4.6 ± 0.3	4.5 ± 0.5	3.5 ± 0.2	2.4 ± 0.2	1.3 ± 0.3	1.1 ± 0.5	0.6 ± 0.1
Mean ± SD (5 ♀)	4.8 ± 0.4	4.3 ± 0.4	3.4 ± 0.1	2.2 ± 0.1	1.6 ± 0.2	0.9 ± 0.2	0.6 ± 0.1
Mean ± SD (n = 10)	4.7 ± 0.4	4.4 ± 0.4	3.4 ± 0.2	2.3 ± 0.2	1.4 ± 0.3	1.0 ± 0.4	0.6 ± 0.1
Range	4.3–5.4	3.9–5.3	3.2–3.7	2.0–2.7	1.1–1.8	0.6–1.8	0.5–0.9

Table 4. Trace and ultratrace elements (mg kg^{-1} wet weight) in edible muscle of *Nephrops norvegicus* (mean ± standard deviation (SD) of males and females, global mean ± SD and range)

Value	Br	Fe	Sr	Zn	Cu	Mn	Se[a]	Rb	Ni	Cr
Mean ± SD (5 ♂)	32.5 ± 0.8	19.9 ± 10.3	16.5 ± 4.1	18.1 ± 8.0	11.1 ± 3.0	0.81 ± 0.36	<0.4	0.40 ± 0.14	0.18 ± 0.10	0.11 ± 0.06
Mean ± SD (5 ♀)	36.3 ± 6.7	20.0 ± 6.6	19.1 ± 6.4	16.7 ± 3.1	10.0 ± 1.1	0.69 ± 0.19	<0.4	0.51 ± 0.10	0.14 ± 0.05	0.14 ± 0.06
Mean ± SD (n = 10)	34.4 ± 4.9	20.0 ± 8.1	17.8 ± 5.3	17.4 ± 5.7	10.6 ± 2.2	0.75 ± 0.28	<0.4	0.46 ± 0.13	0.16 ± 0.08	0.13 ± 0.05
Range	26.8–44.3	7.7–35.4	12.1–29.0	13.8–32.4	8.2–15.5	0.48–1.45	–	0.23–0.67	0.09–0.34	0.05–0.23

[a] Se range always between detection limit (DL) and quantification limit (QL = 1.5 mg kg^{-1} dry weight); error percentage too large to quantify concentration.

significantly higher levels of this element than fish. According to Baden *et al.*,[51] the level of Mn in *N. norvegicus* specimens gives an indication of recent exposure to Mn concentrations in the bottom waters of their habitats. Several authors observed similar contents of Mn in *N. norvegicus*[29,32,44] and other decapod crustaceans.[14,15,17,33] A study by Kress *et al.*[52] showed high Mn levels in some deep-sea crustaceans (*Polycheles tylaphlops, Acnthephyra eximia* and *Aristeus antennatus*) from the southeastern Mediterranean Sea.

Ni and Cr are the last two trace elements studied that can be considered essential, depending on their concentration in tissues. At low levels, Ni is thought to be a factor in hormone, lipid and cell membrane metabolism. Insulin response is increased after Ni ingestion, which may be related to Ni activation of enzymes associated with the breakdown or utilisation of glucose. The most important physiological role of Cr is also to enhance the action of insulin.[40] Generally, the concentrations of these elements are low in seafood (0.01–0.5 mg kg^{-1}).[1,36] In the present study the content of Ni averaged 0.16 mg kg^{-1}, reaching 0.34 mg kg^{-1} in one sample. These levels are in the range of values reported by Carvalho *et al.*[26] in fish species but are lower than those reported by Paéz-Osuna *et al.*[14] and Canli *et al.*[53] in lobster (1.5 mg kg^{-1} dry weight) and prawn (∼2.7 mg kg^{-1} dry weight) respectively. The high values obtained by these two groups of authors are probably due to environmental metal pollution in the studied geographical regions. Segar *et al.*[37] also detected high amounts of Ni in mollusc species. Studies performed on bivalve and cephalopod species from New Caledonia lagoon also showed high levels of Ni.[54,55] These high values can be explained by the intense land-based Ni-mining activity in the area and also by urban development and lack of efficient wastewater treatment. Levels of Cr were similar to those of Ni, with a mean value of 0.13 mg kg^{-1} and a range from 0.05 to 0.23 mg kg^{-1}. Some authors reported similar results in crustaceans.[4,14,15,42] Fish species showed higher levels of Cr in some studies (1–2.4 mg kg^{-1} dry weight),[26,56] but a study by Agusa *et al.*[49] on fish from Southeast Asia showed values in the same range as those obtained in the present study. The low values found for Ni and Cr in *N. norvegicus* edible muscle probably indicate a non-contaminated catching area.

Se is considered both an essential element and a toxic element depending on its concentration. It is a component of the enzyme glutathione peroxidase in human and animal tissues, and Se deficiency is implicated in cardiovascular disease and higher risk of cancer of several organs such as the liver and lungs.[40] In contrast, Se excess can have toxic effects,[57] among which is the promotion of dental caries.[4] Some studies reported a role of Se against the toxicity of Hg, more precisely methyl mercury.[4,57–59] Although Se was detected in *N. norvegicus*, the values were near the limit of quantification (1.5 mg kg^{-1} dry weight) and showed a large error in variability. However, it can be estimated that levels were around 0.4 mg kg^{-1}, which is within the range observed in several seafood species.[1,19,57,60–62]

Dietary reference intake (DRI) is a general term encompassing a set of reference values for specific nutrients: estimated average requirement (EAR), recommended dietary allowance (RDA), adequate intake (AI) and tolerable upper intake level (UL).[63] Some suggestions have been made for mineral reference values.[1,2,4,40,63] Those proposed by Belitz *et al.*[2] and IOM[63] are given in Table 5. Taking into consideration the levels found in the present study, the contribution of each element to the DRI is also shown in Table 5. These results allow one to conclude that, in general, *N. norvegicus* is a good source of minerals, particularly Cu, Zn, Mg, Se and P, with Cu and Se being above the DRI. However, the ULs set by IOM[63] for Cu (10 mg day^{-1}) and Se (0.4 mg day^{-1}) are above the values obtained in the present study (1.7 mg Cu and <0.06 mg Se in a 160 g portion). Additionally, the Mn and Ni values are minimal, ranging between 3 and 7%. The Na/K ratio is high, which can indicate an increased risk of developing high blood pressure and cardiovascular disease.[64]

Table 6 presents data for the non-essential metals Hg, Cd and Pb. To date, no essential function in human life has been attributed to these metals.[58] Furthermore, their connection with several diseases is well known. Elemental Hg, inorganic Hg (Hg^{2+}) and, in particular, organic forms such as methyl mercuric ion (CH_3Hg^+) are considered neurotoxic, with the brain being the principal target

Table 5. Dietary reference intake (DRI) and contribution of most studied elements in *Nephrops norvegicus* (160 g portion)

Value	Cl	Na	K	P	S	Ca	Mg	Fe	Zn	Cu	Mn	Se	Ni	Cr
DRI[a] (mg)	2300[b]	1500[b]	4700[b]	700[c]	800–1000[b]	1000[b]	310–420[c]	8–18[c]	8–11[c]	0.9[c]	1.8–2.3[b]	0.055[c]	1.0[d]	0.025–0.035[b]
% of DRI in *N. norvegicus*	33	47	12	53	22–28	16	29–31	18–40	25–35	188	5–7	116[e]	3	59–83

[a] Estimated for adults.[2,63]
[b] Adequate intake (AI).
[c] Recommended dietary allowance (RDA).
[d] Tolerable upper intake level (UL).
[e] Based on 0.40 mg kg^{-1}.

Table 6. Non-essential elements (mg kg^{-1} wet weight) in edible muscle of *Nephrops norvegicus* (mean ± standard deviation (SD) of males and females, global mean ± SD and range)

Value	Hg	Cd	Pb
Mean ± SD (5 ♂)	0.37 ± 0.15	0.11 ± 0.02	0.05 ± 0.01
Mean ± SD (5 ♀)	0.43 ± 0.18	0.09 ± 0.05	0.05 ± 0.01
Mean ± SD (n = 10)	0.40 ± 0.16	0.10 ± 0.04	0.05 ± 0.01
Range	0.21–0.73	0.03–0.15	0.04–0.06

organ. The most pronounced damage caused by Cd occurs in the kidneys, but high Cd levels are also associated with coronary artery disease, hypertension, emphysema and other chronic pulmonary diseases. Pb also has many adverse health effects, e.g. toxicity to renal, endocrine and skeletal systems, but its primary effect is on the central nervous system.[59] It is thought that these three elements are also cancerigenic. Depending on the habitat and species examined, different levels of Hg can be detected. It was the most abundant toxic metal in *N. norvegicus*, with a mean content of 0.40 mg kg^{-1}. Two samples exceeded the permitted value of 0.50 mg kg^{-1},[5,6] reaching about 0.70 mg kg^{-1}. Capelli *et al.*[44] found similar contents of Hg in *N. norvegicus*. A study by Barghigiani and De Ranieri[65] on the same species revealed similar values for minor length classes but larger values for major length classes. Another study by Drava *et al.*[17] detected higher levels in red shrimp (*A. antennatus*). However, in other crustaceans, lower concentrations were found.[19,57,66,67] The mean contents of Cd and Pb detected in the present study were 0.10 and 0.05 mg kg^{-1} respectively. These values are significantly lower than the limits set by the EU[5,6] (0.50 mg kg^{-1} for both elements). Several authors reported higher concentrations in some crustacean species, but those samples were harvested from waters considered polluted.[15,53,68,69] Others found similar or lower values of Cd[11,14,17,19,44,66] and Pb[17,19,66] compared with the present study. In a study of Hg and Cd uptake from seawater and food by *N. norvegicus*,[70] all treatments resulted in the accumulation of both elements. However, the tissue distribution of the metals differed significantly among treatments. In the case of edible muscle the levels obtained for Hg and Cd in *N. norvegicus* can be attributed to diet or habitat waters. However, Cd only reached its highest concentration in the hepatopancreas. As for essential elements, no significant gender differences were detected in *N. norvegicus* regarding Hg, Pb and Cd levels ($P > 0.05$). This was also found by Canli and Furness[13] in tail muscle tissue of the same species.

Provisional tolerable weekly intakes (PTWIs) of 5, 7 and 25 µg kg^{-1} were established for Hg, Cd and Pb respectively.[71,72] Taking into consideration an average weekly consumption of seafood products in Portugal of 1120 g,[73] an average human BW of 60 kg and the average Hg, Cd and Pb levels found in this study (Table 6), the estimated weekly intakes of Cd (1.9 µg kg^{-1} BW) and Pb (0.9 µg kg^{-1} BW) are lower than their established PTWIs. In contrast, the PTWI for Hg is exceeded (7.5 µg kg^{-1} BW) if *N. norvegicus* is consumed in this way (160 g portion × 7 days). However, it is very unlikely that a person will consume that quantity of this species in a single week, so the value can be considered overestimated.

Positive Pearson correlations occurred between some elements, the most significant being those of Mn with Na (R^2 = 0.878, P = 0.000), Ca (R^2 = 0.746, P = 0.001), Mg (R^2 = 0.867, P = 0.000), Fe (R^2 = 0.742, P = 0.001) and Ni (R^2 = 0.815, P = 0.000). These correlations can be explained by the similar chemistry of the Mn and Mg ions[4] and probably Ca and Na and the electrochemical affinity of Mn with other transition metals such as Fe and Ni.

CONCLUSIONS

Nephrops norvegicus can be a good source of protein and have a rich mineral content. The major elements present were Cl > Na > K > P > S > Ca > Mg followed by trace elements Br > Fe > Sr ≈ Zn > Cu > Mn > Rb > Se > Ni > Cr. Considering the DRI, this species may constitute a good supply of several essential elements such as Cu, Zn, Mg and P. However, the Na/K ratio is high, which can be a concern for consumers with cardiovascular diseases. The contents of Pb and Cd are low and consumption of *N. norvegicus* is not a concern in this respect. Regarding the PTWI for Hg, the studied species should be consumed in moderation, preferably no more than four 160 g meals per week.

ACKNOWLEDGEMENTS

The authors thank PNAB/EU Data Collection Regulation, in particular Dr Cristina Silva and Dr Francisco Leote, for providing *N. norvegicus* species and their advice on the biological parameters. We also wish to thank to Dr Amparo Gonçalves for her help with the statistical analysis. This work was supported by project 22-05-01-FDR-00041 'Benefícios e Perigos Associados aos Produtos da Pesca e Aquicultura', PO Mare.

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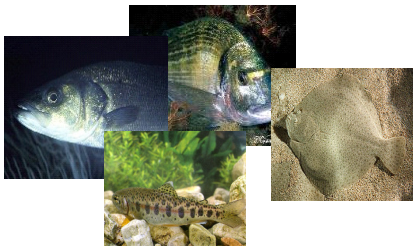
Paper III



PROXIMATE COMPOSITION AND MINERAL CONTENT OF FARMED FISH PRODUCED IN PORTUGAL

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ABSTRACT

Farmed gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*), rainbow trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima)* were analysed in order to characterize proximate composition and essential and toxic elements concentrations. All species presented a high protein level, around 20 g $(100\ g)^{-1}$ and a mineral content of about 1 g $(100\ g)^{-1}$. Similar patterns of macro, trace and ultra trace elements were observed for all studied fish species, although, some different levels occurred among them. The main elements were K, Na, P, Mg and Ca, followed by Zn, Fe, Cu, Cr, Mn and Ni. Cadmium, Hg and Pb concentrations, obtained in this study, allow concluding that these species do not present a risk for human consumption. In addition, they contain almost all essential elements at concentrations sufficient to suit the dietary reference intake. Nevertheless, *P. maxima* contribution for elemental dietary reference intake is minor in comparison to the other three species.

Keywords: *Sparus aurata; Dicentrarchus labrax; Oncorhynchus mykiss; Psetta maxima*; proximate composition; mineral content.

1. Introduction

Fish is a good source of proteins and lipids of high biological value, with long chain polyunsaturated fatty acids, and also liposoluble vitamins (Belitz, 2004) and essential elements (Oehlenschäger, 1997). Epidemiological studies indicate that populations with a fish diet have a lower risk of coronary heart disease, hypertension and cancer (Simopolpoulos, 1997). Lack of essential elements, like Na, K, P, Ca, Mg, Mn, Fe, Cu and Zn leads to improper or poor enzyme mediated metabolic functions and results in organ malfunctions, chronical diseases and finally in death (Oehlenschäger, 1997). However, in spite of fish being considered a food product indispensable in a balanced diet, such products can, to a certain extent, be contaminated with some chemicals coming from several sources (industrial waste discharge and other anthropogenic activities). Some of these chemicals that can be accumulated are Hg, Cd and Pb. These elements can be toxic even in low concentrations, causing severe human health disorders (Francesconi, 2007).

Nowadays, aquaculture comprises an important alternative to those traditional forms of fish stock. In Portugal, farmed fish play, even today, a small part comparing with fisheries (EC, 2008). According Food and Agriculture Organization of the United Nations (FAO), in the year of 2030, aquaculture will dominate the market and probably only less than the half of consumed fish products will come of traditional fisheries (FAO, 2003). Among farmed fish species in EU, gilthead sea bream (*Sparus aurata* Linnaeus 1758), European sea bass (*Dicentrarchus labrax* Linnaeus 1758), rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) and turbot (*Psetta maxima* Linnaeus 1758) observed special attention. Their productions have been increasing in the last years, being generally commercialised as fresh fish (Testi et al., 2006).

In this context, the aim of this study was to determine the proximate composition and the essential and toxic element contents in the edible muscle of gilthead sea bream, European sea bass, rainbow trout and turbot collected in Portuguese fish farms. Additionally, these values were compared with the dietary reference intakes (DRI) and maximum limit levels.

2. Materials and methods

2.1 Raw material and sample preparation

S. aurata, *D.* labrax, *O. mykiss* and *P. maxima* were purchased from several commercial farms located in Portugal. Immediately after collection, farmed fish were stored in ice inside an insulated box and transferred to the laboratory where specimens were weighed, measured (total body length) and processed. Mean length, mean weight and number of sampled fish are presented in **Table 1**. Muscle tissue from each fish was minced, mixed and homogenised in a food blender. Homogenised samples were vacuum-sealed in clean plastic bags, coded for easy identification, and stored at –21 ºC until required for analysis.

2.2 Sample analysis

Proximate composition - protein (nitrogen x 6.25; Kjeldahl method), fat (Soxhlet extraction with ethyl ether), moisture (sample dried overnight at 105ºC), and ash (sample incineration at 500 º C to constant weight) - was done according to Association of Official Analytical Chemists (AOAC) methodologies (AOAC, 1998). Analytical data for proximate composition are reported as g (100 g) $^{-1}$ on a wet weight basis. One certified reference material was tested (n = 4) in the same conditions as the samples, in order to assess analytical method accuracy: SMRD-2000 (Canned matrix meat) from Swedish Meats R & D and Scan Foods/ National Food Administration. The values of

the reference material were 1.63 ± 0.05 (1.62 ± 0.06), 14.3 ± 0.4 (14.4 ± 0.1), 68.8 ± 0.1 (68.7 ± 0.1) and 2.65 ± 0.07 (2.69 ± 0.03) for nitrogen, fat, moisture and ash, respectively. The values of present measurements are given in parentheses. The values obtained were in good agreement with the certified values.

Potassium (K), sodium (Na), magnesium (Mg), calcium (Ca), iron (Fe), copper (Cu), zinc (Zn), manganese (Mn), chromium (Cr), nickel (Ni), cadmium (Cd) and lead (Pb) analysis was based on the methods described by Jorhem (2000). Muscle samples (5 or 10 g wet weight) were dried and then ashed at 500 ºC with gradual increase in temperature. Nitric acid 65 % (w/w) was added, and the solution obtained was evaporated to dryness. The final residue was dissolved in nitric acid 15 % (v/v). Determination of K, Na, Mg, Ca, Fe, Zn, Cu, Mn, Cr, Ni, Cd and Pb was performed by flame atomic absorption spectrometry (FAAS) in a spectrometer model Spectr AA-20 with deuterium background correction (Varian Australia, Mulgrave, Victoria, Australia).

Phosphorus was determined spectrophotometrically according to an ISO standard (1996), in an UNICAM UV 5 220 spectrophotometer (ATI UNICAM, Cambridge, UK). Samples (5 g) were incinerated and dissolved in HCl (20 % w/v). Colorimetric measurements of the absorbance of a yellow compound from the reaction of P with a mixture of ammonium vanadate and ammonium molybdate were performed.

Total mercury (Hg) was determined by cold vapour atomic absorption spectrometry (CVAAS) using Coleman model MAS-50D mercury analyser system (Bacharach, Inc., Pittsburgh, USA). Of each specimen, 1 g of homogenised muscle tissue was digested with concentrated sulphuric acid. Then, Hg in the sample (Hg^0 and Hg_2^{2+}) was oxidised, with potassium permanganate 5 % (w/v), to Hg^{2+}. After reducing the Hg^{2+} to Hg^0 with

stannous chloride 20 % (w/v), the volatile Hg^0 was bubbled into the closed system of the MAS 50D analyser (λ=253.7 nm). The method used was developed by Hatch and Ott (1968) and described in detail by Joiris et al. (1991).

All analyses were carried out in duplicate, using the external calibration method. Detection limits (calculated by residual standard deviation from linear regression) (DL, mg kg^{-1}, wet wt) were 0.01 (K), 0.09 (Na), 0.01 (P), 0.02 (Mg), 0.08 (Ca), 0.06 (Zn), 0.32 (Fe), 0.02 (Cu), 0.01 (Mn), 0.02 (Ni), 0.09 (Cr), 0.01 (Cd), 0.02 (Pb) and 0.01 for Hg.

All laboratory ware was cleaned with HNO_3 (10 %) or HCl (20 %) for 24-48 h and rinsed with ultra pure water (18.2 MΩcm). All chemical were reagent grade. Commercial standard solutions (1000 mg L^{-1}) for all elements were used (Merck).

Analytical data for elements are reported as g kg^{-1} or mg kg^{-1} on a wet weight basis.

Four certified reference materials were tested in the same conditions as the samples, in order to assess analytical method accuracy: LUTS-1 (Non defatted lobster hepatopancreas), TORT-2 (Lobster hepatopancreas), DORM-2 (Dogfish muscle) from National Research Council of Canada and SMRD-2000 (Canned matrix meat) from Swedish Meats R & D and Scan Foods/ National Food Administration. The determined values for all elements were in good agreement with the certified values (**Table 2**).

2.3 Statistical analysis

To test the normality and the homogeneity of variance of data, the Kolmogrov-Smirnov's test and Levene's *F*-test, were used, respectively. Data, which corroborate these assumptions, were analysed by the ANOVA distribution to determine the difference between the mineral levels in muscle tissue sampled from farmed fish. When those conditions were not verified, data were tested non-parametrically with Mann-

Whitney's U-test. Statistical significance was considered when $p<0.05$. All data analysis was performed using STATISTICA (Statsoft, Inc. USA, 2000).

3. Results and discussion

Proximate composition for the four studied fish is reported in **Table 3**. Data relative to macro and trace/ultra trace elements are presented in **Tables 4** and **5**, respectively. Similar patterns were observed for these two groups of elements for the four farmed fish species, suggesting that each element may have the same physiological importance for all organisms. In general terms, the lowest element concentrations were found in *P. maxima*. The main elements were K, Na, P, Mg and Ca, followed by Zn, Fe, Cu, Cr, Mn and Ni. Each group will be discussed separately. Considering the elemental concentrations found in the present work, DRI percentages for each element, in a 160 g portion (a *per capita* fish consumption of 57.1 kg, which represents ≈ 160 g fish person^{-1} day^{-1} (FAO,2007) are calculated. Histograms are shown in Figures 1 and 2, in order to compare DRI percentages in the four farmed fish species. Total concentrations of Hg, Pb and Cd, metals considered toxic and regulated by the EU Commission (EU, 2006, 2008), are summarised in **Table 6**.

3.1 Proximate composition

The results obtained for proximate composition were similar to those reported by several authors (Alasalvar et al., 2002; Erkan and Özden, 2007; Huidobro et al., 2001; Testi et al., 2006). Average moisture content ranged between 67.4 and 75.3 g (100 g)$^{-1}$, for *D. labrax* and *O. mykiss*, respectively. As expected, fat content was inversely proportional to moisture content and the highest value was found in *D. labrax* (13.1 g (100 g)$^{-1}$). The different fat levels are probably due to the dietary history of each fish

species. All study farmed fish presented a high protein level, around 20 g (100 g)$^{-1}$, which is in accordance with Belitz (2004). Ash content was superior to 1 g (100 g)$^{-1}$ in all samples, reaching 1.40 g (100 g)$^{-1}$ in *S. aurata.* The lowest level was found in *P.maxima*, however the value is in accordance with the results reported by some authors (Busetto et al., 2008; Özogul et al., 2006).

3.2. Macro elements (K, P, Na, Mg, Ca)

These elements are considered essential and their main functions include skeletal structure, maintenance of colloidal system and regulation of acid-base equilibrium. Potassium was the most abundant element in the four studied fish species; the minimum value was obtained for P. maxima, about 3.0 g kg^{-1} and the largest concentration was found in *D. labrax* (5.7 g kg^{-1}). No significant differences were found between levels of K in *D. labrax* and *O. mykiss*. *S. aurata* showed an intermediate level, around 4.7 g kg^{-1}. These values are similar to those reported by Orban et al. (2000) and Erkan et al. (2007) in *D. puntazzo*, *D. labrax* and *S. aurata*, respectively. The second most abundant element in the studied fish species was P. No significant differences were found for the values of P, among the fish species, with the exception of *P. maxima* that presented the lowest value (1.8 g kg^{-1}). The mean contents were situated between 2.5 and 2.7 g kg^{-1} for *D. labrax, S. aurata* and *O. mykiss*. These values are in agreement with the statement of Oehlenschläger (1997) that the P content in marine fish muscle is on average close to 2 g kg^{-1}. One study of Vlieg et al. (1991) also revealed values around 2.5 g kg^{-1} for trout species (*Salmo trutta* and *Oncorhynchus mykiss*). Nevertheless, the levels obtained in the present study were lower than those reported by other authors (Erkan et al., 2007; Fuentes et al., 2010; Gokoglu et al., 2004) for the same species. In the main elemental profile observed for the studied farmed fish species, Na was the third element. As

confirmed by Oehlenschläger (1997) the levels of Na were lower than K ones. The concentrations of Na found in this study were around 1.0 g kg^{-1}. Sodium levels of *D. labrax* and *O. mykiss* did not show significant differences. Other studies showed lower levels of Na when compared with the present work in *D. labrax*, *S. aurata* (Erkan et al., 2007), and *O. mykiss* (Gokoglu et al., 2004). However, similar contents were reported by Martinez-Valverde et al. (2000) for blue whiting, hake and sole. Magnesium and Ca were the last macro elements found in the elemental profile, being Mg concentrations higher than Ca ones. This fact is in accordance to Oehlenschläger (1997), that, in general, fish contains more Mg than Ca. No significant differences were observed between values of these two elements in *D. labrax* and *O. mykiss* (approximately 0.37 g kg^{-1} and 0.30 g kg^{-1}, respectively). *S. aurata* showed intermediate levels, but Ca concentrations were also not significantly different from the values of the other two species referred above. As with the other macro elements, *P. maxima* showed the lowest amounts of these two metals, 0.24 g kg^{-1} for Mg and 0.11 g kg^{-1} for Ca. Values obtained for Mg were expected, but for Ca they were low when compared with previous research for the same species (Aubourg et al., 2007; Erkan et a., 2007; Gokoglu et al., 2004) or different ones (Martinez-Valverde et al., 2000). Nevertheless, Fuentes et al. (2010) observed Mg levels lower than those obtained in the present study. The differences found among data reported by several authors could be attributed to a variety of factors including availability and type of food, dietary ingredients and reduced activity of the cultured fish.

3.3 Trace and ultra trace elements (Zn, Fe, Cu, Mn, Cr, Ni)

In general, these essential elements are important components of hormones, enzymes and enzyme activators. They are associated with specific proteins in metalloenzymes, which produce a unique catalytic function. Zinc, Fe and Cu were the elements

presenting the highest values of this group, being Zn the most abundant. *P. maxima* showed the highest average content of Zn (6.8 mg kg^{-1}) and *S. aurata* the lowest average value (4.9 mg kg^{-1}); these concentrations were statistically different. No significant differences for Zn levels were observed among *D. labrax, O. mykiss* and *S. aurata*, however Zn level in *O. mykiss* was also not different from *P. maxima* one. In literature, different Zn concentrations in seafood can be found. Some studies performed in farmed fish indicated lower levels than those observed in the present work (Dugo et al., 2006; Erkan et al., 2007; Fuentes et al., 2010) and other studies revealed higher Zn levels when compared to the levels found in this study (Alasalvar et al., 2002; Gokoglu et al., 2004). On the other hand, compared with studies on farmed and wild *P. maxima* (Aubourg et al., 2006), wild trout (Scherz and Kirchhoff, 2006; Vlieg et al., 1991), and wild *S. aurata* (Canli and Atli, 2003; Türkmen et al., 2008), the results of the present work were similar. For example, in a study performed on fish species from Northeast Atlantic (Celik and Oehlenschläger, 2004), the Zn content ranged from 2.1 mg kg^{-1} to 8.7 mg kg^{-1}. This shows that Zn concentrations in fish species are variable and it is not possible to establish a small range of values. Iron levels were around 5 mg kg^{-1} for *D. labrax* and *S. aurata*. Differently from the Zn profile, the lowest Fe content was found in *P. maxima* (2.6 mg kg^{-1}). The average Fe values found in *O. mykiss* and *S. aurata* were not statistically different from the other two species. The lowest Fe content found in *P maxima* can be explained by the low percentage of dark flesh in this fish. In 1995, Lall (1995) collect several Fe data, showing a wide range of values in many marine fish and crustaceans (0.8 to 373 mg kg^{-1}). The author explained that this wide range would be probably due to metal and bone contamination of fishery products and differences in analytical methodology. In fact, according to Oehlenschläger (1997), Fe is present in concentrations of approximately 5.0 mg kg^{-1} in marine fish species. Therefore, levels

found in the present study were in the range found by Oehlenschläger (1997) and other authors (Aubourg et al., 2007; Canli and Atli, 2003; Dural et al., 2007; Fuentes et al., 2010; Gokoglu et al., 2004; Türkmen et al., 2005), for similar fish species. Nevertheless, some authors reported higher levels than those of the present study (Alasalvar et al., 2002; Erkan et al., 2007; Türkmen et al., 2008; Uluozlu et al., 2007; Vlieg et al., 1991). Copper was the third element in the profile of trace elements, except for *P. maxima*. Significant differences were found when comparing the lowest Cu average level, 0.17 mg kg^{-1}, found in *P maxima*, and the highest one, 0.71 mg kg^{-1}, found in *S. aurata*, *D. labrax* and *O. mykiss* showed intermediate levels, approximately 0.45 mg kg^{-1}, being the average value, found in *D. labrax*, not statistically different from the average values found in *S. aurata* and *O. mykiss*. In literature, some comparative studies between wild and farmed fish showed that, in general wild fish present a lower Cu content than farmed fish (Alam et al, 2002; Alasalvar et al., 2002; Aubourg et al., 2007; Ikem and Egilla, 2008). These different contents can be attributed to the different type of food in wild marine environment and farm systems. In fact, Cu levels reported in the present study were higher than those verified by other authors, for the wild fish species (Canli and Atli, 2003; Dural et al., 2007; Fuentes et al., 2010; Gokoglu et al., 2004; Türkmen et al., 2005; Uuozlu et al., 2007).

The three studied essential ultra trace elements were Mn, Cr and Ni. Significant differences were detected between Mn levels in two of the study fish species: *S. aurata* showed the lowest average value (0.14 mg kg^{-1}) and *P. maxima* the highest one (0.32 mg kg^{-1}). The differences in the Mn contents of *D. labrax* and *O. mykiss* were found to be non-significant. Statistically, and regarding Mn values, *D. labrax* and *P. maxima* as well as *O. mykiss* and *S. aurata* were identical. Manganese levels found in the present study were low and inferior to other values reported by some authors for similar farmed

fish species (Alasalvar et al., 2002; Erkan et al., 2007; Gokoglu et al., 2004). Studies performed by Aubourg et al. (2007) in farmed *P. maxima*, Türkmen et al. (2005) in wild *S.aurata* and Vlieg et al. (1991) in *wild O.mykiss*, showed Mn levels comparable to those found in the present work. In contrast, Mn levels in farmed *D. labrax* from Greece and Spain were lower than the values reported by the present study (Fuentes et al., 2010). The levels of Cr were approximately about 0.30 mg kg^{-1}. No significant differences were found among the Cr contents of the four studied fish species. In literature, it is possible to find a wide range of Cr levels in fish species, partially due to environmental pollution. For example, the study performed by Garg and Krishna (2006), in fish from Indian coastal areas, showed values between 0.06 and 29.9 mg kg^{-1}. In fact, results reported in the present work were similar to those reported by some authors in *S. aurata* and *D. labrax* from Mediterrean and Aegean seas (Canli and Atli, 2003; Türkmen et al., 2005, 2009; Uluozlu et al., 2007), but lower or higher than those found by others (Ikem and Egilla, 2008; Türkmen et al., 2008; Tuzen., 2009 or Alam et al., 2002; Alasalvar et al., 2002; Ersoy et al., 2006). Nickel was the ultra trace element that showed the lowest values of the group (between 0.02 and 0.04 mg kg^{-1}). As for Cr, no significant differences among Ni contents were detected for all studied fish species. Studies on Ni levels in farmed fish species similar to those studied in the present work are scarce. One study performed by Alasalvar *et al.* (2002) showed higher Ni concentrations in *D. labrax* than observed in our study. In general, values found in the present study were lower than those reported by others in wild fish species (Türkmen et al., 2005, 2008, 2009; Tuzen, 2009; Uluozlu et al., 2007). These differences are probably due to the environmental pollution affecting the catch areas. Only two studies, one performed in farmed and wild *Cyprinus carpio* from Lake Kasumigaura, Japan

(Alam et al., 2002) and other performed in wild *D. labrax* from Ria de Aveiro, Portugal (Pérez Cid et al., 2001) showed low Ni values, as in the present study.

3.4 Toxic elements (Cd, Hg, Pb)

Cadmium, Hg and Pb do not show any essential function in human life and are considered harmful. In addition, their connection with several diseases is well known. Mercury in its several forms is regarded as neurotoxic, Pb affects the central nervous system and Cd has a deleterious effect upon the kidneys.

The mean values of Cd were quite low, around 0.01 mg kg^{-1}. Nevertheless, significant differences were observed among the Cd levels for four studied fish species. In fact, Cd concentrations in *D. labrax*, were different from those of the other three fish species, and those of *S. aurata* were not different of Cd levels found in *P. maxima* and *O. mykiss*. There were no significant differences among Pb contents of the four studied fish species. The mean values situated between 0.02 and 0.05 mg kg^{-1}. The values obtained for these two metals in the present work are in accordance to those reported by some studies in farmed fish species (Alam et al., 2002; Ersoy et al., 2006; Fernandes et al., 2008; Ferreira et al., 2008). Nevertheless, *D. labrax* from two Sicilian farms (Dugo et al., 2006) and one Greek farm (Alasalvar et al., 2002) showed higher levels than Cd and Pb concentrations found in this study. In general, wild fish showed higher Pb and Cd contents than farmed ones (Canli and Atli, 2003; Dural et al., 2007; Türkmen et al., 2005, 2008, 2009; Tuzen, 2009; Uluozlu et al., 2007). Mercury was the toxic metal that showed the highest levels of this elemental group. The minimum was observed in *S. aurata* (0.04 mg kg^{-1}) and the maximum was attained in *D. labrax* (0.21 mg kg^{-1}), although the mean levels in these two fish species were not statistically different. The mean concentrations presented by *O. mykiss* and *P. maxima* were similar. Due to

intrinsic and extrinsic factors, very different Hg concentrations in seafood can be observed. In farmed fish, feeds are supposed to be the main source of Hg. Literature reporting Hg levels for these four studied fish species is scarce. Some studies performed in other farmed fish species showed similar or lower Hg values than those determined in the present work (Dewailly et al., 2007; Easton et al., 2002; Ikem and Egilla, 2008; Ureña et al., 2007). Tuzen (2009) reported a similar Hg content for *P. maximma* (45 μg kg^{-1}). On the other hand, studies on farmed tuna species revealed higher values than those observed by the present work (Padula et al., 2008; Vizzini et al., 2010). The mean values of Cd, Pb and Hg, observed in the four studied fish species did not surpass the established limits (Cd: 0.05 mg kg^{-1}, Pb: 0.30 mg kg^{-1}, Hg: 0.50 or 1.0 mg kg^{-1}) by EU (EU, 2006, 2008).

3.5. Dietary reference and provisional tolerable weekly element intakes

Since farmed fish are now a significant diet source in south European countries, intake of macro, trace and ultra trace elements is of utmost important. Taking into consideration the proposed levels for DRI by Institute of Medicine of the National Academies of USA (IOM) (2005), the contribution of each studied element to the DRI for the four fish species was calculated (**Figure 1** - macro elements and **Figure 2** - trace and ultra trace elements). Results show that studied farmed fish are a good source of P, contributing to more than 40 - 50 % of the DRI. These fish may also significantly contribute to the DRI of K, Mg and Na, representing 9 – 19 % of the DRI. The Na/K ratio is inferior to 1 which indicates a low risk of developing high blood pressure and cardiovascular disease (Astorga España et al., 2007). Of the various macro elements studied, Ca is the element that shows the lowest percentage contribution for DRI, which is in agreement with the fact that fish, is considered a poor source of Ca (Lall, 1995).

The contributions for DRI of Zn, Fe and Cu are also important. Moreover, Mn and Ni percentages are small, ranging between 1 and 3 %. In all studied fish species, Cr concentrations are above the DRI (data not show in histogram). Until now, no tolerable upper intake level (UL) was set for Cr. However, according to Council for Responsible Nutrition (CRN) (Hathcock, 2004) Cr supplements at levels up to 1,000 μg per day are regarded as safe for adults. This intake is above the results found in this study (0.04 – 0.05 mg Cr in a 160 g portion). In general, *P. maxima* showed the lowest contents of most elements and obviously their contribution for DRI was lower than that of the other three species.

A joint committee of Food and Agriculture Organization/World Health Organization (FAO/WHO) (WHO, 1999, 2003) established provisional tolerable weekly intakes (PTWI) for Cd (7 μg kg^{-1} body weight), Hg (5 μg kg^{-1} body weight) and Pb (25 μg kg^{-1} body weight). Considering a weekly average consumption of seafood products in Portugal of 1120 g (160 g / day) (FAO, 2007), an average human body weight of 60 kg and the average Cd, Hg and Pb concentrations observed in this study (**Table 6**), estimated weekly intakes of the three metals were calculated. The values found for Cd (≈ 0.2 μg kg^{-1} body weight), Hg (1.1 – 2.6 μg kg^{-1} body weight) and Pb (0.4 – 0.9 μg kg^{-1} body weight) are lower than established PTWIs.

4. Conclusions

This study revealed that consumption of farmed *D. labrax*, *S. aurata*, *O. mykiss* and *P. maxima* does not pose a health risk problem to Portuguese from estimated dietary intakes of toxic elements. In addition, they are a good source of essential elements, contributing for the DRI, especially regarding P, K, Mg and Zn. Nevertheless, *P. maxima* contribution for elemental DRI is minor in comparison to the other three

species. This study supports the importance of promoting consumption of fish, and in this context, farmed fish and will also contribute to the Portuguese food composition database.

Acknowledgements

The authors are grateful to staffs of DGV-Lisbon, for the collection of farmed fish samples. They also would like to thank to Amparo Gonçalves for her help with statistical analysis. This work was supported by the project PTDC/SAU-ESA/103825/2008 "Benefícios e riscos associados ao consumo de produtos da pesca: Uma análise de benefício-risco baseada na abundância e bioacessibilidade de n-3 PUFA e Selénio, Mercúrio e Arsénio em produtos crus e cozinhados (GOODFISH)", from FCT.

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Table 1 – Total length range (cm), total weight range (g) and number (n) of farmed fish analyzed.

Species	**n**	**Length (min-max)**	**Weight (min-max)**
D. labrax	10	26.0 – 36.0	217 – 504
O. mykiss	10	24.0 – 31.0	163 – 390
P. maxima	7	31.5 – 38.0	577 – 1043
S. aurata	10	23.0 – 36.5	174 – 736

Table 2 – Results of analysis (n = 4) for some certified reference materials (mean ± sd, in mg kg^{-1}).

		K	Na	P	Mg	Ca	Zn	Fe	Cu	Mn	Ni	Cr	Hg	Pb	Cd
LUTS-1	Certified	948 ± 72	-	-	89.5 ± 4.1	203 ± 33	-	11.6 ± 0.9	15.9 ± 1.2	1.20 ± 0.13	0.200 ± 0.034	-	-	-	-
	Obtained	955 ± 23			90.8 ± 2.2	197 ± 16		12.1 ± 0.4	15.4 ± 0.2	1.28 ± 0.03	0.195 ± 0.009				
SMRD-2000	Certified	1859 ± 85	8533 ± 281	1075 ± 47		70.3 ± 8.3	-	6.33 ± 1.66	-	-	-	-	-	-	-
	Obtained	1938 ± 38	8346 ± 280	1108 ± 11		66.1 ± 7.4		4.94 ± 0.09							
TORT-2	Certified	-	-	-	-	-	180 ± 6	-	-	13.6 ± 1.2	2.50 ± 0.19	-	0.27 ±0.06	0.35 ± 0.13	26.7 ± 0.6
	Obtained						175 ± 1			13.1 ± 0.1	2.37 ± 0.08		0.28 ± 0.00	0.35 ± 0.06	26.8 ± 0.1
DORM-2	Certified	-	-	-	-	-	-	142 ± 10	-	3.66 ± 0.34	-	34.7 ± 5.5	4.64 ± 0.26	-	-
	Obtained							141 ± 4		3.62 ± 0.18		30.6 ± 2.4	4.48 ± 0.12		

Table 3 – Proximate composition (g (100 g)$^{-1}$) of farmed fish muscle (mean ± standard deviation).

	D. labrax	***O. mykiss***	***P. maximma***	***S. aurata***
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Moisture	67.4 ± 1.0	75.1 ± 1.3	75.3 ± 1.1	71.1 ± 0.4
Protein	20.4 ± 0.4	19.3 ± 1.2	19.5 ± 0.8	20.4 ± 1.6
Lipid	11.8 ± 1.8	4.3 ± 1.2	4.6 ± 0.6	7.0 ± 2.1
Ash	1.22 ± 0.00	1.30 ± 0.09	1.12 ± 0.13	1.38 ± 0.03

Table 4 – Macro elements (g kg^{-1} wet basis) in muscle of farmed fish (mean ± standard deviation and range).

	D. labrax		*O. mykiss*		*P. maximma*		*S. aurata*	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Ca	0.29 ± 0.10a*	0.13 – 0.42	0.28 ± 0.11a	0.19 – 0.48	0.11 ± 0.04b	0.04 – 0.16	0.23 ± 0.09a	0.11 – 0.34
K	5.2 ± 0.3a	4.7 – 5.7	5.2 ± 0.2a	4.8 – 5.5	3.2 ± 0.3b	3.0 – 3.8	4.7 ± 0.3c	4.3 – 5.2
Mg	0.37 ± 0.01a	0.35 – 0.39	0.37 ± 0.03a	0.33 – 0.40	0.24 ± 0.03b	0.18 – 0.27	0.34 ± 0.03c	0.30 – 0.38
Na	1.1 ± 0.3ab	0.47 – 1.5	1.7 ± 0.9a	1.1 – 3.9	0.9 ± 0.1b	0.7 – 1.0	1.2 ± 0.5ab	0.50 – 2.3
P	2.5 ± 0.2a	2.2 – 3.2	2.6 ± 0.1a	2.2 – 2.9	1.8 ± 0.2b	1.6 – 2.1	2.7 ± 0.2a	2.2 – 3.3

* Mean ± SD followed by distinct letter, within a row, are significantly different ($p < 0.05$).

Table 5 – Trace and ultra trace elements (mg kg^{-1} wet basis) in muscle of farmed fish (mean ± standard deviation and range).

	D. labrax		*O. mykiss*		*P. maximma*		*S. aurata*	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Cr	0.31 ± 0.08a*	0.20 – 0.50	0.23 ± 0,13a	0.03 – 0,40	0.28 ± 0.08a	0.20 – 0.40	0.31 ± 0.14a	0.01 – 0.60
Cu	0.46 ± 0.10ab	0.32 – 0.63	0.42 ± 0.06b	0.34 – 0.53	0.17 ± 0.05c	0.12 – 0.25	0.71 ± 0.17a	0.49 – 0.97
Fe	5.0 ± 2.0a	3.0 – 9.5	4.5 ± 1.1ab	3.0 – 6.4	2.6 ± 0.4b	2.1 – 3.5	4.8 ± 3.0ab	2.8 – 13.0
Mn	0.30 ± 0.07ab	0.20 – 0.40	0.18 ± 0.06ac	0.13 – 0.33	0.32 ± 0.11b	0.15 – 0.45	0.14 ± 0.02c	0.12 – 0.17
Ni	0.04 ± 0.02a	0.01 – 0.06	0.02 ± 0.00a	0.02 – 0.03	0.02 ± 0.00a	0.02 – 0.03	0.04 ± 0.02a	0.01 – 0.07
Zn	5.2 ± 0.4a	4.8 – 6.0	6.3 ± 1.5ab	4.7 – 9.4	6.8 ± 0.5b	6.0 – 7.4	4.9 ± 1.0a	4.1 – 6.8

* Mean ± SD followed by distinct letter, within a row, are significantly different ($p < 0.05$).

Table 6 – Toxic elements (mg kg^{-1} wet basis) in muscle of farmed fish (mean±standard deviation and range).

	D. labrax		***O. mykiss***		***P. maximma***		***S. aurata***	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Cd	< DL* a	-	0.01 ± 0.01b	0.01 – 0.02	0.01 ± 0.01c	< 0.01 – 0.01	0.01 ± 0.01bc	< DL – 0.02
Hg	0.14 ± 0.04a**	0.08 – 0.21	0.06 ± 0.02b	0.05 – 0.10	0.06 ± 0.03b	0.03 – 0.11	0.11 ± 0.04a	0.04 – 0.17
Pb	0.02 ± 0.02a	< DL – 0.07	0.05 ± 0.02a	0.02 – 0.08	0.05 ± 0.01a	0.03 – 0.07	0.03 ± 0.02a	< DL – 0.09

* DL – Detection Limit; ** Mean ± SD followed by distinct letter, within a row, are significantly different ($p < 0.05$).

Paper IV

Total and organic mercury, selenium and α-tocopherol in some deep-water fish species

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Abstract

BACKGROUND: Mercury is a well-known toxic element present in seafood. Nevertheless some antioxidants, like selenium, as an integral component of the enzyme glutathione peroxidase, and α-tocopherol having a protective role against it, are present as well. The purpose of this study was to evaluate the levels of total mercury (Hg_T), organic mercury (Hg_{Org}), Se and α-tocopherol in some adult deep-water fish species caught off Portuguese waters.

RESULTS: Levels of Hg_T were above EU limits in 6% of *Lophius* spp. and *Aphanopus carbo*, 2% of *Lepidorhombus* spp. and 70% of *Helicolenus dactylopterus*. Hg_{Org} represented the major fraction (more than 84%) of Hg_T in all studied muscles. The selenium concentration in muscle was similar in all species (about 0.4 mg kg^{-1} wet weight) and the highest level of α-tocopherol (70 mg kg^{-1} wet weight) was found in *Aphanopus carbo*.

CONCLUSION: The muscle of all species studied is a good source of selenium, but only can give a small contribution of α-tocopherol. Attending to the provisional tolerable weekly intake established by the WHO/FAO for methylmercury, it appears that the species studied should be consumed sparingly.

Keywords: fish; total and organic mercury; antioxidants; AAS; HPLC; EDXRF

INTRODUCTION

There is a much concern about the amount and quality of the nutrients that are supplied by the diet to maintain human health and well-being.[1] Although seafood products are well known for their contribution to health, there are several types of toxic substance from natural and/or anthropogenic origins that can contaminate them.[2]

Contaminant metals, such as mercury, do not show any essential function in life and, because of their toxicity, are considered harmful elements. These metals can be toxic for humans, even at very low concentrations.[3] They can be assimilated, stored and concentrated by organisms, through the food chain, resulting in physiological damage.[4,5]

Regarding these toxic metal ions, most of the chemical reactions that explain their toxicity at cellular level involve electron transfer, formation of free radicals and their influence on DNA, with possible consequences in mutagenicity, genotoxicity and carcinogenicity.[6] Mercury is a contaminant metal that is naturally present in the environment. This contaminant is present at low level in water systems, but bioconcentrates in the aquatic food chain, reaching its highest level in large and old predatory fish and marine mammals. Organic mercury (Hg_{Org}) is found mainly in fish as methylmercury. The exposure to mercury, especially to organic mercury, results in neurological and renal damage and has potentially harmful effects on cardiovascular diseases as well.[7]

Almost all elements considered to be essential can be found in different levels in edible parts of marine fish, supplying macro and trace elements to the human diet.[1] In addition, there is growing evidence that micronutrient intake has a significant effect on the toxicity and carcinogenesis caused by various chemicals. Micronutrients can affect the toxicity of metals by interacting with them at several points in the body: absorption and excretion, transport, binding to target proteins, metabolism and sequestration of toxic metals and in secondary mechanisms of toxicity.[8] Consequently, if the human diet is deficient in micronutrients it is possible to predispose to toxicity from non-essential metals. Seafood has received attention as a possible modifier of methylmercury distribution in a way that protects organisms exposed

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(*Received 18 March 2008; revised version received 4 July 2008; accepted 11 July 2008*)
DOI: 10.1002/jsfa.3379

 J Sci Food Agric 0022–5142/2008/$30.00

to this form of mercury through the consumption of marine products.[9] Selenium is a required dietary element for health, but it can be toxic at high concentrations. It is an integral component of the enzyme glutathione peroxidase, which, together with vitamin E and catalase and superoxide dismutase enzymes, acts as an antioxidant, thereby protecting cells against oxidative damage.[10,11] Several studies have shown that antioxidants like selenium offer some protection against methylmercury and inorganic mercury,[8,9] thus selenium deficiently accentuates the toxicity caused by heavy metals.[10] Fish fat and liver are significant sources of fat-soluble vitamins, A and D. Also present are vitamins E (tocopherol) and K.[11] Vitamin E is an antioxidant and prevents free radical injury caused by methylmercury.[8]

Vitamins are organic compounds that are indispensable in very small amounts in the diet. They form a heterogeneous group of substances and are vital nutrients. Some have specific and individual functions to promote growth or reproduction, or to maintain health and life.[12] α-Tocopherol has the highest biological activity among the different forms of vitamin E. Since α-tocopherol is the major form detected in marine animal tissue, vitamin E values most likely parallel α-tocopherol values. In biological systems, vitamin E functions as an antioxidant to protect cellular lipid components by donation of the phenolic hydrogen to a free radical, thus inhibiting the chain reaction of free radical propagation.[13] In addition, the *in vitro*, cellular and animal studies, which are impressive both in quantity and quality, leave no doubt that vitamin E, the most important fat-soluble antioxidant, protects animals against various types of oxidative stress.[14] Furthermore, different antioxidants seem to act synergistically, selenium can enhance tocopherol activity and it appears that the protective role of these two antioxidants is cumulative,[10,11] so supplementation with vitamin E might be more effective if combined with other micronutrients.[14]

The main objective of this work was to evaluate the levels of total and organic mercury and some antioxidants in tissues of some deep-water fish species caught off the Portuguese coast and to establish suitable relationships between the animal length and weight and accumulation of these elements. In addition, the comparison of results obtained with maximum levels fixed by a European Commission regulation[15] and with the provisional tolerable weekly intake (PTWI) recommended by the Joint FAO/WHO Expert Committee on Food additives[16,17] and the comparison of antioxidant content in the muscle and their daily intake requirements were a second goal.

EXPERIMENTAL

Sample collection and preparation

For this experiment fish samples were collected between 2004 and 2006. The species *Lophius* spp. (*Lophius piscatorius* and *L. budegassa*, angler and black-bellied angler, respectively) ($n = 33$) and *Helicolenus dactylopterus* (blackbelly rosefish) ($n = 50$) were obtained during several bottom trawl research surveys carried out by the Portuguese Institute for Marine Research (INRB-IP/IPIMAR) along the Portuguese coast. *Aphanopus carbo* (black scabbardfish) ($n = 50$) were caught off Sesimbra and *Lepidorhombus* spp (*Lepidorhombus boscii and L. whiffiagonis*, fourspotted megrim and megrim) ($n = 53$) in the Peniche area. Total length (cm), weight (g) and the sex of all fish were recorded. Muscle of all fish samples and liver of *Lophius* spp. and *Aphanopus carbo* were removed and stored at $-30\,^{\circ}C$ for further analyses. For organic mercury and selenium determination, fish samples were freeze dried for 48 h at $-45\,^{\circ}C$ and low pressure (approximately 10^{-1} atm). Samples were powdered and stored under controlled moisture conditions until further analysis.

Sample analysis

Total and organic mercury (Hg_T and Hg_{org}) were determined by atomic absorption spectrometry using an automatic Hg analyser (Leco apparatus AMA 254, St. Joseph, MI, USA). Hg_{org} was extracted from samples using the method described by Scerbo and Barghigiani.[18] An aliquot of the lyophilised sample (0.2–2 g) was hydrolysed with hydrobromic acid (47% w/w) and then the Hg_{org} species were extracted from the sample with toluene. Finally, a cysteine solution was added to remove the organic mercury compounds from the toluene. Approximately 100 mg of wet tissue (for total Hg) or 100 µL of cysteine extract (for Hg_{org}) was transferred to a sample boat, which was placed in a decomposition tube where it was dried and then thermally decomposed by controlled heating. The final decomposition products pass thought a mercury amalgamator which collects Hg^0. The mercury amalgamator is heated to 700 °C and the mercury is released and detected at 254 nm by absorption spectrometry. An external calibration curve was made with standard solutions prepared by dilution from a 1000 mg l^{-1} mercuric nitrate standard solution (Merck, Darmstadt, Germany). The mercury concentration was within the range 5–400 µg L^{-1}.

The energy dispersive X-ray fluorescence spectrometer (EDXRF) used in this work to quantify selenium consists of an X-ray tube (Philips, PW 1140; 100 kV, 80 mA; Eindhoven, The Netherlands) equipped with a changeable secondary target, in molybdenum. The characteristic radiation emitted by the elements present in the sample was detected by a Si(Li) detector (Oxford, High Wycombe, United Kingdom), with a 30 mm^2 active area and 8 µm beryllium window. The energy resolution was 135 eV at 5.9 keV and the acquisition system was a Nucleus PCA card (High Wycombe, United Kingdom). Quantitative calculations were made by the fundamental

DOI: 10.1002/jsfa

parameters method,[19,20] which makes use of the fundamental parameters such as cross-sections for absorption and X-ray production, transition intensities, fluorescence yields, for example. The relation between the measured peak intensity (I_i) and the concentration of an element C_i is given by the relation $I_i = I_0 m K_i C_i A_i$, where I_0 is the intensity of the X-ray beam, m is the sample thickness (g cm^{-2}), K is an experimental calibration factor which depends on the spectrometer geometry, detector efficiency, detector solid angle and cross-sections for producing characteristic X-rays, and together with I_0 is obtained by analysis of standard reference samples. The standard reference materials used were similar to the samples studied: TORT-2, lobster hepatopancreas reference material; DORM-2, dogfish muscle certified reference material, both from National Research Council (NRC-CNRC), Canada, and freeze dried animal blood from International Atomic Energy Agency (IAEA-A-13), Austria.

A_i is the self-attenuation factor which can be obtained iteratively, considering that 80% of the matrix elements consist on H, C and O.

The peak shape and background are described by a mathematical Gaussian function. At the low-energy side of the peak, this function is replaced by an exponential function. The basic idea of the fitting procedure is to find a set of parameter values which minimise the chi-square value.

The X-ray generator was operated at 50 kV and 20 mA and a typical acquisition time of 1000 s was used. The sample powder was pressed into pellets 2.0 cm in diameter without any chemical treatment. Pellets were then glued onto Mylar film, on a sample holder, and placed directly in the X-ray beam, for determination of elements.

Analysis of α-tocopherol was carried out after lipid extraction following the Bligh and Dyer method.[21] Tocopherols were determined by normal phase high-performance liquid chromatography (HPLC) following the method described by Piironen *et al*.[22] with minor modifications. Total lipids were dissolved in hexane in the concentration of 36 mg lipid mL^{-1} and 20 μL were injected in the HPLC (Jasco model PU-980; Tokyo, Japan), equipped with an automatic injector Jasco (model AS-950-10), and a fluorescence detector (Jasco model FP-1520) (excitation wavelength = 292 nm, emission wavelength = 324 nm), the mobile phase was degassed (Gastor model GT-104; Tokyo, Japan). The column was a Lichrosorb Si 60-5 (250 mm × 3 mm i.d.; Chrompack, Walnut Creek, CA), protected with a guard column (S2)-SS (10 mm × 2 mm i.d., Chrompack). The isocratic mobile phase contained 7 mL L^{-1} of isopropanol in *n*-hexane, with a flow rate of 0.5 mL min^{-1}. The retention time for the identification of α-tocopherol was determined by injection of an α-tocopherol standard. Tocopherols concentration was obtained using the peak integration area of Borwin software 1.2 version (Tokyo, Japan).

An external calibration curve was made with standard solutions prepared by dilution from a 95% α-tocopherol standard solution (Sigma, St Louis, MO, USA). The range of α-tocopherol concentration was within 1–200 μg mL^{-1}.

A minimum of two replicate analyses was performed for each sample and each element analysed. All standards and reagents were of the highest purity. To quantify Hg_T and Hg_{Org}, and α-tocopherol in the sample a calibration curve was made with no fewer than five standards in different concentrations. The moisture was determined by the sample drying technique at 105 °C[23] to convert the results of Hg_{Org} and Se on a wet weight base. Data for α-tocopherol were transformed to mg kg^{-1} wet weight taking into account the level of extract oil from the samples by gram of fresh tissue.

Accuracy was checked by analysis of several certified reference materials (Table 1). From these results it can be concluded that the analysed material was in the range values of the certified material. The detection limits were 0.5 μg g^{-1} (total and organic mercury), 0.6 mg kg^{-1} dry weight (Se) and 2.59 μg mL^{-1} (α-tocopherol).

Table 1. Laboratory performance on standard reference materials for total mercury, methylmercury (measured as organic mercury), selenium and α-tocopherol (mg kg^{-1} dry weight) (n ≥ 4)

	Standard reference material*	Hg_T	Hg_{Org} (methylmercury)	Se	α-Tocopherol
Present work	DORM-2[a]	4.68 ± 0.17	4.23 ± 0.05	–	–
	TORT-2[b]	–	–	5.34 ± 0.43	–
	MA-A-2[c]	–	–	1.5 ± 0.4	–
	CRM-463[d]	2.99 ± 0.02	3.01 ± 0.08	–	–
	CRM 421[e]	–	–	–	95 ± 1
Certified value	DORM-2	4.64 ± 0.26	4.47 ± 0.32	–	–
	TORT-2	–	–	5.63 ± 0.67	–
	MA-A-2	–	–	1.7 ± 0.3	–
	CRM-463	2.85 ± 0.16	3.04 ± 0.16	–	–
	CRM 421	–	–	–	99 ± 6

[a] Dogfish muscle, National Research Council of Canada; [b] lobster hepatopancreas, National Research Council of Canada; [c] fish flesh, International Atomic Energy Agency, Austria; [d] tuna fish, Institute for Reference Materials and Measurement, Belgium; [e] milk powder, Institute for Reference Materials and Measurement, Belgium.

Statistical analysis

To test the normality and the homogeneity of variance of data, the Kolmogrov–Smirnov test and Levene's *F*-test, respectively, were used. Data that corroborate these assumptions were analysed by Student's *t* distribution to determine the difference between sex and all measured parameters (length, weight, total Hg, organic Hg, Se and α-tocopherol). Data that do not assume the normality or homogeneity of variance were log transformed. When the conditions of the Student's *t* distribuition were not verified, data were tested nonparametrically with the Mann–Whitney *U*-test. The Pearson coefficient was calculated to determine the correlation between the obtained values. For all statistical tests the significance level (α) was 0.05. All data analysis was performed using STATISTICA 6 (2003 version) (Stat-sof, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

A total of 186 fish from the North Atlantic Ocean (Portuguese mainland) were examined in this study (Table 2). All fish sampled were adults and their sizes were within the commercial range. Contrary to the behaviour in *Aphanopus carbo* and *Lepidorhombus* spp., *Lophius* species and *Helicolenus dactylopterus* showed significant differences between males and females either with length or weight ($P < 0.05$). In all fish species studied a significantly positive correlation between length and weight ($P < 0.001$) was verified. The correlation coefficients obtained were: $r = 0.9651$ for *Lophius* spp.; $r = 0.9517$ for *Helicolenus dactylopterus*; $r = 0.7923$ for *Aphanopus carbo*; and $r = 0.9806$ for *Lepidorhombus* spp.

Levels of total and organic mercury, selenium and α-tocopherol and the corresponding mean, standard deviations, median, range measured in muscle and liver tissues are summarised in Table 3.

Considering all fish species, the range of Hg_T in the muscle and liver tissue were, respectively, 0.06–1.75 and 0.04–3.96 mg kg^{-1} wet weight. The Hg_T content, either in muscle or liver tissue, was not different ($P > 0.05$) between males and females for every species studied. This fact was also noted by Monteiro *et al.*[24] for *Helicolenus dactylopterus* caught in the sea around the Azores.

Table 2. Number of individuals (*n*), length (cm), weight (g) and sex of the studied species

Species	*n*	Length range	Weight range	Sex
Lophius spp.	22	40–77	950–7050	M
	11	41–95	1100–12100	F
Helicolenus dactylopterus	32	21–36	162–996	M
	18	22–32	190–478	F
Aphanopus carbo	17	88–120	1038–2278	M
	33	90–122	940–3050	F
Lepidorhombus spp.	25	23–46	90–788	M
	28	22–33	74–341	F

M, male; F, female.

Helicolenus dactylopterus Hg_T mean values registered in the present work (mean: 0.66 ± 0.26 mg kg^{-1} wet weight) are higher than those reported by Andersen and Depledge[25] (mean: 0.260 mg kg^{-1} wet weight) and Monteiro *et al.*[24] (mean: 0.29 ± 0.025 mg kg^{-1} wet weight) for the same species caught in the Azores area. However, this different mean level may be due not only to the geographic capture zone, but also to the range size of the specimens analysed in these works (range size: 13.8–29.6 cm noted by Andersen and Depledge,[25] 11.2–47.2 cm noted by Monteiro *et al.*[24] and 21.2–36.0 cm in the present work). It is recognised that, in fish, mercury usually increases with body size and/or age, so larger and/or older fish generally have higher concentrations than do smaller or younger fish. For *Helicolenus dactylopterus*, a significant positive correlation ($P < 0.05$) with Hg_T in the muscle tissue and both length and weight was found (Table 4). This finding has also been verified by other authors.[24,25] According to the Von Bertalanffy growth model for combined sexes of *Helicolenus dactylopterus*, found by Monteiro *et al.*,[24] the sampled fish used in the present study was between 5 and 20 years old. In addition, the same work reported that, during the first 20 years of age, the period of exponential growth, there is a significant increase in the mass of mercury accumulated each year in the muscle tissue. After age 20, the inflection and decrease in growth rate is accompanied by an exponential increase in mercury accumulation. The same author[24] also verified that after age 20 the annual increase in mercury is approximately constant. It was also revealed that higher levels of mercury, when compared with that in other species, could be due to its slower growth and greater longevity, resulting in longer exposure to contamination.

The mean percentage of Hg_{Org} in the muscle of *Helicolenus dactylopterus* was $86 \pm 6\%$. Similar mean percentage (85.9%) for the same tissue and fish species was obtained by Andersen and Deplege.[25]

Aphanopus carbo is a deep-water species, living up to a maximum of 12 years[26] and is considered a large carnivorous predator.[27] The mean total mercury concentration in the muscle for this species (0.63 ± 0.27 mg kg^{-1} wet weight) was lower than the values reported by other authors. The study by Afonso *et al.*[28] exhibited mean levels near 0.9 mg kg^{-1} wet weight in *Aphanopus carbo* caught off both Madeira and the Azores archipelagos. Nevertheless, in that study the maximum level found in the areas of sampling was lower (around 1.4 mg kg^{-1} wet weight) when compared with the specimens sampled in Sesimbra (off the Portuguese mainland) of the present study (maximum was 1.75 mg kg^{-1} wet weight). Renzoni *et al.*[29] reported a Hg_T maximum concentration in *Aphanopus carbo* caught in the Madeira archipelago, near to those verified in the present study (1.8 mg kg^{-1} wet weight). In the liver of *Aphanopus carbo* the

J Sci Food Agric **88**:2543–2550 (2008)
DOI: 10.1002/jsfa

Table 3. Concentrations of total (mg kg^{-1} wet weight) and organic mercury (%), selenium (mg kg^{-1} wet weight) and α-tocopherol (mg kg^{-1} wet weight) in tissues of the species studied

Species and tissue	Hg_T	Hg_{Org}	Selenium	α-Tocopherol
Lophius spp.				
Muscle				
Mean ± SD	0.43 ± 0.32	84 ± 6	0.43 ± 0.22	1.1 ± 1.4
Median	0.32	86	0.34	0.5
Range	0.06–1.51	73–91	0.21–0.90	0.2–5.0
n	33	10	10	10
Liver				
Mean ± SD	0.21 ± 0.16	75 ± 8	1.42 ± 0.64	ND
Median	0.17	76	1.43	
Range	0.04–0.68	61–85	0.71–2.69	
n	33	10	1	
Helicolenus dactylopterus				
Muscle				
Mean ± SD	0.66 ± 0.26	86 ± 6	0.36 ± 0.07	4.4 ± 5.0
Median	0.65	84	0.35	2.9
Range	0.10–1.14	81–99	0.28–0.48	0.1–16.8
n	50	11	9	10
Aphanopus carbo				
Muscle				
Mean ± SD	0.63 ± 0.27	86 ± 5	0.46 ± 0.13	15.9 ± 20.3
Median	0.58	87	0.44	9.3
Range	0.14–1.75	80–95	0.33–0.81	2.8–70.3
n	50	10	10	10
Liver				
Mean ± SD	1.31 ± 0.88	34 ± 7	3.86 ± 0.96	ND
Median	1.22	34	3.41	
Range	0.11–3.96	18–42	2.77–5.78	
n	50	10	9	
Lepidorhombus spp.				
Muscle				
Mean ± SD	0.33 ± 0.24	87 ± 7	0.41 ± 0.09	6.6 ± 8.6
Median	0.25	87	0.42	4.2
Range	0.10–1.22	71–96	0.24–0.57	0.1–26.1
n	53	13	12	8

n, number of fish analysed; ND, not determined; SD, standard deviation.

Table 4. Correlations among length, weight and levels of total and organic mercury, selenium and α-tocopherol in tissues of the species studied

	Lophius spp.		*Helicolenus dactylopterus*	*Aphanopus carbo*		*Lepidorhombus* spp.
	Muscle	Liver	Muscle	Muscle	Liver	Muscle
Length with						
Total Hg	NS	0.622†	0.339*	0.522†	0.511***	0.492†
α-Tocopherol	NS	NS	NS	NS	NS	0.746*
Weight with						
Total Hg	NS	0.610†	0.309*	NS	0.329*	0.428***
Selenium	NS	NS	NS	0.762**	0.924†	NS
α-Tocopherol	0.650*	NS	NS	NS	NS	NS

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; † $P < 0.0001$; NS, not significant.

Hg_T mean content was 1.31 ± 0.88 mg kg^{-1} wet weight with a maximum of 3.96 mg kg^{-1} wet weight. In accord with the studies by Afonso *et al.*[28] and Bebianno *et al.*,[30] these levels indicate that mercury accumulation is greater in liver than in muscle (liver:muscle ratio > 1). This fact can be explained by uncontaminated fish that recently migrated into a mercury-polluted area.[31] Nevertheless, the percentage of Hg_{Org} was lower in the liver ($34 \pm 7\%$) than in the muscle ($86 \pm 5\%$). Riisgård and Hansen,[31] in a study of mercury accumulation and elimination in flounders force fed with contaminated food, registered equal

concentrations of organic and inorganic mercury and found that organic mercury is rapidly incorporated in blood and accumulated in the muscle tissue. Much less inorganic mercury is accumulated in blood or muscle but retained in the liver and eventually excreted via liver bile. The same work also supports the notion that organic mercury is biotransformed to inorganic mercury in the liver. This fact probably explains the low level of organic mercury in the liver when compared to the amount of inorganic mercury. In the present study and taking into account the age–length relationships found by Morales-Nin *et al.*,[26] *Aphanopus carbo* was between 4 and 10 years. In contrast to the studies by Afonso *et al.*[28] and Renzoni *et al.*,[29] the present *Aphanopus carbo* showed a significant positive correlation for the level of Hg_T in muscle, either with length or weight, and in liver tissue, with weight. Bebianno *et al.*[30] noted that total mercury increased exponentially with size (total length, cm) in the muscle and liver and the pattern of mercury was similar between tissues. A positive correlation between both mercury levels in the muscle and liver was also verified ($r = 0.592$, $P < 0.0001$).

Lophius species are voracious predators that feed on a wide variety of small and juvenile fish[32] and can live up to age 20.[33] By referring to the results obtained by Landa *et al.*[33] in a study concerning the growth of *Lophius piscatorius* and *L. budegassa*, the fish used in the present work was more than 4 years of age. *Lepidorhombus* is a visual day feeder, which feeds on prey such as fish that move quickly, although the smaller specimens are more dependent on smaller prey such as crustaceans.[34] Both *Lepidorhombus boscii*[35] and *L. whiffiagonis*[36] live up age 10. Storelli and Marcotrigiano[32] identified the concentrations of total mercury in *Lophius piscatorius and L. budegassa* and in *Lepidorhombus boscii*. Such concentrations showed a higher maximum in the *Lophius* species (2.22 mg kg^{-1} wet weight for *L. piscatorius* and 1.62 mg kg^{-1} wet weight for *L. budegassa*) and a lower maximum in *Lepidorhombus boscii* (0.92 mg kg^{-1} wet weight) when compared to the maximum level obtained in the present work (1.51 mg kg^{-1} wet weight for *Lophius* spp. and 1.22 mg kg^{-1} wet weight for *Lepidorhombus* spp.). This difference may be due to the different geographical zone (the samples were from the southern Adriatic sea along the Apulian coast) and in the case of *Lepidorhombus boscii* also due to the lower weight of the samples (maximum weight was 225.8 g) when compared to the weight of the *Lepidorhombus* spp. in the present study (maximum weight was 788 g). In contrast to the result verified in *Aphanopus carbo*, the mean concentration of Hg_T in the muscle of *Lophius* spp. (0.43 ± 0.32 mg kg^{-1} wet weight) was higher than in liver (0.21 ± 0.16 mg kg^{-1} wet weight). So the liver:muscle ratio is <1. This may occur when mercury is no longer taken up, the liver concentration decreases and muscle level after some time may exceed the liver level. Nevertheless, this ratio is a somewhat problematic parameter because chronically contaminated fish can also show a liver:muscle ratio <1.[31] The mean percentage of Hg_{Org} for *Lophius* species was $84 \pm 6\%$ and $75 \pm 8\%$, respectively, for muscle and liver. This percentage was $87 \pm 7\%$ in the muscle of *Lepidorhombus* spp. Except for muscle of *Lophius* spp., Hg_T had a significant positive correlation with length and weight in both tissues (muscle and liver) species. Pellegrini and Barghigiani[34] and Barghigiani *et al.*[37] also found a positive correlation between mercury levels and weight in the muscle of *Lepidorhombus boscii*. Storelli and Marcotrigiano[32] found in *Lophius piscatorius* and *L. budegassa* a mercury increase with length.

The mean concentrations of selenium were quite constant and for all species the range in the muscle tissue was between 0.21 and 0.90 mg kg^{-1} wet weight. Similar values were described by other authors.[10,38] The greatest levels were found in the liver of the *Lophius* spp. (range 0.71–2.69 mg kg^{-1} wet weight) and *Aphanopus carbo* (range 2.77–5.78 mg kg^{-1} wet weight). *Aphanopus carbo* weight was positively correlated with levels of selenium in the muscle ($P < 0.01$) and liver ($P < 0.0001$). Mean contents of α-tocopherol ranged from 1.1 to 15.9 mg kg^{-1} wet weight in *Lophius* spp. and *Aphanopus carbo*, respectively. Similar contents of α-tocopherol in marine fish flesh were reported by other authors.[13,39] The best source of α-tocopherol was *Aphanopus carbo*, reaching a maximum of 70.3 mg kg^{-1} wet weight. Nevertheless, fish flesh contains low to modest amounts of vitamin E when compared with other products.[13] α-Tocopherol showed a significant positive correlation ($P < 0.05$) with *Lepidorhombus* spp. length and *Lophius* spp. weight. No statistical significant differences ($P > 0.05$) between sex and the analysed levels of the two antioxidants (selenium and α-tocopherol) were verified.

No significant correlations were found between Hg_T and selenium or α-tocopherol in the muscle of any studied species. This fact was also verified by Barghigiani *et al.*[37] and Plessi *et al.*[40] among levels of mercury and selenium evaluated in *Lepidorhombus boscii, Lophius piscatorius* and in several other fish species. However, in liver a significant negative correlation was found between selenium and the percentage of Hg_{Org} ($r = -0.823$; $P < 0.01$).

The molar ratio Se:Hg in the muscle was similar among species and varied from 1.7 (*Helicolenus dactylopterus*) to 3.7 (*Lophius* spp.). Similar ratios were presented by other authors.[25,38,40] This ratio in the liver tissue was 21.4 for *Lophius* spp. and 26.8 in *Aphanopus carbo*. So, the selenium concentration is higher than mercury in both tissues on a molar basis. The ratio 1:1 among selenium and mercury in liver and other tissues has been described.[5]

No significant correlation was obtained between selenium and α-tocopherol in the muscle, but the trend was negative. According to De Silva and

DOI: 10.1002/jsfa

Andersen[41] the vitamin E requirements are greater in selenium-depleted fish, which is in agreement with the results obtained here.

The species studied are considered deep-water, long-lived (more than 5 years) and the higher trophic level position may also contribute for the accumulation of contaminant metals in their tissues. The efficient accumulation of organic mercury in the muscle tissue (nearly 90%) and its lack of elimination, results in an increasing mercury concentration with both age and trophic level in the marine grazing food-chain.[31] This fact may explain the higher level of organic mercury in the muscle tissue of the studied species (organic mercury mean percentage above 84%).

Different organisms can bioaccumulate mercury at varying concentrations and eventually to harmful levels, when their normal parameters are exceeded, thus exposing the human population who consume seafood to a potential danger.[32] According to a European Commission regulation[15] the limit fixed for this metal in the species studied is 1.0 mg kg^{-1} wet weight with the exception of *Helicolenus dactylopterus*, which t have a maximum allowed level of 0.50 mg kg^{-1} wet weight. Taking this into account, around 6% of *Lophius* spp. and *Aphanopus carbo*, 2% of *Lepidorhombus* spp. and 70% of *Helicolenus dactylopterus* have concentrations of total mercury above the limit established by the EU regulation.

The Joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) recommends a provisional tolerable weekly intake (PTWI) of 5 μg kg^{-1} body weight for total mercury[16] and 1.6 μg kg^{-1} body weight for methylmercury.[17] For the antioxidants, adequate intakes of 30–70 μg Se day^{-1} and around 14 mg vitamin E day^{-1} have been estimated for adults.[11]

Portugal is one of the European countries that has the highest annual *per capita* fish consumption, around 57.1 kg,[42] which represents 156 g fish person^{-1} day^{-1}. In addition, it should be noted that the consumption is based on a high number of species. The estimated daily intake (μg kg^{-1} body weight) for each fish species studied was calculated based on the mean levels obtained for total and organic mercury, and considering an adult of 60 kg and the daily Portuguese consumption of fish *per capita* (Table 5). Taking these results into consideration, landings, import–export balance and the Portuguese population and taking account of the PTWI established by WHO,[16,17] the fish species studied have to be consumed in moderation, desirably not more than once a week. So, these results lead to the conclusion that mercury intake is a problem which justifies more attention. On the other hand, in contrast to α-tocopherol, which only provides a small contribution to the daily need, the muscle of the fishes studied is a good source of selenium and could largely supply the requirement.

Table 5. Total and organic mercury mean concentrations (mg kg^{-1} wet weight) and estimated daily intake (μg kg^{-1} body weight) for an adult of 60 kg and considering a daily fish consumption of 156 g

Species	Hg_T	Estimated daily intake	Hg_{Org}	Estimated daily intake
Lophius spp.	0.43	1.1	0.36	0.9
Helicolenus dactylopterus	0.66	1.7	0.57	1.5
Aphanopus carbo	0.63	1.6	0.55	1.4
Lepidorhombus spp.	0.33	0.9	0.29	0.8

ACKNOWLEDGEMENTS

The authors thank the financial support from QCA III – PO MARE ''Valorização e Inovação em Produtos da Pesca – 22-05-01- FDR-0006 – Vigilância, Segurança e Qualidade Alimentar'' project and to Dr Narcisa Bandarra, Vanessa Ferraria and Patricia Anacleto for the technical support in the determination of selenium and α-tocopherol. The authors also thank the scientists of the U-REMS of the INRB-IP/IPIMAR, who supplied the fish and gave advice on the biological parameters.

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Paper V

STOCK STRUCTURE AND QUALITY OF BLACK SCABBARDFISH IN THE SOUTHERN NE ATLANTIC
L.S. Gordo (ed.)

Scientia Marina 73S2
December 2009, 77-88, Barcelona (Spain)
ISSN: 0214-8358
doi: 10.3989/scimar.2009.73s2077

Mercury, cadmium and lead in black scabbardfish (*Aphanopus carbo* Lowe, 1839) from mainland Portugal and the Azores and Madeira archipelagos

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SUMMARY: Total mercury (THg), cadmium (Cd) and lead (Pb) concentrations were investigated in muscle, liver and gonad tissue of black scabbardfish (*Aphanopus carbo)* from the southern northeast Atlantic at three Portuguese locations: the mainland and the archipelagos of the Azores and Madeira. Particular emphasis was placed on the comparison of metal levels between geographic locations. Other factors such as size, sex and sexual maturity were also investigated. The median concentrations (mg/kg ww) of THg showed a decreasing trend in the order liver>muscle>gonad. Cadmium levels decreased in the order liver>gonad>muscle and Pb levels decreased in the order gonad>liver>muscle. According to the EU limits, the concentration observed in some specimens, particularly those of THg, raises some food safety concerns. In all the tissues the concentrations of each toxic metal differed between locations. In particular: i) THg concentrations in specimens from Madeira were significantly higher than those from the Azores and the mainland; ii) Cd levels in specimens from the mainland were significantly higher than those from Madeira and the Azores; and iii) for all analysed tissues Pb levels were significantly higher in specimens from the Azores than in those from the mainland. The differences in toxic metal levels between locations were further discussed in relation to aspects of species dynamics and the influence of the main environmental features at each location.

Keywords: total mercury, cadmium, lead, muscle, gonad, liver, *Aphanopus carbo*, Northeast Atlantic.

RESUMEN: Mercurio, cadmio y plomo en el sable negro (*Aphanopus carbo* Lowe, 1839) de Portugal Continental y de los Archipiélagos de Madeira y Azores. – Se investigaron las concentraciones de mercurio total (THg), cadmio (Cd) y plomo (Pb) en músculo, hígado y gónada de sable negro en muestras recogidas en tres regiones del sur del Atlántico noreste: Portugal continental y archipiélagos de Madeira y Azores. Se puso especial énfasis en la comparación entre regiones. También fueron analizados los factores talla, sexo y madurez sexual. Una tendencia decreciente fue observada en las concentraciones medias (mg/kg ww) de THg entre hígado, músculo y gónadas. Los niveles de Cd decrecieron entre hígado, gónadas y músculo, y en caso de los niveles de Pb, decrecieron entre gónadas, hígado y músculo. De acuerdo con las regulaciones de la UE sobre el consumo de esta especie, las concentraciones observadas en algunos especímenes, particularmente de THg, han suscitado inquietudes relacionadas con la seguridad alimentaria. El factor región fue significativo en las diferencias entre los tres metales para todos los tejidos. Particularmente: i) la concentración de THg en especímenes de Madeira fue significativamente más alta que en Azores y en el continente; ii) los niveles de Cd en especímenes del Continente fueron

significativamente más altos que en Madeira y en Azores; y iii) en todos los tejidos analizados, los niveles de Pb fueron significativamente más elevados en Azores que en el continente. Estas discrepancias en los niveles tóxicos entre localizaciones son discutidas en relación con aspectos de dinámica de la especie y la influencia de características ambientales de cada región.

Palabras clave: mercurio total, cadmio, plomo, músculo, gónada, hígado, *Aphanopus carbo*, Atlántico Nordeste.

INTRODUCTION

Most fish species accumulate in their tissues contaminants present in the environment and in food (Dugo *et al*., 2006). Among the trace elements of general concern, total mercury (THg), cadmium (Cd) and lead (Pb) are of major potential ecotoxic significance in the marine environment (Falcó *et al.*, 2006; Storelli, 2008). Although these metals do not play any known metabolic function, they can cause damage in the human body, interfering in the functioning of many of its basic systems: renal, cardiovascular, gastrointestinal, endocrine, nervous, etc. (Pérez-Cid *et al.*, 2001). Mercury is a highly toxic metal that occurs naturally in the environment as a result of metal volatilisation associated with volcanic events or cinnabar deposits (mercuric sulphide, HgS). The anthropogenic contribution to environmental mercury is derived from metal extraction (e.g. gold mining) and from its direct or indirect industrial uses. Unlike cadmium and lead, inorganic mercury can be methylated and form organic mercury compounds covalently bound to carbon, such as methylmercury (CH_3Hg), which is one of its most toxic forms (Hrudey *et al.*, 1996). In addition, mercury is the only known metal that consistently biomagnifies through the food chain, i.e. predators accumulate higher mercury concentrations than those found in their prey (Monteiro *et al*., 1996). Cadmium is a stable, ubiquitous toxic metal that is not abundant in its pure state in the environment. It is a by-product of zinc, lead, and copper mining and smelting that enters the organism through the diet and is concentrated mostly in the kidneys and the liver (EPA, 1999). Fossil fuel combustion is the major source of pollutant lead in the atmosphere. Later it enters the marine environment through the atmospheric deposition by the wet and dry removal process (Paterson, 1987). Lead can accumulate in the bone, affect the central nervous system and interfere with the metabolism of hemoglobin. Living organisms tend to bioaccumulate mercury, cadmium and lead since they absorb the toxic substances at a greater rate than they are metabolised or excreted. As a result, bioaccumulation of toxic metals poses a serious threat to animal health, including that of humans.

The growing concerns about the presence of toxic metals in food led the European Commission to establish legal limits for several chemical contaminants. For black scabbardfish (*Aphanopus* carbo), maximum permissible levels of mercury, cadmium and lead are 1.0 mg/kg, 0.05 mg/kg and 0.30 mg/kg, respectively (EU 2006, 2008).

The black scabbardfish (family Trichuridae) is a top predator that inhabits the NE Atlantic slopes around isolated island groups and seamounts between 200 and 1600 m in depth (Parin, 1986; Martins and Ferreira, 1995). Juveniles are mesopelagic and adults are benthopelagic, migrating to midwater at night to feed on fish, squid and crustaceans (Merrett and Haedrich, 1997). This species is appreciated by fish consumers in Portugal, especially in the Madeira archipelago.

The aim of this study was to characterise the levels of mercury, cadmium and lead in muscle, gonad and liver of black scabbardfish caught off three different Portuguese regions (the mainland, the Azores and Madeira). Factors such as region, length, sex, and maturity stage that can influence the variability of metal concentrations in black scabbardfish in those three tissues were also investigated. Lastly, metal levels obtained in tissues were compared with established limits defined by the European Community Regulation (2006, 2008).

MATERIALS AND METHODS

Black scabbardfish samples were obtained on a routine monthly basis at the landing ports of Sesimbra (mainland Portugal) and Funchal (Madeira). They were obtained on an irregular basis at Santa Maria (the Azores) (Fig. 1), since there is no fishery targeting the species in this archipelago. For the purpose of this study, samples were collected in February-March, June-July and October-November (the spawning period in Madeiran waters, Figueiredo *et al*., 2003) from May 2005 to December 2007 but, due to problems involving sampling dispatch, it was only possible to sample two seasons in the Azores and Madeira. For each individual, total length (mm), total weight (g) and sex were recorded. Maturity

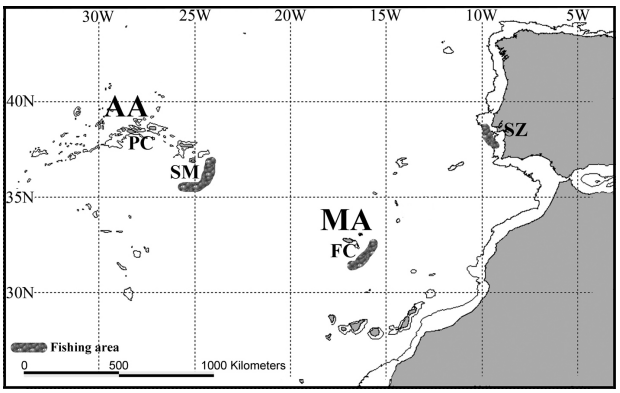

FIG. 1. – Map of the southern northeast Atlantic with the sampling locations of black scabbardfish and the 1000 m isobath. AA, Azores Archipelago; FC, Funchal; MA, Madeira Archipelago; PC, Pico Island; SM, Santa Maria Island; SZ, Sesimbra (mainland Portugal).

stage was assigned for each sampled individual according to the scale proposed by Gordo *et al*. (2000). The otoliths were removed and age was assessed by counting annuli in sectioned otoliths (Vieira *et al*., 2009). Samples of dorsal muscle (post-opercular region), gonad and liver were collected (±10 g), stored in a plastic bag and preserved at -20ºC. The frozen samples were sent to two laboratories (DOP-IMAR, UAç and INRB, IP/L-IPIMAR) for mercury, and cadmium and lead analysis, respectively. The number of samples analysed by each laboratory was different because the procedure for cadmium and lead analysis uses much more material than the method for THg analysis. Prior to the analysis, samples were defrosted at room temperature and homogenised in a food blender. All laboratory ware was cleaned with HNO_3 (10%) for 48 h and rinsed with ultrapure water (18.2 MΩ cm) to avoid contamination. Chemical reagents were pro-analysis or superior. Mercury, cadmium and lead concentrations were reported as milligrams per kilogram on a wet weight basis (mg/kg ww). All analyses were carried out at least in duplicate; an external calibration method was used for quantitative analysis.

Total mercury analysis

Total mercury (THg) was determined by cold vapour atomic absorption spectrophotometry (CVAAS) according to the procedures described by Hatch and Ott, (1968). Tissue sub-samples (0.2-1 g ww) were subjected to a wet mineralisation by digestion in sulphuric acid and subsequently oxidised to Hg^{2+}, using potassium permanganate. Mercury quantification was performed with a Bacharach Coleman Model 50D Mercury Analyser System. The Hg^{2+} was reduced to Hg^0 by adding stannous chloride. The volatile Hg^0 was bubbled into the closed system of the analyser and the absorption was measured (wavelength at 253.7 nm). The detection limit (expressed in mg/kg ww) was 0.012. Total mercury concentrations in the sample were determined in duplicate using an external calibration. The linear calibration curve was obtained by measuring the absorbance of six standards, with different concentrations, which were prepared by dilution from a 1000 mg l^{-1} mercuric chloride solution (Merck, Germany). Analytical quality control was provided through within- and between-laboratory quality control procedures employed throughout the study period. The accuracy of the method (expressed as relative error) was within 10%, and was monitored throughout the analysis of the certified reference materials (dogfish muscle, DORM-2; dogfish liver, DOLT-2) from the National Research Council of Canada, Ottawa. The results obtained in this study (4.49 ± 0.25 mg/kg; 2.14 ± 0.16 mg/kg) were in the range of the certified values (4.64 ± 0.26 mg/kg and 2.14 ± 0.28 mg/kg) for DORM-2 and DOLT-2, respectively. The precision (or reproducibility) of the method (expressed as the coefficient of variation of duplicates within and between batches) was within the usual 10% for total mercury determinations in biological samples (Saltzman *et al.*, 1983). Interference with sensitivity due to matrix and pre-treatment were assessed by the method of standard additions before the wet mineralisation digestion, and the average recoveries of added inorganic mercury was 96.8% (s.d. =11.3, n=12).

Cadmium and lead analysis

Determination of cadmium and lead levels was performed by flame atomic absorption spectrometry (FAAS), according to the procedures described by Jorhem (2000). Edible portion samples (10 g) were dry-ashed at 500ºC through a gradual temperature increase. Ash was dissolved in concentrated nitric acid and the solution obtained was evaporated to dryness. The final residue was dissolved once again with 5 ml nitric acid 15% (v/v) and transferred to 10-ml volumetric flasks, and final volumes were adjusted with ultrapure water. Quantification of these elements was performed using a Spectr AA-20 Varian spectrophotometer with deuterium background correction (λ = 228.8 nm for Cd and λ = 217.0 nm

for Pb). Detection limits (expressed in mg/kg ww) were 0.02 (Cd) and 0.01 (Pb). Certified reference material TORT-2 (Lobster hepatopancreas), from the National Research Council of Canada, was tested in the same conditions as the samples in order to assess analytical method accuracy. The results obtained in this study (0.35 ± 0.06 mg/kg for Pb and 26.8 ± 0.1 mg/kg for Cd) were in the range of the certified values (0.35 ± 0.13 mg/kg for Pb and 26.7 ± 0.6 mg/kg for Cd). Concentrations of Pb and Cd were calculated from linear calibration plots obtained by measurement of the absorbance of six standard solutions. These solutions were prepared by dilution of 1000 mg l^{-1} lead and cadmium nitrate solutions (Merck, Germany).

Statistical analysis

For muscle, liver and gonad samples, the differences in log-transformed THg, Cd and Pb concentrations between the classes of region, sex and maturity stage were explored through box plots. Data logarithmic transformation was applied to reduce the wide range of values and thus facilitate the joint plot of the three tissues for each factor. To evaluate the contribution of region, sex, maturity stage and covariate length, univariate general linear models (GLM) were adjusted to data for each combination of tissue and toxic metal. Multiple comparisons of estimated marginal means of metal concentrations between different locations were performed using the least significant difference test (LSD test). These tests were used to evaluate the statistical significance of the differences in concentration between locations. In all statistical analyses the level of significance was set at 0.05.

RESULTS

The range of total length (mm); the sample size by sex; the maturity stages; the total number of specimens analysed by region; the mean, range age

TABLE 1. – Summary of the descriptive statistics of the mercury analyses for the three tissues from the Azores, Madeira and Mainland: range of total length (mm); number of specimens by sex; maturity stages available for each geographic region; number of specimens sampled (N); mean and age range (years) and number of specimens aged; and median and range of total mercury concentration (mg/kg ww).

	Azores	Madeira	Mainland
Total length	625 - 1370	1165 - 1481	840 - 1293
Sex (F/M)	84 / 43	50 / 4	57 / 64
Maturity stage	I, II, III, IV, V	II, III, IV, V	I, II
N	135	54	121
Age	8 (6-12; n=35)	12 (10-15; n=20)	8 (5-12; n=104)
Total mercury			
Gonad	0.19 (0.03 - 2.74)	0.41 (0.07 - 1.40)	0.18 (0.05 - 0.94)
Liver	1.62 (0.43 - 45.9)	5.83 (1.10 - 50.23)	1.56 (0.42 - 7.15)
Muscle	0.71 (0.27 - 2.19)	1.45 (0.52 - 2.76)	0.69 (0.27 - 1.4)

TABLE 2. – Summary of the descriptive statistics of the cadmium and lead analyses for the three tissues from the Azores, Madeira and Mainland: range of total length (mm), number of specimens by sex, maturity stages available for each geographic region; number of specimens sampled (N) and median and range of lead and cadmium concentration (mg/kg ww).

Region	Azores	Madeira	Mainland
Total length	945 - 1225	1117 - 1481	834 - 1330
Sex (F/M)	23 / 16	32 / 23	120 / 100
Maturity stage	I, II, III, IV	II, III, IV, V	I, II, III
n	39	55	220
Cadmium			
Gonad	0.19 (0.04 - 0.22)	0.1 (0.03 - 0.71)	0.26 (0.04 - 1.30)
Liver	3.15 (0.58 - 13.67)	9.2 (2.63 - 19.81)	8.08 (2.56 - 30.24)
Muscle	<0.02* (<0.02*- 0.04)	<0.02* (<0.02*- 0.02)	0.02 (<0.02*- 0.08)
Lead			
Gonad	0.15 (0.07 - 1.15)	0.05 (<0.01*- 0.17)	0.05 (0.02 - 0.67)
Liver	0.1 (0.03 - 0.22)	0.03 (<0.01*- 0.06)	0.04 (<0.01*- 0.51)
Muscle	0.05 (0.03 - 0.11)	0.04 (0.03 - 0.12)	0.04 (<0.01*- 0.15)

* values below the detection limit: Cd = 0.02 mg/kg; Pb = 0.01 mg/kg

TABLE 3. – Significance of p-values for each factor considered in linear models adjusted to total mercury, cadmium and lead levels in gonad, liver and muscle.

Metal	Tissue	R^2	Region	Sex	Maturity stage	Length
Total mercury	Gonad	0.19	<0.001	ns	<0.001	<0.001
	Liver	0.3	<0.001	<0.001	<0.001	<0.001
	Muscle	0.59	<0.001	<0.001	<0.001	<0.001
Cadmium	Gonad	0.27	<0.05	ns	<0.05	ns
	Liver	0.19	<0.01	ns	ns	ns
	Muscle	0.1	<0.001	ns	ns	ns
Lead	Gonad	0.36	<0.001	ns	ns	ns
	Liver	0.19	<0.05	ns	ns	ns
	Muscle	0.08	<0.01	ns	ns	ns

ns = not significant (p>0.05)

(years) and the number of specimens aged; and the median and range of metal concentrations are summarised in Tables 1 (mercury) and 2 (cadmium and lead). There were differences in the length ranges between geographic locations: specimens from Madeira were larger (total length >1150 mm) than those from the Azores and the mainland (Tables 1 and 2). The sex ratio estimates varied between locations (Table 1). Maturity stages (MS) were not all found at each of the three locations, and in particular no mature individuals were observed off mainland Portugal (lack of MS IV and V).

Mercury

Concentrations of total mercury differed between tissues: they were highest in the liver, and decreased from muscle to gonad (Table 1). The adjusted GLM (Table 3) showed that the factors region, maturity stage and covariate length explained a fairly high percentage of the variance in mercury concentrations in liver (R^2 = 0.30) and muscle (R^2 = 0.59). For gonad this variance was lower (R^2 =0.19). Total mercury levels were significantly different between regions and maturity stages ($p<0.001$ in both cases) for all tissues. Length was also significant for all tissues ($p<0.001$). The levels of total mercury differed between sexes in liver and muscle ($p<0.001$ for both), but no differences were observed in gonad ($p= 0.93$).

For the three tissues, the highest total mercury levels were registered off Madeira and the lowest off the mainland (Fig. 2a). The total mercury levels in liver (maximum of 50.23 mg/kg ww recorded in one specimen from Madeira) were commonly much higher than those in the other two tissues (Table 1). Total mercury in gonad showed the lowest levels, with a minimum (0.03 mg/kg) and a maximum (2.74 mg/kg) in specimens from the Azores. Accordingly, the levels registered for the muscle were higher, respectively 1.45, 0.71 and 0.69 mg/kg ww for Madeira, the Azores and the mainland (Table 1). The LSD test indicated that total mercury means in muscle from specimens caught off the Azores and the mainland were significantly lower ($p<0.001$) than those from specimens caught off Madeira (Table 4). In gonad and liver, means were not statistically different between the mainland and the Azores. For muscle and liver the differences between Madeira and the mainland and between Madeira and the Azores were statistically significant ($p<0.001$; Table 4). The mean total mercury levels in muscle and liver were significantly higher ($p<0.001$) in females than in males (Fig. 2b, Table 3). Although males showed higher mean mercury concentrations in gonad than females, this difference was not statistically significant (Fig. 2b; Table 3). Total mercury levels increased from maturity stage I to maturity stage IV and decreased in the post-spawning stage (MS V; Fig. 2c). The highest variability of total mercury levels was registered for maturity stage II (Fig. 2c).

Cadmium

In all three regions the cadmium concentration was higher in liver tissue than in gonad or muscle (Table 2 and Fig. 3a). The highest cadmium concentration was found in liver of fish caught off the mainland (30.24 mg/kg ww) and the lowest (below 0.02 mg/kg ww) in muscle from specimens of all three regions. The cadmium concentration in gonads ranged from 0.03 (Madeira) to 1.30 mg/kg ww (mainland).

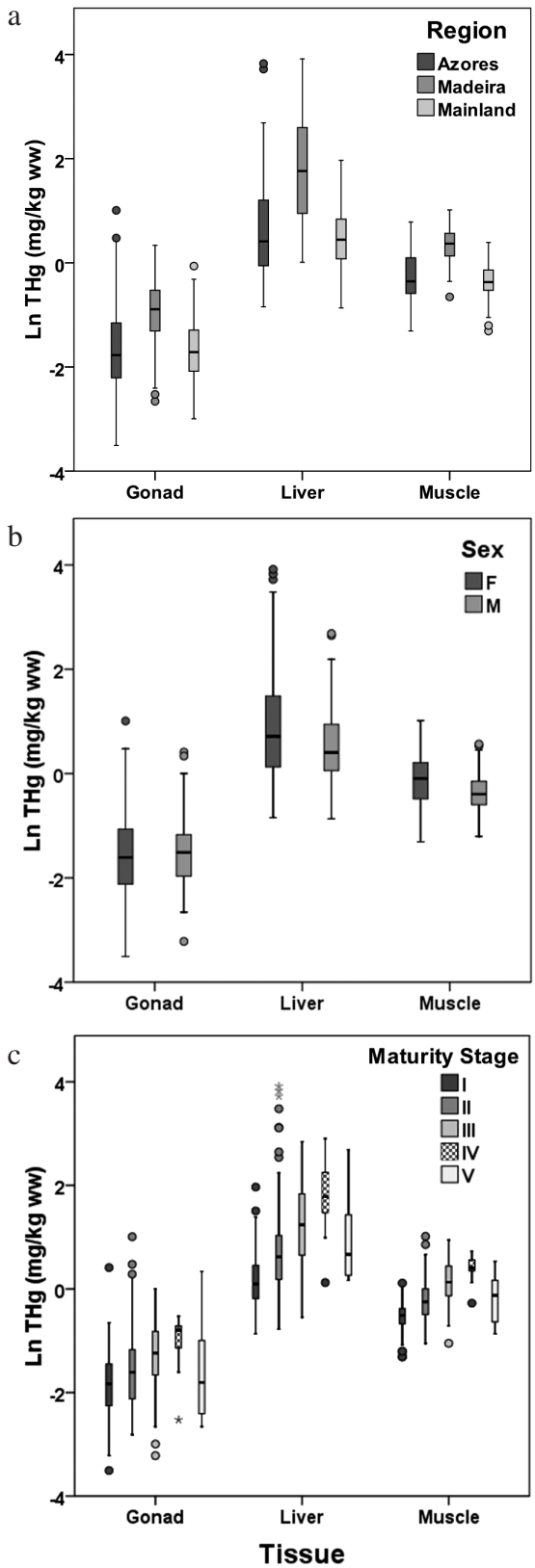

FIG. 2. – Box plots of total mercury concentrations by (a) region, (b) sex, and (c) maturity stage. Each box plot presents the median, the 1st and 3rd quartiles, the maximum and the minimum. The circles correspond to the outliers and the asterisks are the extreme outliers.

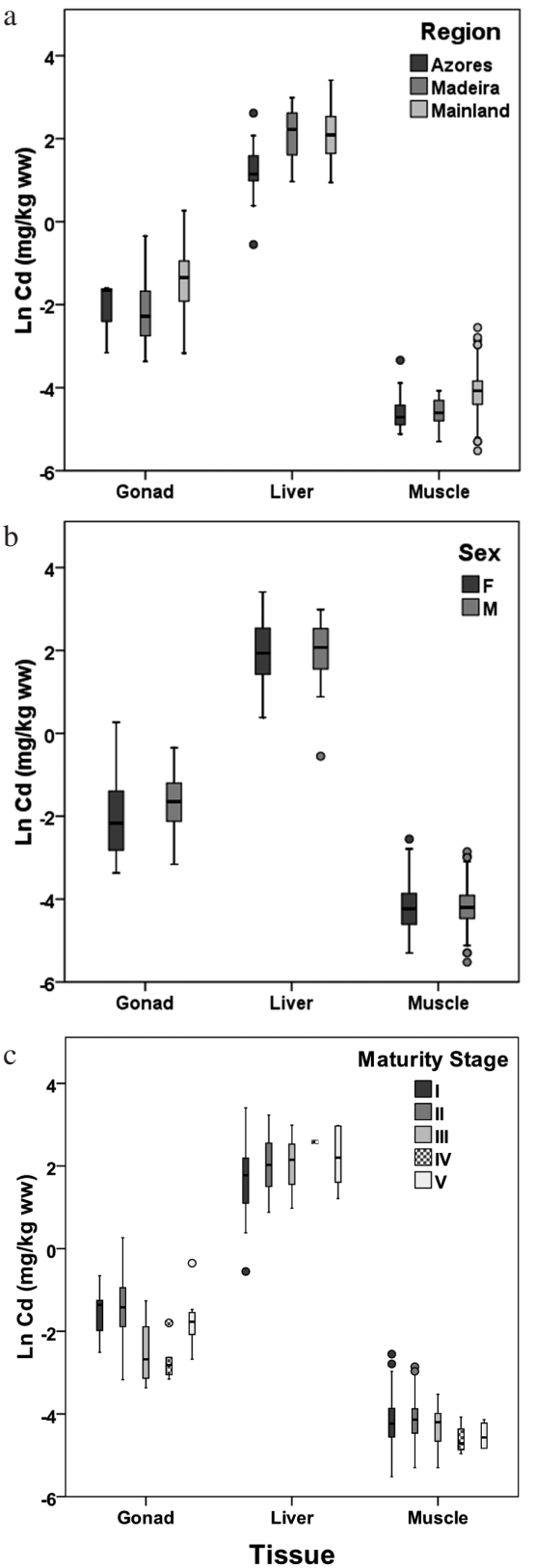

FIG. 3. – Box plots of cadmium concentrations by (a) region, (b) sex, and (c) maturity stage. Each box plot presents the median, the 1st and 3rd quartiles, the maximum and the minimum. The circles correspond to the outliers and the asterisks are the extreme outliers.

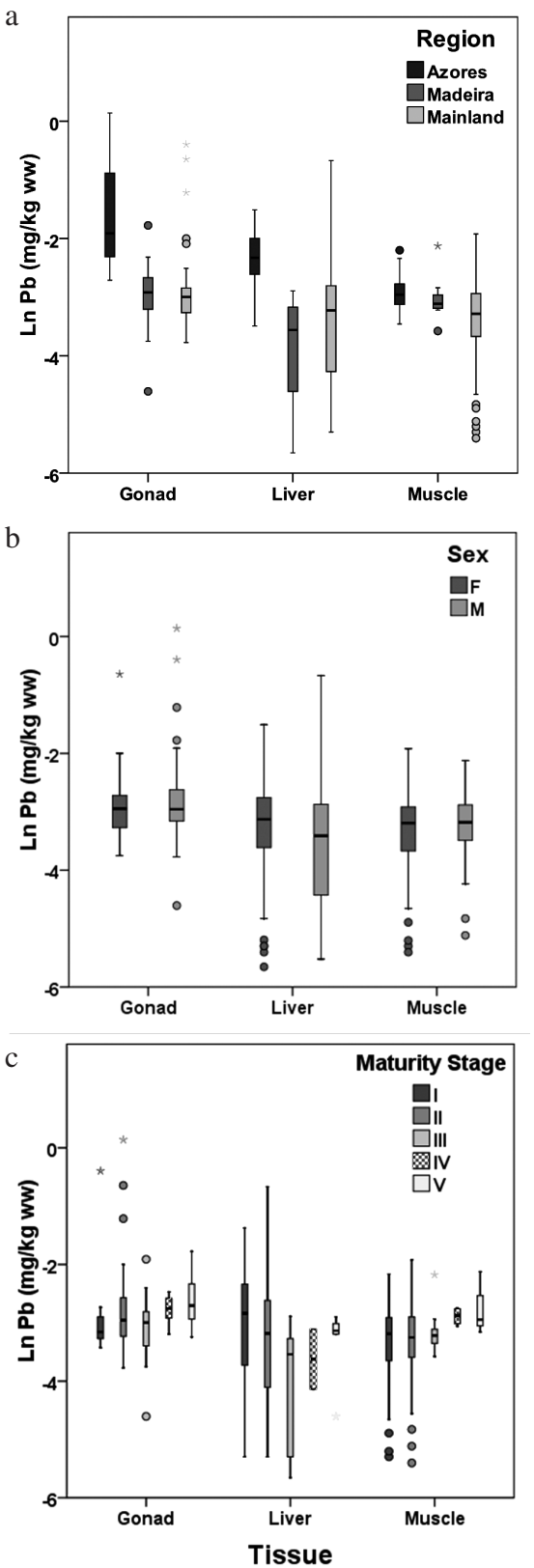

FIG. 4. – Box plots of lead concentrations by (a) region, (b) sex, and (c) maturity stage. Each box plot presents the median, the 1st and 3rd quartiles, the maximum and the minimum. The circles correspond to the outliers and the asterisks are the extreme outliers.

The adjusted GLM (Table 3) showed that region, maturity stage and length explained a low percentage of the variance of cadmium concentrations in the three tissues (liver; $R^2 = 0.19$; muscle, $R^2 = 0.10$ and gonad, $R^2 = 0.27$). For all the tissues, the adjusted GLM (Table 3) revealed that region was a significant factor in explaining concentration differences in muscle ($p<0.001$), liver ($p<0.01$) and gonad ($p<0.05$). The maturity stage was only significant in the case of gonad ($p<0.05$). The pairwise comparison indicated that cadmium mean concentrations in muscle and liver differed significantly ($p<0.001$) between the Azores and the mainland (Table 4). For gonad tissue, significant differences ($p<0.01$) were found only between Madeira and the mainland.

Male gonad showed higher median cadmium levels than female gonad, but the difference was not statistically significant ($p>0.05$) (Fig. 3b, Table 3). Differences between maturity stages were detected for gonad ($p<0.05$, Table 3). Maturity stages III and IV showed the lowest median levels and in the remaining stages median values were similar (Fig. 3c).

Lead

In general, lead showed a decreasing gradient from gonad to liver and lastly to muscle (Table 2). The region was the only factor that explained the variance in lead concentrations for all analysed tissues (liver, $R^2 = 0.19$; muscle, $R^2 = 0.08$ and gonad, $R^2 = 0.36$) (Table 3), of which the median lead levels were highest for the Azores (Table 2). The maximum concentration (1.15 mg/kg ww) was observed in the gonad of a specimen from the Azores and the minimum (<0.01 mg/kg ww) in the liver of specimens from Madeira and the mainland. The median concentrations in muscle were similar within all the regions (0.04-0.05 mg/kg ww; Table 2). The pairwise comparisons (Table 4) showed that lead levels in liver and gonad differed significantly between Madeira and the Azores ($p<0.05$ and $p<0.001$, respectively). The mainland and the Azores differed from each other in terms of concentrations in all three tissues ($p<0.001$ for gonad and muscle; $p<0.05$ for liver; Table 4). Lead levels in all three tissues did not differ between sexes (Fig. 4b). In liver, the ranges of lead levels for the first three maturity stages (I, II and III) were fairly wide, exhibiting a decreasing trend from stage I to IV. Median levels of stage V were similar to those of stage II (Fig. 4c).

TABLE 4. – Multiple comparisons between total mercury, cadmium and lead estimated marginal means by region for each tissue (mean difference; standard error; significance of p-value).

Metal	Tissue	Azores *vs* Madeira	Azores *vs* Mainland	Madeira *vs* Mainland
Total mercury	Gonad	-0.15; 0.05 (***)	0.02; 0.04 (ns)	0.17; 0.06 (**)
	Liver	-3.81; 0.90 (***)	0.41; 0.72 (ns)	4.22; 1.09 (***)
	Muscle	-0.32; 0.05 (***)	-0.11; 0.04 (**)	0.21; 0.07 (***)
Cadmium	Gonad	0.00; 0.11 (ns)	-0.20; 0.12 (ns)	-0.20; 0.07 (**)
	Liver	-7.01; 2.81 (*)	-5.79; 1.74 (***)	1.22; 2.65 (ns)
	Muscle	0.00; 0.00 (ns)	-0.01; 0.00 (***)	-0.01; 0.00 (*)
Lead	Gonad	0.44; 0.09 (***)	0.37; 0.09 (***)	-0.07; 0.06 (ns)
	Liver	0.08; 0.04 (*)	0.05; 0.02 (*)	-0.03; 0.03 (ns)
	Muscle	0.01; 0.01 (ns)	0.02; 0.00 (***)	0.00;0.01 (ns)

Significance (p-values): (ns) $p \geq 0.05$; (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$

DISCUSSION

Copper, zinc and iron are essential for fish metabolism but mercury, cadmium and lead have no known role in biological systems. These non-essential metals use a similar metabolic pathway to that of essential minerals, being assimilated by fish from water, food or sediment, and consequently accumulating in their tissues (Canli and Atli, 2003). The concentration of mercury is enhanced by the high availability of its stable organic form below the thermocline. This form, especially methylmercury, occurs in the cold deep waters of the north Atlantic and is assimilated by organisms four times more efficiently than the inorganic form (Mason *et al*., 1995).

In general, deepwater species show an increased potential for accumulating trace metals (Gordon *et al*., 1995). It has also been reported that mercury accumulation in fish increases with trophic level, age, size and depth (Riisgård and Hansen, 1990; Monteiro *et al*., 1991; Joiris *et al.*, 1995; Magalhães *et al.,* 2007). This is the case for *A. carbo*, a voracious predator, living at depths of around 1200-1700 m and exhibiting fairly rapid growth rates in the first years of life (Vieira *et al.*, this issue). The median mercury concentrations obtained in this study are in agreement with the levels reported in the literature (Afonso *et al*., 2007, 2008 and Bebiano *et al*., 2007). The median total mercury concentrations found in muscle tissues from *A. carbo* were approximately 5-10 times higher than those reported in other benthopelagic species caught in more northern areas of the Atlantic (Mormede and Davis, 2001b; Cronin *et al*., 1998). Moreover, our results were within a similar range to those obtained for other deep-water species captured off the Azores (Magalhães *et al.*, 2007) and mainland Portugal (Afonso *et al*., 2008), and for hydrothermal vent fish species at the Mid-Atlantic Ridge (Martins *et al*., 2006).

Total mercury levels observed in black scabbardfish caught off the Azores and Madeira were significantly higher than those caught off the mainland. Potential sources of mercury are the volcanism associated with the expansion of the Mid-Atlantic Ridge and the occurrence of mercury-enriched Mediterranean water, which flows into the North Atlantic at depths below 1000 m (Johnson and Stevens, 2000). However, the greatest source of mercury seems to be the atmospheric deposition of elemental mercury associated with the global cycle by way of long-range transport (Monteiro *et al.*, 1996).

While little is known about trace metal composition of water masses in the northeast Atlantic, these archipelagos are also characterised by highly dynamic oceanographic, atmospheric and geological processes, namely the North Atlantic gyre, the Azores front, the upwelling off the Iberian Peninsula and the Canary Islands current (Pollard and Pu, 1985), which can contribute to the dispersal and concentration of pollutants in these areas.

The higher total mercury levels found in specimens from Madeira in comparison with those from the Azores might be related to the size of the specimens analysed. The length of specimens from Madeira was greater (total length >1150 mm) than that of specimens from the Azores, and a higher percentage of females (93%) was observed in Madeira individuals. Mercury concentrations in tissues increased with fish length (data not shown), so the larger (older) fish showed higher concentrations than the smaller (young) ones. Females showed higher mercury concentrations than males, a result that can be related to differences in growth rates between sexes (Vieira *et al*., 2009). As mercury accumulation is

age-dependent, the faster-growing sex (males) exhibit lower concentrations due to higher dilution of pollutant as a result of tissue growth. In addition, the higher total mercury concentrations were found in the highest maturity stages and mostly corresponded to specimens from Madeira, which were also the largest individuals analysed. These differences in maturity stages between regions reflect the reproductive dynamics of this species in the different regions, which is characterised by the inexistence of mature individuals off the mainland (MS: IV and V) and their occurrence only off Madeira (Neves *et al*., 2009). These observations might constitute a better explanation for the higher mercury concentrations found in Madeiran specimens than in the mainland individuals.

Similarly to mercury, the enrichment of cadmium in the deep sea suggests higher concentrations in benthic than in epipelagic biota (Mormede and Davies, 2001a). The cadmium concentrations in muscle of deep-water fish are generally low in comparison to levels found in the liver (Bustamante *et al*., 2003). In the present study, median levels of cadmium in the liver of black scabbardfish were 450 times higher than those found in the muscle, in agreement with the results obtained for other deep-water species (Cronin *et al*., 1998; Hornung *et al*., 1993; Mormede and Davies, 2001b) and those reported for the same species by Afonso *et al*. (2007). Cadmium contents were significantly higher in liver and in muscle in mainland individuals than in Azores individuals, and significantly higher in muscle and in gonads than in Madeiran individuals. These findings were expected because this metal is mostly derived from anthropogenic sources (e.g. metallurgical industries).

Lead concentrations in fish muscle are generally lower than in liver, a pattern that is also observed for cadmium (Mormede and Davies, 2001a). In the present study, lead levels found in muscle samples from the three Portuguese regions were of the same magnitude as those reported by Afonso *et al*. (2007) and Cronin *et al*. (1998). Our results were also consistent with those observed by Mormede and Davies (2001a) in several deep-water species caught off the Rockall Trough (west Scotland). In agreement with Afonso *et al*. (2007), we obtained higher lead levels in the Azorean than in the Madeiran specimens, but similar levels between Madeira and the mainland. Furthermore, it was observed that the majority of lead levels in the muscle of specimens collected off the mainland were below the detection limit of this method (<0.01 mg/kg ww). The lead levels in gonads and liver were 3 times higher in Azorean specimens than in those found in the other two regions. Though the Azores are distant from a known anthropogenic source of metal pollution, it is also conceivable that the input of lead may originate from remote sources of industrial pollution or from mineral dusts by way of "long-range atmospheric transport". Furthermore, arsenic and other elements such as lead originating in the NW African Sahara have been found in material collected from Azorean "dust-events" (Reis *et al.*, 2002), and may later settle down in the deep sea. In addition, hydrothermal activity associated with nearby shallow- and deep-vent waters can directly contribute to this metal enrichment in deep-water species from this oceanic region (Wallenstein *et al*., 2009; Cosson *et al*., 2008).

It is generally accepted that metal concentrations in the organism reflect the exposure in the aquatic environment and the intake through diet (Mormede and Davies, 2001a). A probable reason for the highly significant region effect observed in this study might be the differences in the diet of black scabbardfish from these three geographic areas. Studies on stomach contents of this species in the study region have so far been inconclusive, mainly due to the characteristics of the fishery. In longline fisheries the specimens remain attached to the gear long enough for the stomach contents to be completely digested, resulting in a high rate of emptiness (93%) (Freitas,1998; Anon, 2000). Nevertheless, older published data have described the existence of lower mesopelagic and upper bathypelagic fish species (e.g. Maul,1961) and more recently Freitas (1998) found crustacean species, cephalopods and fish from the meso- and bathypelagic zones in the stomachs of *A.carbo* captured in Madeira waters. There is also evidence that black scabbardfish from the mainland feed on the crustacean *Aristeus antennatus* (I. Figueiredo, *pers. com.*), so a preferred crustacean diet might explain the higher cadmium concentrations found in mainland specimens than in speciments from the Azores and Madeira, given the fact that crustaceans tend to accumulate cadmium, mainly in the hepatopancreas (ATSDR, 2008). A cephalopod-based diet could also contribute to the intake of toxic metals in black scabbardfish since they also prey on these invertebrates (Nakamura and Parin, 1993, Muus *et al*., 1998). In a recent study, Lourenço

et al. (2009) showed significant concentrations of cadmium (0.04–0.33 mg/kg ww), lead (0.02–0.09 mg/kg ww) and mercury (0.05–0.12 mg/kg ww) in three different species of cephalopods caught off the Portuguese coast. The variability of metal concentrations can be attributed to the availability of different prey (crustaceans, cephalopods and/or fishes) in the Portuguese regions studied. However, without additional data on dietary composition, it is not possible to explain these geographic differences based only on the black scabbardfish diet.

Regarding the metals distribution in the different tissues, it is recognised that the mechanisms by which fish regulate the accumulation of metals may be linked to the metal absorption or elimination processes, which in turn seem related to tissue and organ characteristics (Reinfelder *et al*., 1998). Black scabbardfish has higher levels of mercury and cadmium in liver than in the other analysed tissues. The high concentrations found in this organ can be explained by the liver's ability to accumulate large amounts of these metals, through the formation of a soluble metal-binding protein, and by its important role in storage, redistribution, detoxification, and transformation of pollutants (Evans *et al*., 1993; Mormede and Davies, 2001a,b).

The low concentrations of lead in liver and muscle have their origins in several mechanisms that are involved in this metal intake by fishes. Remarkably, there are no known specific metal-binding cytosolic proteins in the liver with high affinity for lead. Thus, rather than being accumulated in the liver, the absorbed lead is quickly distributed to other tissues (Gašpić *et al*., 2002) such as gonad tissues, as evidenced in this study. To our knowledge, this is the first study that reports on levels of total mercury, cadmium and lead in gonad of black scabbardfish. The inclusion of this organ in ecotoxicological studies allows a better understanding of metal distribution throughout the fish's lifespan.

With regard to public health, muscle is the most important tissue used to estimate metal concentrations since it is mainly used for human consumption. However, the toxic metal content in liver and female gonad should still be an issue of concern because these organs are also consumed in the Madeira archipelago. The median concentrations of mercury found in muscle of black scabbardfish from both the mainland and the Azores were below the limit established by the EU (1.0 mg kg^{-1} ww), whereas the median concentration in specimens from Madeira were above this limit. For all the regions, the median total mercury concentrations were above 1.0 mg kg^{-1} ww in liver and below this limit in gonads. Median lead concentrations were under the limit value (0.30 mg/kg ww) established by the EU (2006, 2008). The limit of 0.05 mg/kg ww established by the EU (2006, 2008) for cadmium was exceeded in both liver and gonads, but it was not reached in muscle in any of the regions studied.

In view of the high levels of mercury found and some results observed for the other two metals, it is not safe to assume that this species may be consumed regularly and without restrictions. It is advisable to perform a risk analysis in order to evaluate the PTWI (provisional tolerable weekly intake) of black scabbardfish in the overall diet of Portuguese consumers.

Taken together, the presence of high mercury levels in tissues from this benthopelagic species is expected and is related not only to the biochemistry of this metal but also to the ecological and biological characteristics of the species. *A.carbo* lives at great depths, occupies a high trophic level, is long-lived and is a predator subjected to longer burdens of this contaminant during its lifetime as a consequence of a diet rich in meso- and bathypelagic species.

Regarding cadmium and lead levels, the specimens landed on the mainland were significantly different from those landed in the Azores. Cadmium concentrations were higher in the mainland specimens, whereas lead concentrations were higher in the Azorean specimens. Contrary to the described distribution pattern of mercury, the region is the only factor that explains the observed variability of cadmium and lead levels. This may be related to the absence of biomagnification of these metals. As for cadmium and lead levels, the differences between maturity stages can be related to the energetic and chemical composition oscillations that occur during the reproductive cycle of this species.

In summary, the similarities and dissimilarities observed between regions might be due to differences in trace metal contents in the water, species physiology, and feeding preferences of the fish inhabiting these three Portuguese regions. In view of the fact that the exploration of deep-sea fish stocks has increased greatly, particularly in the North Atlantic, it is important to monitor the levels of metals in abiotic and biotic compartments of these marine ecosystems. Extending our knowledge to the biological cycle of *A. carbo*, including its feeding

ecology and sampling in other geographic areas, may not only provide a better understanding of toxic metals bioaccumulation mechanisms but also help to identify black scabbardfish populations within its geographic distribution.

ACKNOWLEDGEMENTS

This work was supported by Fundação para a Ciência e Tecnologia (project POCTI/CVT/46851/2002) and by the "Programa Nacional de Recolha de Dados da Pesca". We are grateful to Dália Reis, Ângela Canha and João Gil Pereira (DOP/Uaç-Azores) for the sampling and the reproduction and growth analyses in the Azores, and to Sara Reis and Joana Vasconcelos (DSIP-Madeira) for the sampling in Madeira. We also thank Maria Magalhães, Raul Bettencourt and Ruth Higgins for their support and critical reading of the manuscript. We are also grateful to the anonimous reviewers and the english reviewer for proof-reading of the manuscript.

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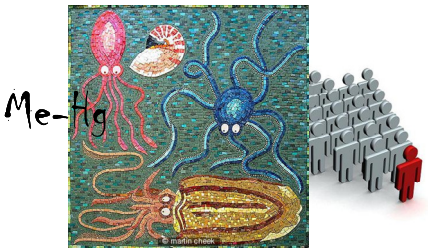
Scient. ed.: F. Maynou.
Received December 19, 2008. Accepted October 26, 2009.
Published online December 22, 2009.

Paper VI



Risk Assessment of Methylmercury Intake through Cephalopods Consumption

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ABSTRACT

The intake of methyl-Hg through consumption of three common cephalopod species, cuttlefish (*Sepia officinalis*), squid (*Loligo vulgaris*), and octopus (*Octopus vulgaris*), in Portugal as well as the associated probability of exceeding the provisional tolerable weekly intake (PTWI) were estimated by combining methyl-Hg contamination levels in these three cephalopods with constructed consumption scenarios or with a hypothesized consumption distribution for the general Portuguese population.

It was found that squid presents no serious health concern with respect to methyl-Hg, but, cuttlefish and octopus consumption should not exceed two 150 g meals per week. Moreover, the methyl-Hg risk assessment for Portuguese consumers revealed no danger concerning the observed cephalopods consumption levels. Tail estimation (TE) estimator was more accurate for lower probabilities, rendering accurate risk estimations different from zero. However, for higher probabilities, the much simpler plug-in (PI) estimator could be applied. Additionally, the limitations of a deterministic approach were shown.

KEYWORDS: methylmercury, cuttlefish, octopus, squid, risk assessment, extreme value theory

1. INTRODUCTION

During the last decades, the health benefits of a diet rich in seafood have been extensively recognized. In particular, ω3 polyunsaturated fatty acids (ω3-PUFA) are associated with decreased morbidity and mortality due to various diseases. Several epidemiological studies[(1)] have found a reduced risk of coronary heart disease, hypertension, stroke and consequent further effects due to the beneficial effects of these fatty acids on lipids —

namely, cholesterol—, blood pressure, eicosanoids and coagulation rate. For instance, the World Health Organization (WHO) and Food and Agriculture Organization (FAO) recommend a regular fish consumption of 1-2 servings per week in order to provide an equivalent of 200-500 mg of the ω3-PUFA eicosapentaenoic and docosahexaenoic acids.[2] However, seafood can also be a source of pernicious contaminants: PCBs, dioxins, residues of pesticides and contaminant metals.

Cephalopods, in particular, can be a source of contaminant metals, since they may accumulate these metals in their tissues.[3,4] A recent paper has found significant levels of various toxic metals in cephalopods, such as octopus or squid, caught in Portuguese waters.[5] On the other hand, cephalopods are one of the most important seafood groups captured in Europe.[6] Concerning Portuguese consumers, cephalopods had a share of approximately 8 % of the wholesale market sales. Furthermore, these cephalopods correspond to an economic value (in terms of auction transaction) approximately three times more important, representing a quarter of the market.[7]

Among contaminant metals, mercury is not known to play any metabolic function and can be very toxic to humans, even at very low concentrations.[8] WHO and FAO have established as provisional tolerable weekly intake (PTWI) value 5 μg/kg for total mercury.[9] For methylmercury (methyl-Hg), a highly toxic organic compound of mercury, given its ready and easy absorption by the gastrointestinal tract, WHO and FAO reduced in 2003 the PTWI from 3.3 (two thirds of the total mercury) to 1.6 μg/kg.[10] Therefore, a quantitative assessment of the methyl-Hg intake by populations eating large amounts of cephalopods as well by the general population should be carried out in order to have more information about the contribution of each species to a given methyl-Hg intake.[11] This

concern is even greater for the cephalopods consumption in Southern Europe and, particularly, in Portugal, because these populations frequently consume cephalopods, mainly cuttlefish, octopus, and squid.[12]

Contaminant assessment can be performed by combining food consumption data or, in the absence of this information, consumption estimates and plausible scenarios with contaminant concentration levels, according to different approaches: deterministic or probabilistic. In the former, a single 'worst case' point estimate of each input variable (consumption and contamination level) is used. In the latter, the variability of all input variables can be accounted for by using appropriate probability distributions with appropriate parameters.[11] This approach is increasingly being used for the assessment of contaminants in foods.[11,13,14] Its output is a probability distribution providing some insight into the variability of the assessed intake in a population. Thus, in this way it is possible to estimate the probability that the individual exposure to a specific heavy metal exceeds the PTWI. In a large population, an accurate estimation of this quantity is very important since even a difference of 1 ‰ involves a large number of individuals. However, the estimation of this probability highly depends on the tail behaviour of the distribution, since PTWI values are usually higher than most individual intakes. The main statistical tool for this is the extreme value theory (EVT).[15] This tool has been used in very different areas, such as hydrology or finance.[16] In these areas, extreme values are of crucial importance. For contamination and consumption data, extreme values are also very important, since risk mainly concerns highly polluted food or high consumptions. EVT properly addresses these extremes because it takes full account of the very high (or very low) observed values.[15] The principle is to model the tail of an exposure distribution by a Pareto type distribution, characterized by a Pareto index, which can be interpreted as a risk index.[15] This index is

estimated by the Hill estimator, which, in turn, shows a large instability, producing the so called 'Hill-horror plot'.[16] This instability may be greatly improved by using bias correction techniques,[17,18] hence, ensuring the achievement of more reliable estimates of the population at risk.

Therefore, the main objective of this paper is to estimate the methyl-Hg intake through the consumption of three cephalopod species, cuttlefish, octopus, and squid, in Portugal and, thus, to estimate the portion of a given population (whether the whole Portuguese population or sub-populations displaying specific consumption patterns) at risk.

2. MATERIAL AND METHODS

2.1. Raw Data

The individuals of the studied species, cuttlefish (*Sepia officinalis*), squid (*Loligo vulgaris*), and octopus (*Octopus vulgaris*) were adults and were caught in Portuguese continental waters with the purpose of ensuring a representative sampling of the cephalopod products marketed in Portugal. The weight ranged between 540 and 3100 g (a sample size of 37 individuals), 65 and 1200 g (26), 430 and 4900 g (43), for cuttlefish, squid, and octopus, respectively. Total Hg content was determined by cold vapour atomic absorption spectrometry[19] in a Bacharach Coleman MAS-50D Mercury Analyser. Methyl-Hg was extracted by a previously described method[20] and its level determined by atomic absorption spectrometry in a LECO AMA 254 Automatic Mercury Analyser. Moreover, whereas total Hg content was determined in all individuals, methyl-Hg was determined only in 10 individuals of each species. All contents were expressed in mg/kg of fresh weight. Furthermore, for those individuals whose methyl-Hg content was not determined, it was assumed that the ratio between methyl-Hg and Hg contents was identical to that

calculated for the 10 individuals of each species, that is, 90.7, 86.6, and 83.9 %, for cuttlefish, squid, and octopus, respectively.

Apparent consumption data was estimated from cephalopods capture totals in Portuguese waters (data from Portuguese National Statistics Institute, Instituto Nacional de Estatística[21]) and trade data (data from EUROSTAT).[22] Thus, import values were added to capture volumes and export values were subtracted. Afterwards, the attained apparent consumption values were divided by the population[23] and admitting that two thirds of the cephalopods weight would be surely edible, it was possible to estimate weekly *per capita* consumptions of 10.2, 31.5, and 30.4 g/(week.person), for cuttlefish, squid, and octopus, respectively.

2.2. Raw Data Processing

Probability distributions were fitted to the contamination and consumption data. Using all data and imposing as previous condition that methyl-Hg contents must be positive values, the best probability distributions, according to χ^2 tests for goodness of fit of the observed distributions to theoretical ones, were achieved with the software @ RISK® – advanced risk analysis for spreadsheets, from Palisade Corporation (Ithaca, NY, USA), version 4.5, 2005. For consumption, log-normal distributions with means of 10.2, 31.5, and 30.4 g/(week.person), and truncated in zero (an enforced minimum) were selected for cuttlefish, squid, and octopus, respectively. In **Figure 1**, the distribution for octopus is presented. Hence, a significant frequency at near zero was achieved with this solution, accounting for that portion of the population which does not eat cephalopod products. Log-normal distributions are widely used distributional forms in probabilistic assessment[11] and seem to

reflect the consumption patterns of cephalopod products in Portugal. Moreover, in the United States, using more complete data on the overall consumption of fish by the general population (from a survey conducted by the National Marine Fisheries Service), strong log-normal fits were found.[24]

2.3. Intake Levels Calculation

A distribution of the intake levels of methyl-Hg for cuttlefish, squid, and octopus can be achieved by combining the consumption log-normal distributions and the methyl-Hg content distributions for each studied cephalopod species. Alternatively, the latter distributions can be combined with a given weekly personal consumption (encompassing specific scenarios, from one portion to three portions of 150 g per week). In order to attain the intake distributions, values of each distribution curve are randomly sampled according to the Monte-Carlo method.[25] In this method, a given randomly selected cephalopod consumption from a virtual consumer (or a precise value of a specific scenario) is multiplied by the contaminant content in the corresponding cephalopod species, also randomly selected. This procedure is repeated many times with the software @ RISK®, rendering a random sampling size of 10000. Output values are divided by an average body weight, 60 kg,[10] producing a distribution of weekly toxic metal intakes expressed in μg per week and kg of body weight. For this approach, the random sampling size must be largely superior to the sum of all sample sizes. This condition is fulfilled because 10000 is much larger than the sum of 43 (maximum number of contaminant analyses) with 2 (effectively introduced consumption data, that is, average and standard deviation).

2.4. Risk Assessment

Methyl-Hg risks to human health are assessed by comparing the weekly intake level with the recommended safe exposure level, the methyl-Hg PTWI.[26] There are two alternatives for estimating the risk: the plug-in (PI) and the tail estimation (TE) based estimators.[15] The mathematical formulas and methodology were extensively presented in a previous paper.[12] TE estimation is based on the EVT, which is focused on the tail estimation of the distribution of the heavy metals intakes. Accordingly, the distribution tail is fitted to a Pareto law, which consists in estimating parameters C and γ for 'sufficiently large' numbers. These numbers are necessarily the k largest observed values of the output distribution. Estimation of the parameters can be done according to the formulas:

$$\gamma(k) = H_{k,n} = \frac{1}{k} \times \sum_{i=1}^{k} \log \frac{X_{n-i+1,n}}{X_{n-k,n}}$$

$$C(k) = \frac{k}{n} \times (X_{n-k,n})^{1/H_{k,n}}$$

where,

$H_{k,n}$ – Hill estimator;

k – number of considered largest values in the sample;

n – sample size.

The Hill estimator[16] is very sensitive to the choice of k. In fact, its bias increases with k, whereas its variance decreases with k. This bias problem is explained by the perturbation of data results through a slowly varying function, which causes deviations from a Pareto law.[27] So, a bias corrected Hill estimator may be required. For bias correction, the Hill estimator must be interpreted as an estimator of the QQ plot slope perturbed by a small

deviation induced by the L(x).[15] The estimation of the bias corrected parameters is done through the least squares method applied to the expression[18,27]:

$$Log(Z_i) = \mu + D_1 \times \left(\frac{i}{n}\right)^{\beta_1}$$

where,

$Z_i = i \times (Log(X_{n-i+1,n})-Log(X_{n-i,n}))$, following an exponential distribution;

$\mu = Log\gamma + \mu_0$ (being μ_0 the constant of Euler);

$D_1=-\beta_1 \times C^{-\beta 1} \times D$;

$\beta_1 = 1$.[28]

In this way, the bias corrected estimator of γ is calculated. These estimations can be done for different values of k, enabling to find the optimal sample fraction k* by minimizing the asymptotic mean squared error of the Hill estimator.[15] With optimal k*, the risk of exceeding the PTWI, $P(X_i>PTWI)$, may be estimated in a more accurate way.

3. RESULTS

3.1. Distribution of Me-Hg Content in Cuttlefish, Octopus, and Squid

The best fits for the methyl-Hg content in the three cephalopod species according to χ^2 test for goodness of fit of the observed distributions to theoretical ones were attained with extreme value distributions (**Table I**). All these distributions were truncated at zero in order to avoid negative values. This solution was preferred to truncating at the limit of detection, since some of the experimental data were below that limit. The fitted distributions means and standard deviations were identical to the same parameters of the raw data itself. The order of the methyl-Hg content means was the following one: cuttlefish>octopus>squid.

3.2. Risk Assessment for Cephalopods in Specific Scenarios

Hypothesizing specific scenarios for cuttlefish, octopus, and squid consumption and using the methyl-Hg distributions (**Table I**), it was possible to obtain intake distributions whose variability only depends upon the methyl-Hg contents found in each cephalopod species (**Table II**).

The average weekly intake of methyl-Hg per kg of body weight was always below the methyl-Hg PTWI (1.6 μg/(week×kg body weight)) even for the worst case scenario, a consumption of three portions of 150 g of cuttlefish per week. But, besides the average values, other values were possible since some specimens may be more contaminated with methyl-Hg than other. Therefore, for an adequate risk assessment, the probability of exceeding the PTWI, $P(X_i>PTWI)$, was estimated. The PI and the TE estimators were used for this objective.

Regarding the PI estimator, methyl-Hg weekly intake distribution values never exceeded the PTWI for squid in all hypothesized scenarios, leading to a null probability. Likewise, for the consumption of only one portion of 150 g of any cephalopod species, the maximum values of the 10000 values of the methyl-Hg intake distributions were always below the methyl-Hg PTWI, thus entailing a null probability. This illustrates a clear disadvantage of this estimator: risk cannot be evaluated if PTWI is too large when compared to the higher observed values.[15] Since a null risk does not exist, precise quantification requires another estimation method, that is, the TE estimation. However, for cuttlefish and octopus consumptions of two and three portions per week, risk estimated by PI method was different from zero. Moreover, this risk had an exponential progression, since, for instance,

a 50 % increase of the methyl-Hg average intake (from 0.4726 to 0.7114 μg/(week×kg body weight)) led to a $P(X_i>PTWI)$ almost 18 times higher (from 0.10 % to 1.75 %). This last probability was undoubtedly non-negligible. Thus, someone consuming three portions of octopus each week is already exposed to some methyl-Hg risk and the same applies to cuttlefish. These risks albeit low have already some significance: approximately one in 57 and one in 164 consumers (eating three portions per week), for octopus and cuttlefish, respectively, may be above PTWI. This may be the case of a consumer that eats octopus/cuttlefish from a particularly polluted area or a cephalopod whose specific traits (size, maturation, sex, etc.) may entail a higher level of contamination by methyl-Hg.

On the other hand, a more accurate estimation of $P(X_i>PTWI)$ requires a more complex estimator based on EVT, the TE estimator. The k largest observed values are crucial for TE, thus, an optimal k* must be chosen. This is done according to theoretical assumptions and mathematical estimations (see Material and Methods, Risk Assessment), where minimization of the Hill estimator's variance and of its bias determines the choice of k*. This calculation yielded TE estimator probabilities all different from zero. Nevertheless, for one portion per week, $P(X_i>PTWI)$ values were very low, not exceeding 0.00019 % (1:526316). The same applies to all squid consumption scenarios. As with PI estimator, with higher average methyl-Hg intakes, an exponential progression of the probabilities was observed. Interestingly, for the consumption of three weekly portions of cuttlefish and octopus, TE estimator yielded $P(X_i>PTWI)$ values almost identical to those achieved with the PI estimator.

3.3. Risk Assessment for Cephalopods in the Portuguese Population

The estimated maximum weekly intake of methyl-Hg per kg of body weight was always below the PTWI, hence, PI estimates of $P(X_i>PTWI)$ were zero (**Table III**). Once again, this means that the PI estimator is flawed, since, when the PTWI is larger than the highest observed (estimated) intake value, $P(X_i>PTWI)$ becomes zero, but, a null risk never exists. The TE estimator offers an alternative. As explained above, this required calculation of bias corrected Hill estimators of the risk index γ, because of the substantial instability observed with the uncorrected Hill estimators (**Figure 2**). However, such instability was lower than in other studies because consumption values were taken from a single and simple distribution and not from a consumption survey, thus, the heterogeneity of consumption behaviours behind an enhanced instability was lacking.[15] Additionally, the variance factor of AMSE was important for all cephalopod species, thus entailing k* values (**Table III**) higher than those determined in a previous study.[29] In the case of the octopus, this meant using the largest 335 intake values of the output distributions for estimating the bias corrected Hill estimator, which, in turn, enabled to calculate the associated probability of exceeding the methyl-Hg PTWI. This probability was extremely low for cuttlefish and squid, less than one in one million. However, for octopus, the TE estimator yielded a relatively higher risk, since a $P(X_i>PTWI)$ of 1:21000 (0.0048 %) was estimated (**Table III** and **Figure 1**).

4. DISCUSSION

With respect to risk assessment in specific scenarios, various studies have already pointed out the relevance of a moderate consumption of some seafood species, such as black

scabbardfish,[29,30] as a result of a high methyl-Hg content. However, for cephalopods, there are no recommendations of moderate consumption. In fact, no research, to the knowledge of the authors, has examined the risks associated to methyl-Hg in cephalopods. Hence, no consumption recommendations have been issued. This study may help to change this situation, since it clearly shows that the risk of exceeding the methyl-Hg PTWI is non-negligible for cuttlefish or octopus consumption frequencies equal to or higher than three 150 g meals every week. Thus, whereas for squid no consumption restriction seems necessary, for the other two species an ideal maximal consumption of two 150 g meals per week must be advised. However, it is not to be excluded the formulation of a further restriction of the cephalopods consumption frequencies if other contaminants imparting higher health risks to consumption of this seafood are found. Namely, this may be the case for cadmium (Cd), since it has been reported that Cd levels in octopus sometimes exceeded the recommended EU limit of 1.0 $mg.kg^{-1}$ while total Hg levels never surpassed the 0.5 $mg.kg^{-1}$ EU limit.[5] Effectively, molluscs very often accumulate Cd in digestive glands,[3] being found lower amounts in the arms and mantle.[31] Therefore, future studies should also assess the Cd risk associated to octopus consumption.

Additionally, the estimated risks associated to cephalopods consumption must be compounded with those of other seafood also presenting substantial levels of methyl-Hg. Therefore, someone consuming two cuttlefish or octopus meals per week is taking a non-negligible risk and, for consumers eating seafood several times every week, consumption should be even more restrained and the addition of meals with fish, specially, from deep waters, should be avoided.

Another interesting point is the comparison between the probabilities of exceeding the methyl-Hg PTWI for cuttlefish and octopus consumption and the average methyl-Hg levels

in these species. In fact, whereas cuttlefish presents higher contamination levels than octopus (0.106 vs 0.094 mg/kg), $P(X_i>PTWI)$ values are systematically higher for octopus, regardless of the used estimator (**Table II**). This is due to the parameters of the contaminant distributions, since both are extreme value distributions. The high β scale parameter of the octopus methyl-Hg distribution is the main cause of this mathematical phenomenon (**Table I**). The octopus methyl-Hg distribution albeit presenting a lower average than the cuttlefish methyl-Hg distribution has a much heavier tail than the latter distribution, as can be proved by the PI estimations —for instance, for three meals per week, the cuttlefish distribution of 10000 points shows 61 above the PTWI, while the octopus distribution of 10000 points has 175 above the PTWI.

The combination of specific scenarios with the variability of methyl-Hg contents in cephalopods is a semi-deterministic approach.[11] This provides a more realistic assessment of risk. In fact, the non-negligible risks obtained for cuttlefish and octopus compare with the absence of total Hg risk reported in the literature.[5] Latter authors used a full deterministic approach, that is, the average Hg content in cuttlefish and octopus was multiplied by an assumed consumption frequency of seven meals per week. Although this scenario was more extreme than those considered in this study, it was concluded that estimated Hg weekly intake was much lower than established PTWI,[5] thus leaving implicit that no risk arises from Hg content in cephalopods. Of course, this work concerns methyl-Hg (PTWI: 1.6 μg/(week×kg body weight)) and not total Hg (PTWI: 5.0 μg/(week×kg body weight)) and methyl-Hg in this study is, at least, 80 % of total Hg content. Nevertheless, this example highlights the great importance of taking any data variability into consideration in order to reach a sound and more rigorous risk assessment.

The comparison between both estimators (PI and TE) also provides some insight into the advantages and drawbacks of each estimator. The PI estimator does not allow a precise quantification when risk is small, since a null risk does not exist. Indeed, this conclusion was also mentioned by other studies,[15] which also state that a small sample size impairs the rigour of the PI method. On the contrary, the TE estimator allows a much more accurate quantification of risk in such circumstances. Nevertheless, for higher probabilities, there was a remarkable convergence of both estimators, which seems to support the adequacy of the much simpler PI estimator for a probability range between 0.1 and 2 %.

Nonetheless, the assumption of specific cephalopod consumption frequencies is not enough if a proper risk assessment in any given population is intended. It is deterministic on the side of consumption, since, for this input variable, a single point is used (for instance, 3 meals of octopus per week). However, there is a distribution of consumption values. Unfortunately, given the absence of detailed consumption surveys (differentiating among eaten seafood species) in Portugal, such distribution had to be hypothesized using a limited array of information. Therefore, log-normal distributions with means equal to the calculated Portuguese cephalopod consumption averages were considered instead. Hence, combining these consumption distributions with the contaminants distributions, it was possible to obtain in this full probabilistic approach more realistic distributions of the weekly intake of methyl-Hg in Portugal and to improve the associated risk assessment.

Concerning cuttlefish and squid, given the size of the Portuguese population, 10617575,[23] risk may be considered negligible. With respect to octopus, risk is still small (**Figure 3**), but may be considered as non-negligible, since a risk of exceeding the methyl-Hg PTWI of 1:21000 represents approximately 500 individuals in Portugal. However, it must be emphasized that the established PTWI level already integrates a safety buffer related to

those individuals more sensitive to the health effects of a high methyl-Hg intake. Furthermore, such individuals are a minority in the population. Accordingly, it can be accepted a negligible risk associated to the consumption of octopus *per se* in the Portuguese population. Moreover, the relatively higher risk ascribed to octopus is due to the combination of high consumption levels (30.4 g/(week.person)) with relatively high contamination levels (0.094 mg/kg). Though cuttlefish presents an even higher methyl-Hg average content (0.106 mg/kg), its consumption levels are lower (only 10.2 g/(week.person)). On the other hand, squid presents higher consumption levels (31.5 g/(week.person)), but its contamination by methyl-Hg is very low (0.035 mg/kg).

Once again, it should be remembered that these estimated risks for the consumption of cephalopods must be combined with risk arising from the consumption of other seafood species and food items in general. So, there is a greater risk in the Portuguese population of surpassing methyl-Hg PTWI through food consumption than that risk estimated for octopus only. In fact, in the French population, the probability of exceeding the methyl-Hg PTWI through food consumption was substantially higher, at least, in the most favourable scenario, 7.40 %.[15] Moreover, Portuguese consumers also eat other food items with relatively high methyl-Hg levels, such as black scabbardfish. A previous study[29] has shown that the consumption of this fish species in Portugal entails a methyl-Hg risk of 1.19 or 1.81 %, according to the PI and TE methods, respectively. Additionally, another study[12] using the five most consumed fish species in Portugal produced a probability of exceeding the methyl-Hg PTWI as a result of fish consumption of 6.71 %. Accordingly, methyl-Hg in food has to be treated as an important public health concern and further studies on the subject are warranted.

In all this discussion of the results, the underlying assumptions should not be forgotten. Namely, one hypothesis underlying the utilization of available data is that individuals are subjected to a constant exposure over time and keep the same consumption behaviour over their lifetime. This is a strong assumption which cannot be prevented, but might be relaxed by combining used methods with some ideas proposed by other authors,[32,33] if time series of consumption are observed. These methods were compared and discussed in a previous study.[34] Additionally, it is hypothesized that occasional short-term excursions above the methyl-Hg PTWI would present no major health effect, provided that the average intake over long periods does not exceed the PTWI. Accordingly, the parameter of interest could be viewed as the probability of occasional short-term excursions above the PTWI rather than a true probability to develop a disease as a result of the exposure to methyl-Hg.

Therefore, as main results of this study, it can be mentioned that while squid presents no serious health concern with respect to methyl-Hg, cuttlefish and octopus consumption should be restrained to two 150 g meals per week. Moreover, the methyl-Hg risk assessment for Portuguese consumers revealed no danger concerning the observed cuttlefish and squid consumption levels. On the other hand, octopus consumption by Portuguese consumers presented a relatively higher methyl-Hg risk (1:21000). However, given the various assumptions used in this study, it cannot be considered a public health problem. Nonetheless, this methyl-Hg risk associated to octopus consumption in Portugal can be compounded by the risk of other foods containing significant methyl-Hg, thus making any new studies on methyl-Hg risk warranted.

TE estimator was more accurate for lower probabilities, namely for the assessment of risk associated to one or two meals per week or in the Portuguese population in general, rendering accurate risk estimations different from zero. However, for higher probabilities

(roughly between 0.1 and 2 %), there was a remarkable convergence of both estimators, supporting the adequacy of applying the much simpler PI estimator. Additionally, the limitations of a deterministic approach were shown, being of great importance the assumption of variability for the two input variables, contamination and consumption levels.

ACKNOWLEDGMENTS

This research was funded by Project Qalibra (Quality of Life - Integrated Benefit and Risk Analysis - 022597). Author Carlos Cardoso would like to thank FCT PhD grant (ref. SFRH/BD/36455/2007). Furthermore, the authors would like to thank Jessica Tressou, PhD (INA-PG, Paris, France) for her excellent technical support to the study.

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LEGENDS TO TABLES

Table I – Best fit distributions for methylmercury content in cuttlefish, octopus, and squid.

Table II – Specific cephalopods consumption scenarios, average methylmercury weekly intakes and associated probability of exceeding PTWI.

Table III – Methylmercury intake and associated risk assessment for the Portuguese population.

LEGENDS TO FIGURES

Figure 1 – Distributions of methyl-Hg levels in octopus and of octopus consumption levels in the Portuguese population.

Figure 2 – Variation of the Hill estimator and of the bias corrected Hill estimator of the risk index γ with k (for methyl-Hg weekly intake through octopus consumption in the Portuguese population).

Figure 3 – Comparison between the PI and the TE estimators for the methyl-Hg intake through octopus consumption in the Portuguese population.

Table I – Best fit distributions for methylmercury content in cuttlefish, octopus, and squid.

Parameters	Cuttlefish	Octopus	Squid
Type of distribution	Extreme value (truncated at 0)	Extreme value (truncated at 0)	Extreme value (truncated at 0)
α	0.090881	0.074644	0.026793
β	0.023222	0.034275	0.014166
Mean (mg/kg)	0.106	0.094	0.035
Standard deviation (mg/kg)	0.044	0.043	0.017

Table II – Specific cephalopods consumption scenarios, average methylmercury weekly intakes and associated probability of exceeding PTWI.

Cephalopods consumption scenarios	Cephalopod species	Average methyl-Hg weekly intake (μg/(week×kg bw))	$P(X_i>PTWI)$ (%)	
			PI Estimator	TE Estimator
1 portion of 150 g per week	Cuttlefish	0.2607	0.00	0.000029
	Octopus	0.2363	0.00	0.00019
	Squid	0.0865	0.00	0.00000099
2 portions of 150 g per week	Cuttlefish	0.5217	0.01	0.036
	Octopus	0.4726	0.10	0.12
	Squid	0.1756	0.00	0.0000099
3 portions of 150 g per week	Cuttlefish	0.7818	0.61	0.46
	Octopus	0.7114	1.75	1.71
	Squid	0.2598	0.00	0.00086

Table III – Methylmercury intake and associated risk assessment for the Portuguese population.

Caphalopod species	Average methyl-Hg weekly intake (μg/(week×kg bw))	Maximum methyl-Hg weekly intake (μg/(week×kg bw))	k*	$\hat{\gamma}_{k^*}$	P(X_i>PTWI) (%)	
					PI Estimator	TE Estimator
Cuttlefish	0.0173	0.4784	256	0.300	0.00	0.000067
Octopus	0.0473	1.1384	335	0.345	0.00	0.0048
Squid	0.0179	0.3535	218	0.289	0.00	0.000069

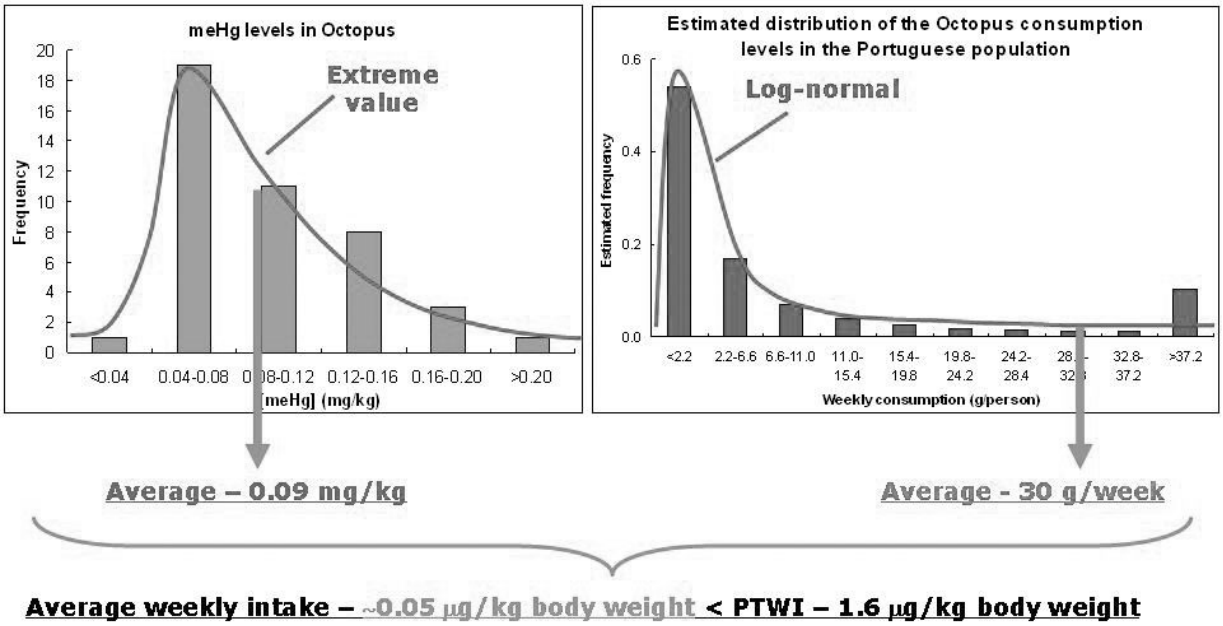

Figure 1 – Distributions of methyl-Hg levels and consumption levels in the Portuguese population for octopus.

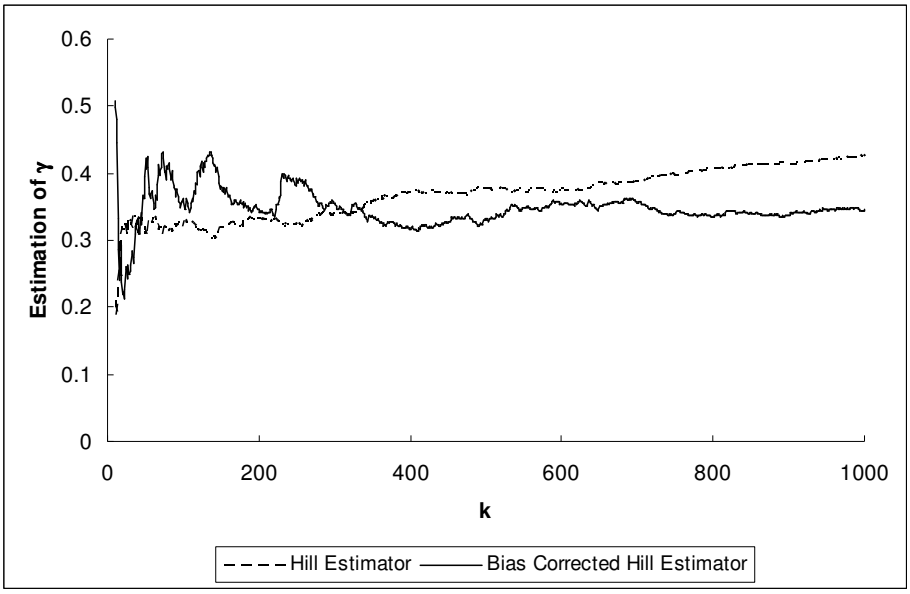

Figure 2 – Variation of the Hill estimator and of the bias corrected Hill estimator of the risk index γ with k (for methyl-Hg weekly intake through octopus consumption in the Portuguese population).

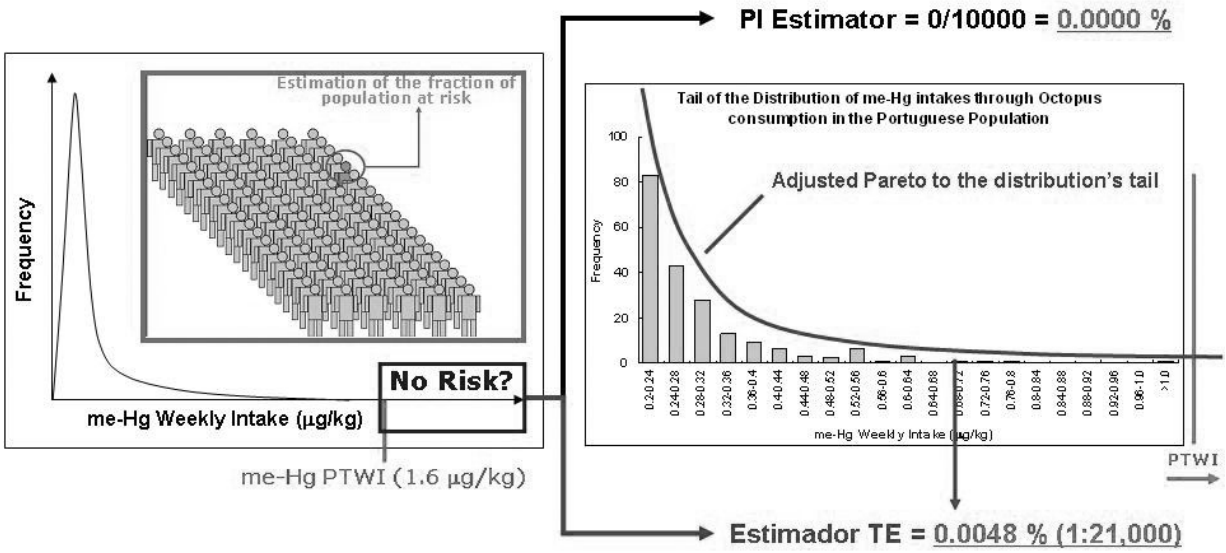

Figure 3 – Comparison between the PI and the TE estimators for the methyl-Hg intake through octopus consumption in the Portuguese population.

Paper VII

Journal of Aquatic Food Product Technology, 18:32–45, 2009

ISSN: 1049-8850 print/1547-0636 online
DOI: 10.1080/10498850802581088

Total Arsenic Content in Seafood Consumed in Portugal

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Marine organisms are known to accumulate arsenic (As) in the range of 1–100 mg kg^{-1} from their environment and food sources. The majority of arsenic is present as organoarsenic compounds, metabolized from inorganic arsenic present in seawater or accumulated from food sources such as algae or other fish. The aim of this study was to evaluate total arsenic content in the muscle of eight fish species, three cephalopods, and one crustacean consumed in Portugal. The measurement of total arsenic (10 individuals of each species) was carried out by EDXRF (Energy Dispersive X-Ray Fluorescence Method). The highest concentrations were found in Norway lobster (30.3 ± 8.7 mg kg^{-1} wet weight), followed by common octopus (25.9 ± 8.4 mg kg^{-1} wet weight). Within fish species, wild gilthead sea bream, anglerfish, and megrim species presented values around 12 mg kg,$^{-1}$ and in the others the average was lower than 10 mg kg^{-1}. Taking into consideration the tolerable daily intake recommended by the World Health Organization (WHO; 0.05 mg As kg^{-1} body weight), the obtained results of total arsenic in the studied species do not represent a hazard for human consumption.

KEYWORDS arsenic (As), fish, cephalopods, crustacean, Energy Dispersive X-Ray Fluorescence (EDXRF)

This work was supported by the project 22-05-01-FRD-00041 "Benefícios e perigos associados aos produtos da pesca (BENPER)," PO Mare.

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INTRODUCTION

Arsenic (As) is an important and ubiquitous element which has become one of the toxic trace elements under concern in recent decades (Karadjova et al., 2007). This element has no known beneficial biological function, and the long-term exposure may be harmful to health because of its acute and chronic toxicity (FSA, 2005, 2006).

The aquatic environment is important in the global cycling of arsenic. The background level of arsenic in marine environments is slightly higher (2–3 mg/l) than in freshwater and terrestrial environments (Moore and Ramamoorthy, 1985 and Phillips, 1990 in Klarić et al., 2004). Total human intake of arsenic occurs mainly via the food chain and depends on the quantity of seafood consumption (Suñer et al., 1999; Cava-Montesinos et al., 2005). Research has shown that marine organisms have the ability to accumulate arsenic naturally present in seawater and food, rather than only accumulating arsenic due to local pollution (Eisler, 1994 in ATSDR, 2000). Moreover, atmospheric deposits, output from rivers, and upwelling from marine sediments lead to enrichment of arsenic in marine organisms (Neff, 1997). In general, arsenic is present in high concentrations (orders of micrograms per gram) in seafood (Li et al., 2003). The arsenic levels in fish and shellfish are usually about 4–5 ppm, but may be as high as 170 ppm (Bennett, 1986, NAS, 1977b, and Schroeder and Balassa, 1966 in ATSDR, 2000). It is known that intrinsic factors such as growth (size, weight), age, gender, sexual maturity, physiology, and stress influence the accumulation of trace metals in marine organisms (Phillips and Rainbow, 1993). For that reason, issues related to arsenic content in aquatic organisms and sea fish, in particular, have attracted considerable attention (Lai et al., 2001). And therefore, suitable dietary guidelines are needed so that safe consumption is ensured.

In seafood, arsenic can be found in various chemical forms (chemical species) and oxidation states that differ in their degree of toxicity and the pathologies associated with them (Muñoz et al., 2000; Li et al., 2003). As a general rule, inorganic arsenic, which predominates in seawater and sediments, is the most toxic form which can damage DNA and cause cancer. Most of the arsenic in seafood is present in nontoxic organic forms, such as arsenobetaine (Lai et al., 1999), that are metabolically inert and easily eliminated by the human body (Fattorini et al., 2004) and, therefore, represent a low risk to the consumers of fishery products (Neff, 1997). No more than 2–10% of the total arsenic is potentially harmful as a toxic form (Fowler, 1983 and Freiberg, 1988 in Klarić et al., 2004). Since it is difficult to reliably measure the forms of arsenic present, most surveys measure total arsenic.

At the present time, the Food and Drug Administration (FDA, 1993) accords the highest priority (maximum concentration) to arsenic in its program

on toxic elements in foods. The guidance level for crustacea is 76 mg kg^{-1} and 86 mg $kg^{-1}$for clams, oysters, and mussels.

The aim of this work was to determine the total arsenic content in common species of fish, a crustacean, and cephalopods consumed in Portugal. Moreover, this study provides information regarding this element in a wide range of samples of fresh fish. Muscle was chosen for analysis because it is the major tissue consumed by humans and constitutes a significant percentage of the organism's body mass.

MATERIAL AND METHODS

Sample Collection

The samples analyzed in this study were selected on the basis of their high domestic consumption by the general Portuguese population. In total, about 120 samples of eight fish species, three cephalopods, and one crustacean were analyzed. The studied species were sardine (*Sardina pilchardus*), hake (*Merluccius merluccius*), wild and cultured gilthead sea bream (*Sparus aurata*), anglerfish species (*Lophius piscatorius* and *L. budegassa*), black scabbardfish (*Aphanopus carbo*), blackbelly rosefish (*Helicolenus dactylopterus*), megrim species (*Lepidorhombus whiffiagonis* and *L. boscii*), cuttlefish (*Sepia officinalis*), European squid (*Loligo vulgaris*), common octopus (*Octopus vulgaris*), and Norway lobster (*Nephrops norvegicus*). Species were obtained during several bottom trawl research surveys carried out by the INRB-IP/IPIMAR, in the Northeast Atlantic Ocean along the Portuguese coast during 2004–2005. Only wild gilthead sea bream was bought into one of the most important auction markets—Peniche in November 2005. Cultured gilthead sea bream were delivered by Portuguese aquacultures in 2004–2005.

Sample Preparation

Before analysis, samples were prepared by separating the edible portion from the inedible (guts, scales, heads, and bones). Only the commonly edible part (muscle) of the animal was selected for analysis. The number of individuals of each species can be found in Table 1. Samples were homogenized in a food blender, frozen and freeze-dried for 48 h at −45°C and low pressure (approximately 10^{-1} atm). Further, samples were powdered and stored at −20°C under controlled humidity conditions until analysis.

Analytical Methods

Total concentrations of As were determined by Energy Dispersive X-Ray Fluorescence Method (EDXRF). The energy resolution was 135 eV at

TABLE 1 Total Content of Arsenic and Range in Muscle of Several Species of Fish, Cephalopods, and a Crustacean Consumed in Portugal

Species	*n*[a]	Total As[b](mg kg^{-1})	Range (mg kg^{-1})
Crustacean			
Norway lobster (*Nephrops norvegicus*)	10	32.2 ± 8.21	23.1–51.2
Cephalopods			
Cuttlefish (*Sepia officinalis*)	10	19.8 ± 4.44	14.2–27.5
European squid (*Loligo vulgaris*)	10	4.0 ± 0.45	3.5–4.9
Common octopus (*Octopus vulgaris*)	10	25.9 ± 8.36	16.2–44.7
Fish			
Sardine (*Sardina pilchardus*)	10	4.9 ± 0.41	4.2–5.6
Hake (*Merluccius merluccius*)	10	6.7 ± 1.41	4.7–8.4
Black scabbardfish (*Aphanopus carbo*)	10	2.9 ± 1.00	1.7–4.8
Anglerfish species (*Lophius* sp)	10	11.7 ± 4.63	4.4–20.3
Blackbelly rosefish (*Helicolenus dactylopterus*)	9	4.4 ± 0.81	2.8–5.8
Megrim species (*Lepidorhombus* sp)	12	11.6 ± 4.74	6.4–21.4
Wild gilthead sea bream (*Sparus aurata*)	8	10.7 ± 6.60	4.2–22.8
Cultured gilthead sea bream (*Sparus aurata*)	10	4.0 ± 0.47	3.2–4.7

[a]*n:* number of samples analyzed.
[b]The values are expressed as mean ± standard deviation.

5.9 keV and the acquisition system was a Nucleus PCA card. Quantitative calculations were made by the fundamental parameters method (Carvalho et al., 2005). Experimental parameters were obtained by calibration, using standard reference materials. The X-ray generator was operated at 50 kV and 20 mA, and a typical acquisition time of 1000 s was used.

The samples were prepared by mixing and pressing the powder into pellets 2.0 cm in diameter without any chemical pretreatment. Each pellet was glued onto a Mylar film, on a sample holder, and placed directly in the X-ray beam for element determination. For each sample, a minimum of two replicates were made in order to reduce the risk of analytical error.

The accuracy of measurements was tested by the analysis of certified reference materials, MA-A-2 (fish flesh, obtained from the International Atomic Energy Agency), SRM 1566 (oyster tissue, obtained from the National Bureau of Standards), TORT-2 (lobster hepatopancreas, obtained from National Research Council of Canada), and SRM 1571 (Orchard leaves, obtained from National Institute of Standards and Technology). The values obtained were 2.5 ± 0.7, 13.5 ± 1.5, 21.3 ± 1.8 and 13.9 ± 1.5 μg g^{-1}, dry weight, which is in accordance with the certified ones (2.6 ± 0.1, 13.4 ± 1.9, 21.6 ± 1.8, and 14.0 ± 2.0 μg g^{-1}), respectively. Detection limit (DL) was 0.7 μg g^{-1}. Concentrations of arsenic are presented as mg kg^{-1} on a wet weight basis.

Statistical Analysis

The statistical treatment of the results was performed using Software STATISTICA, version 6.0 (StatSoft, Inc., 2003). The Kolmogorov–Smirnov test (or the Shapiro-Wilk's W test) and the Levene's test were used to check the normality and the homogeneity of variance of data, respectively (Zar, 1999). Comparisons were made using the Kruskall–Wallis test when the conditions were nonparametric (a violation of the test of homogeneity of variances assumption). Correlations between total arsenic concentrations and total length were analyzed for each species separately with Pearson correlation coefficients for linear correlation. The significance level for statistical analyses was always tested at $\alpha = 0.05$ ($p < .05$).

RESULTS AND DISCUSSION

Analysis of Seafood Samples

Results for the determination of total arsenic in the seafood species analyzed in this study are shown in Table 1. In all cases, number of samples analyzed, average values ± standard deviation, and range in which they lie are shown.

The arsenic content (mg kg^{-1}, wet weight [ww]) in the species analyzed was in the range of 1.7–22.8 for fish, 3.5–44.7 for cephalopods, and 23.1–51.2 for crustacean (Table 1 and Figure 1). Median levels of arsenic in these three groups were 5.1, 17.0, and 31.0 mg kg^{-1} ww, respectively (Figure 1). This variation of arsenic in marine organisms is due to many factors, such as species, geographic location (Anke et al., 1997 and Navarro et al., 1992a in Delgado-Andrade et al., 2003), temperature, salinity of the water, as well as weight (Norin et al., 1985 in Storelli and Marcotrigiano, 2000).

The highest mean total As concentration was found in the Norway lobster *Nephrops norvegicus* (32.2 mg kg^{-1}, ww). Moreover, this species showed the widest range within all species (Table 1). It has been reported that total As concentrations in the tissues of crustaceans and molluscs are generally higher than those found in marine fish (Phillips, 1990 in De Gieter et al., 2002). According to ATSDR (2000), the arsenic is mainly accumulated in the exoskeleton of invertebrates, which means a higher concentration of As is found in crustaceans. For prawns, arsenic values range from 9 to 19 mg kg^{-1}; for shrimps, from <1 to 40 mg kg^{-1}; and for crabs, from <1 to 70 mg kg^{-1} (Norin et al., 1985 in Storelli and Marcotrigiano, 2000). The levels reported in the literature for crustaceans also present a very wide range of 2.31–149.16 μg g^{-1}, dw (Brooke and Evans, 1981; Flanjak, 1982; Vlieg et al., 1991; Attar et al., 1992; Larsen et al., 1993; López et al., 1994; Muñoz et al., 1999, 2000). High arsenic levels in the muscle of these bottom dwellers can be associated with their habitat being closer to the muddy sediments, which are always higher in arsenic than surface waters (Byrd,

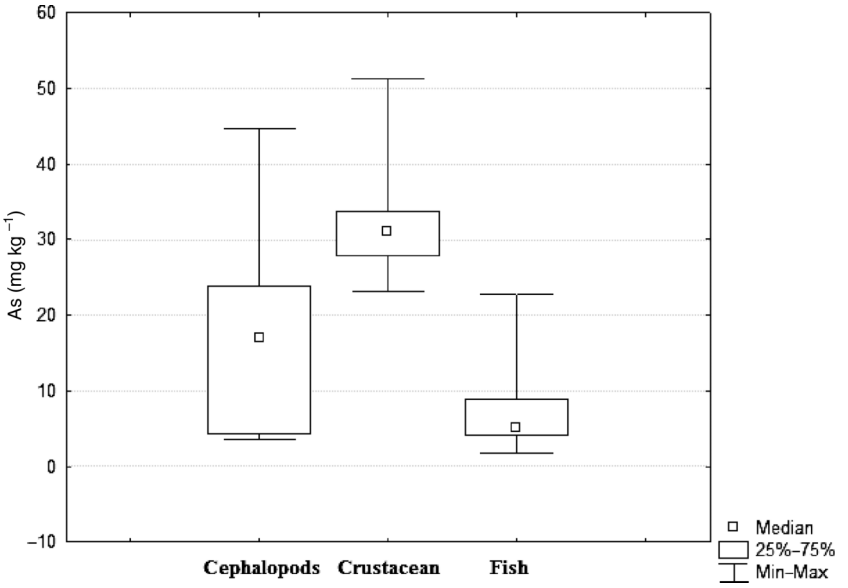

FIGURE 1 Total arsenic distribution (mg kg^{-1}) in 3 groups of fish, cephalopods, and a crustacean.

1988 and Trembley and Gobeil, 1990 in Storelli and Marcotrigiano, 2000). Another reason is that this decapod feeds on polychaetes, crustaceans, molluscs, echinoderms, and detritus (Figueiredo and Thomas, 1967 in Krång & Rosenqvist, 2006; Baden et al., 1990), which consequently present high levels of arsenic. Therefore, habitat and feeding are other important factors contributing to the variability of arsenic in marine organisms. The samples in this present work had relatively large sizes, which may also explain the high levels of arsenic. In fact, the significant differences found ($p < .05$) in total arsenic concentrations among Norway lobster, European squid, and almost all species studied (except for anglerfish, megrim, and wild gilthead sea bream) also relate to the different diet and habitat in which they live.

The levels for common octopus and cuttlefish are the highest in the group (25.9 and 19.8 mg kg^{-1}, ww, respectively) of cephalopods, while the European squid exhibited the lowest concentration (4.0 mg kg^{-1}, ww). Cephalopods are carnivorous, active predators; and because they have very high feeding rates, most of the arsenic can be assumed to be incorporated by the diet (Villanueva and Bustamante, 2006). In the case of *Octopus vulgaris*, the largest contents may be due to the fact that these species are characterized by feeding primarily on gastropods and bivalves, which are filterers and consequently present high levels of arsenic; and secondly, are benthic species living in direct contact with the substratum, which represents another possible pathway for arsenic accumulation (Seixas et al., 2005). On the other hand, the European squid feeds on fish, crustaceans,

and cephalopods; but fish is the major and sometimes the only component, so the concentrations of arsenic are lower in this species compared to other cephalopods. The common octopus and cuttlefish are the only species that do not show significant differences ($p < .05$) within the group of cephalopods.

The highest element level in fish samples was observed in anglerfish and megrim species, with mean values of 11.7 and 11.6 mg kg^{-1} ww, respectively. The analyzed samples of black scabbardfish presented lower levels (2.9 mg kg^{-1}, ww), followed by the samples of cultured gilthead sea bream (4.0 mg kg^{-1}, ww), blackbelly rosefish (4.4 mg kg^{-1}, ww), and sardine (4.9 mg kg^{-1}, ww). Marked variation in total arsenic concentrations observed in fish samples reflects the variation among species.

The high concentrations found in different species of anglerfish and megrim may be principally due to different feeding habits. In fact, anglerfish is a bathydemersal fish that is found on sandy and muddy bottoms feeding mostly on other fish and being half buried in the sediment waiting for their prey. Megrim is a benthic fish that occurs on soft bottoms and feeds mainly on bivalves and small bottom-dwelling fishes as well as squids and crustaceans that generally show high levels of arsenic. In addition, Survey on Arsenic in Food (MAFF, 1982) recognized that flatfishes, which are bottom feeders and live on or close to the sea bed, are readily exposed to the greater quantities of arsenic that accumulate in sediments than other species. So, it seems that marine organisms acquire their arsenic burden through the food chain (Norin et al., 1985 in Storelli and Marcotrigiano, 2000). But in contrast to Hg, for example, As seems not to biomagnify or bioaccumulate (De Gieter et al., 2002). Hence, black scabbardfish showed low levels of arsenic despite being a top predator that feeds on crustaceans and cephalopods. Thus, and according to an extensive study of the factors affecting bioaccumulation of arsenic in two streams in western Maryland in 1997–1998, no evidence of biomagnifications was found since arsenic concentrations in organisms tend to decrease with increasing trophic level (Mason et al., 2000). In this group, the differences between the concentrations found in the megrim in respect to black scabbardfish and cultured gilthead sea bream were significant ($p < .05$). The same is true for the anglerfish and the latter two fish species.

The arsenic levels in wild gilthead sea bream (10.7 mg kg^{-1}, ww) are in general much higher than in the cultured (4.0 mg kg^{-1}, ww; Table 1), which may be due to a different diet, especially in cultured fish where feeds are used. Nevertheless, wild gilthead sea bream was not statistically different from all other species ($p > .05$).

Comparison with Literature Data

Related publications on total arsenic concentrations in several species of fish, cephalopods, and crustaceans can be observed in Table 2. The levels

TABLE 2 Reported Data on Arsenic Concentration in Several Seafood Species

Species	Sample size	Range (mg kg^{-1})	Total As (mg kg^{-1})	Reference
Crustaceans	11	1.69–137.32	35.12 ± 38.77	Muñoz et al. (2000)
Scampi (*Nephrops norvegicus*)	60	–	3.87	FSA (2005)
Lobster (*Nephrops norvegicus*)	5	0.10–12.58	5.05	FSA (2006)
Lobster (*Jasus edwardsii*)	–	–	50.7 ± 18.6	Fabris et al. (2006)
Cuttlefish (*Sepia esculenta*)	20	2.45–5.33	–	Falcó et al. (2006)
Cuttlefish (*Sepia officinalis*)	3	–	75.9 ± 2.2*	Lavilla et al. (2008)
European squid (*Loligo vulgaris*)	12	1.13–42.78	13.06 ± 13.11	Muñoz et al. (2000)
	20	1.41–4.74	–	Falcó et al. (2006)
Common octopus (*Octopus vulgaris*)	6	–	103 ± 38*	Seixas et al. (2005)
Sardine (*Sardina pilchardus*)	20	3.53–3.94	–	Falcó et al. (2006)
	11	5.76–36.44	20.05 ± 9.15	Muñoz et al. (2000)
	60	–	3.43	FSA (2005)
	5	0.81–1.82	1.2	FSA (2006)
Hake (*Merluccius merluccius*)	20	3.22–4.55	–	Falcó et al. (2006)
	12	4.55–39.75	12.44 ± 10.45	Muñoz et al. (2000)
	21	6.15–15.9	9.7 ± 3.08	Storelli and Marcotrigiano (2000)
	60	–	2.02	FSA (2005)
	3	–	16.0 ± 0.6	Lavilla et al. (2008)
Black scabbardfish (*Aphanopus carbo*)	54	<0.02–26.49	1.25 ± 3.49[a]	Mormede and Davies (2001)
Anglerfish (*Lophius piscatorius*)	38	2.70–21.47	8.63 ± 4.77[a]	Mormede and Davies (2001)
	–	4.1–13.7	–	De Gieter et al. (2002)
Blackbelly rosefish (*Helicolenus dactylopterus*)	8	3.91–29.69	–	Amato et al. (2006)
Megrim (*Lepidorhombus whiffiagonis*)	12	4.14–67.38	28.45 ± 17.71	Muñoz et al. (2000)
	3	–	42.5 ± 1.3	Lavilla et al. (2008)
Wild gilthead sea bream (*Sparus aurata*)	–	–	5.26 ± 0.09*	Schaeffer et al. (2005)
Farmed gilthead sea bream (*Sparus aurata*)	60	–	2.11	FSA (2005)
Wild sea bass (*Dicentrarchus labrax*)	60	–	1.14	FSA (2005)
Farmed sea bass (*Dicentrarchus labrax*)	60	–	0.91	FSA (2005)
Wild yellow perch (*Perca flavescens*)	9	–	0.99 ± 0.11*	González et al. (2006)
Farmed yellow perch (*Perca flavescens*)	9	–	1.42 ± 0.32*	González et al. (2006)

All figures refer to wet weight except those indicated by *(values related to dry weight).
[a]This number refer to median ± standard deviation.

reported in the literature for crustaceans also present a very wide range of 1.69–137.32 mg kg^{-1}, ww (Muñoz et al., 2000). Moreover, in the case of Norway lobster, much higher concentrations were obtained in the present study than in other surveys conducted by the Food Standards Agency (FSA, 2005, 2006). In contrast, in another study conducted in Australia (Fabris et al., 2006), the mean content was slightly higher (50.7 ± 18.6 mg kg^{-1}, ww) and in agreement with our results.

The concentrations found in muscle tissue of hake, anglerfish, and cuttlefish are similar to those referred, respectively, by Storelli and Marcotrigiano (2000), De Gieter et al. (2002), and Lavilla et al. (2008). Moreover, values of arsenic given in a report performed on the Portuguese coast with common octopus (Seixas et al., 2005) are in the same order as those found in this study. In addition, the samples of anglerfish present levels similar to those reported in the literature (Mormede and Davies, 2001; De Gieter et al., 2002).

Compared to the results found by Falcó et al. (2006) in species that are among the most consumed by the general Spanish population, the present work showed higher levels for sardine, hake, cuttlefish, and European squid. In contrast to this, arsenic concentrations found in our study were lower compared with another study in Spain (Muñoz et al., 2000) for the same species. These authors also found high levels of total arsenic in megrim (28.45 ± 17.71 mg kg^{-1}, ww) and a wide range of the data (4.14–67.38 mg kg^{-1}, ww). Even larger values were found in the work done by Lavilla et al. (2008). The high values of arsenic often reported in the literature may be due to the fact that the species could have been captured in highly contaminated areas.

In two studies (Schaeffer et al., 2005; FSA, 2005) of wild and farmed sea bream, the arsenic levels found (5.26 mg kg^{-1}, dw and 2.11 mg kg^{-1}, ww, respectively) were below those obtained in the present study. Moreover, arsenic concentrations found in our work were higher in wild and cultured sea bream muscle when compared to other important species from aquaculture, such as sea bass, *Dicentrarchus labrux,* and yellow perch, *Perca flavescens* (FSA, 2005; González et al., 2006).

There are few data in the literature for total arsenic in black scabbardfish and in blackbelly rosefish, but they vary within a range of <0.02–26.49 mg kg^{-1}, ww (Mormede and Davies, 2001) and 3.91–29.69 mg kg^{-1}, ww (Amato et al., 2006), respectively. These levels showed a very wide dispersion when compared to our results (1.71–4.80 and 2.84–5.78 mg kg^{-1}, ww, respectively).

Correlations

There were no significant correlations ($p > .05$) between total arsenic and body length in almost all species (Table 3). Only in the case of sardine, *Sardina pilchardus*, was a significant (positive) relationship ($p < .05$) found (Table 3). This means that the levels of this element usually increase in

TABLE 3 Pearson's Correlations (*R*) Between Concentrations of Total Arsenic and Length in Different Species of Fish, Cephalopods, and a Crustacean

Species	Length range (mm)	Correlation (As-length)
Cuttlefish	165.0–315.0	0.5673 (p = .087)
European squid	130.0–420.0	0.5298 (p = .115)
Common octopus	115.0–245.0	0.3919 (p = .263)
Norway lobster	29.5–59.5	0.6104 (p = .061)
Sardine	16.5–23.5	**0.7573 (p = .011)**
Hake	21.3–61.5	0.0388 (p = .915)
Black scabbardfish	88.2–122.0	−0.2854 (p = .424)
Anglerfish species	43.1–77.0	−0.3923 (p = .262)
Blackbelly rosefish	25.3–36.0	−0.2300 (p = .552)
Megrim species	21.5–46.5	0.5445 (p = .067)
Wild gilthead sea bream	34.5–57.5	0.3100 (p = .455)
Cultured gilthead sea bream	23.0–36.5	0.4672 (p = .173)

Probability values (p) are in parentheses. Significant correlations are indicated in boldface.

sardine with body size, so larger and/or older fish generally have higher concentrations than smaller or younger fish (Storelli et al., 2002). However, no other correlations were evident, which means that total arsenic concentrations are not related to body size but probably due to the small sample size.

Legislation and Health Considerations

At the international level, very few countries have published guidelines/legislation to regulate the maximum concentration of total arsenic in seafood products (British Food Manufacturing Industries Research Association, 1993). The Joint FAO/WHO Expert Committee (1983) imposed a limit of 0.1 mg kg^{-1}, ww for total arsenic in food (Muñoz et al., 2000; De Gieter et al., 2002), and the Arsenic in Food Regulations (SI 1959 no. 831 in FSA, 2005), in the UK, lays down a general limit of 1 mg kg^{-1}. Hong Kong has the most tolerant legislation in the world, with maximum concentrations of 6 mg kg^{-1}, ww for fish and fish products and 10 mg kg^{-1}, ww for shellfish and shellfish products (Phillips et al., 1982 in Muñoz et al., 2000). The Food and Drug Administration (FDA) suggests a limit of 76 mg kg^{-1} for crustacea (FDA, 1993). There is presently no Europe-wide regulation of arsenic in food (FSA, 2005).

Considering the tolerable daily intake recommended by WHO (0.05 mg As kg^{-1} body weight; WHO, 1983), the obtained results of total arsenic in the studied species do not represent a hazard for human consumption. Only the Norway lobster exceeded this value. However, taking into account the FDA (1993) consideration, most of the arsenic compounds found are methylated, and therefore are relatively low in toxicity and do not present a concern to the consumer.

CONCLUSIONS

The levels of total arsenic varied widely among the studied species, which may be due to different dietary habits and environmental characteristics. The results suggest that concentrations of this element are lower in fish than in crustaceans. It is also worth pointing out that farmed gilthead sea bream presented levels of total arsenic much lower than the wild one. From this work we also conclude that the technique, EDXRF, is suitable for this kind of study. It is easy to use, nondestructive, and the samples do not require any chemical treatment and therefore avoid possible contamination.

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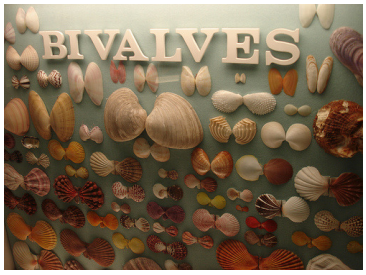
Paper VIII



Seafood research from fish to dish

Quality, safety and processing of wild and farmed fish

edited by

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C. Jacobsen
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Wageningen Academic
Publishers

ISBN-10: 90-8686-005-2
ISBN-13: 978-90-8686-005-0

Cover design: Oddvar Dahl, Fiskeriforskning
Cover photo: Frank Gregersen, Fiskeriforskning

First published, 2006

Wageningen Academic Publishers
The Netherlands, 2006

Concentrations of mercury, lead and cadmium in bivalves from the Portuguese coast

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Abstract

The accumulation of heavy metals in bivalve tissues is due to their morphological and biological characteristics associated to the habitat. In Portugal, these species are important in terms of economic value and local consumption. The purpose of this work was to evaluate the levels of some heavy metals which can be toxic for humans at elevated concentrations in bivalves collected in 2004. Total mercury levels were lower than the maximum legal limit by EU (0.5 mg/kg wet weight). Regarding lead and cadmium levels, most samples did not exceeded the legal maximum limits (1.5 mg/kg wet weight and 1.0 mg/kg wet weight, respectively). As exceptions some furrow shell and Portuguese oyster samples from Tagus and Sado estuaries could be identified.

Keywords: mercury, lead, cadmium, bivalve molluscs, production areas

Introduction

Contamination of the marine environment by heavy metals has risen in recent years due to the global population increase and industrial development (Arellano and others 1999). This is a serious problem due to their toxicity and their ability to accumulate in the biota (Islam and Tanaka 2004). Metals like mercury (Hg), cadmium (Cd) or lead (Pb) seem not to participate in any metabolic functions (Dallinger 1995; Suzuki and Suzuki 1996). These elements can provoke changes in the central nervous, cardiovascular or respiratory systems. They may be mutagenic and they may promote the risk of cancer, in both, animals and human beings (Vercruyse 1984).

Estuaries and near-shore marine waters can be considered a space of great importance for the survival of a large variety of plants, animals and marine species (Castro and others 1999), but they are very vulnerable to all kinds of influences (Blasco and others 1999). These zones have become extensively degraded over the recent years (Chase and others 2001) since these productive and sensitive areas are often directly and most seriously affected and exposed due to their proximity of sources of pollution (Arellano and others 1999; Cohen and others 2001). The main sources of metals, resulting from various processes like underwater geothermal activities, geological weathering, industrial processing of metals, use of metals and metal components, leaching from dumps and fertilisers, atmospheric deposition, animal excretion and the discharge of human sewage (Wright and Mason 1999). Consequently, a determination of metal concentrations in organisms should be part of any assessment and monitoring program in these risk zones.

Sedentary molluscs, like bivalves, are the animals most often used for screening of metal contamination (Phillips 1977). They have the ability to accumulate and concentrate metals found in their environment by several orders of magnitude above the background levels. However, physiological changes, like life cycle, can play a role in the regulation mechanisms of heavy metals in bivalves (Bebiano 1995).

In Portugal bivalve molluscs are much appreciated by consumers and there are many production areas along estuaries and coastal zones. The most representative molluscs are some clam species, like grooved carpet shell, carpet shell or European razor clam, common cockle and mussel, oysters, furrow shell and wedge shell (Table 1)

The main aim of this study was to evaluate heavy metal contamination in whole body tissues of several mollusc bivalves. Various estuaries and coastal zones in Portugal, exposed to different metal load, were chosen and the concentrations of Hg, Cd and Pb were measured.

Material and methods

Material

Bivalves species used in this study were collected in 2004 at various production areas localised along the Portuguese coastal zone and estuaries. Common name, scientific name, number of analysed samples and production area are described in detail in Table 1. The collected molluscs were immediately transferred to the laboratory at 2 °C in isotherm conditions. They were not depurated prior to processing. From each sampling, 20-30 bivalves were analysed. Bivalves were

Table 1. Species analysed (common and scientific name, number of samples analysed and production area).

Common name	Scientific name	Samples analysed (n)	Production area
Grooved carpet shell	*Ruditapes decussatus*	249	Ria Formosa Lagon; Alvôr Estuary; Óbidos Lagoon
Carpet shell	*Venerupis Senegalensis*	33	Tagus Estuary; Óbidos Lagoon; Aveiro Estuary
Thick trough shell	*Spisula solida*	16	Aveiro Estuary; Mouth of Douro River
Cockle	*Cerastoderma edule*	54	Sado Estuary; Óbidos Lagoon; Aveiro Estuary; Mouth of Lima River
Furrow shell	*Scrobicularia plana*	29	Tagus estuary; Sado Estuary
European razor clam	*Solen marginatus*	25	Sado Estuary; Óbidos Lagoon; Aveiro Estuary
Mussel	*Mytilus edulis*	105	Ria Formosa Lagoon; Mira Estuary; Tagus Estuary; Óbidos Lagoon; Aveiro Estuary; Mouth of Lima River
Portuguese oyster	*Crassostrea angulata*	22	Mira Estuary; Sado Estuary; Aveiro Estuary
Giant cupped oyster	*Crassostrea gigas*	8	Alvôr Estuary
Wedge shell	*Donax trunculus*	5	Sado Estuary
Smooth calista	*Callista chione*	8	Sado Estuary

washed to eliminate sediment and debris, using potable water and the excess water in their mantle was drained. Stainless steel scalped blades were used to cut open the animals and to remove the whole body tissue, which was homogenised by a food blender with stainless steel cutters and stored in plastic bags at 5 °C for some hours prior to analysis.

Analytical methods

Total Hg was determined by cold vapour atomic absorption spectrometry (CVAAS) (Bacharad MAS 50D) according to Lawson and Keikwood (1980). The levels of Pb and Cd were measured by flame atomic absorption spectrometry (Varian, Spectr AA-20 with deuterium background correction) in agreement with the procedures described by Jorhem and others (2000). All analyses were carried out in duplicate, using the external calibration method. Tuna fish (CRM 463) and cod muscle (CRM 422) certified standard reference materials from BCR (Bureau Communitaire de Référence) were used to prove the accuracy of the methods. The determined values of Hg, Pb and Cd were in good agreement with the certified values (Table 2). Detection limits (calculated by residual standard deviation from linear regression) were: 0.01 mg kg^{-1} for Hg, 0.05 mg kg^{-1} for Pb and 0.01 mg kg^{-1} for Cd. All data were reported on mg kg^{-1} wet weight basis.

Results and discussion

Metal concentrations in whole body tissues of bivalve molluscs studied are shown in Table 3.

Mercury levels analysed were always lower than 0.1 mg kg^{-1}. The highest mercury concentrations were found in carpet shell (0.08 mg kg^{-1}) and furrow shell (0.07 mg kg^{-1}) from Tagus Estuary and in European razor clam (0.06 mg kg^{-1}) from Aveiro Estuary, however, these results are far below the legal limit of 0.5 mg kg^{-1} established by EU (2001). Several authors reported similar values like Wright and Mason (1999) in the Stour estuary for mussel and cockle, de Mora and others (2004) in different oysters species from the Gulf and Gulf of Oman and Usero and others (1996, 1997, 2005) for striped venus (*Chamelea gallina*), grooved carpet shell or wedge shell from the Atlantic coast of Southern Spain.

Some of the furrow shell samples sampled off Tagus Estuary exceeded the legal limit for lead (1.5 mg kg^{-1}), set for these molluscs (EU 2002). Similar Pb concentrations were also found in furrow shell samples from the river Gualdalquivir estuary after the disaster in the Aznalcóllar mine (Blasco and others, 1999). Highest values were observed by Ruiz and Saiz-Salinas (2000) in the same bivalve from the Bilbao estuary, caused by the 1989-90 droughts and in Tagus estuary by França and others (2005). Pb values registered in the other Portuguese production areas were quite low, indicating a lower contamination by this metal in those areas.

Table 2. Mercury, lead and cadmium (mg kg^{-1}) concentrations in certified standard reference materials determined in the present study (n=5).

Certified standard reference material		Hg	Pb	Cd
CRM 463 (European Com.)	Certified	2.85 ± 0.16	-	-
	Found	2.88 ± 0.22	-	-
CRM 422 (European Com.)	Certified	-	0.085 ± 0.015	0.017 ± 0.002
	Found	-	0.081 ± 0.002	0.019 ± 0.001

Table 3. Concentrations of mercury, lead and cadmium (means ± S.D. and range in mg kg^{-1} wet weight) in whole body tissues of bivalve molluscs.

Species	Mercury	Lead	Cadmium
Grooved carpet shell	0.02 ± 0.00	0.1 ± 0.1	0.02 ± 0.01
	(0.02-0.02)	(< 0.1-0.2)	(< 0.01-0.08)
Carpet shell	0.04 ± 0.02	0.2 ± 0.3	0.05 ± 0.02
	(0.02-0.08)	(< 0.1-1.3)	(< 0.01-0.09)
Thick trough shell	0.02 ± 0.00	0.1 ± 0.1	0.06 ± 0.01
	(0.01-0.02)	(< 0.1-0.2)	(0.04-0.08)
Smooth calista	0.03 ± 0.00	0.1 ± 0.1	0.06 ± 0.01
	(0.03-0.03)	(< 0.1-0.2)	(0.04-0.07)
Furrow shell	0.04 ± 0.01	1.4 ± 1.1	0.03 ± 0.02
	(0.03-0.07)	(< 0.1-3.0)	(0.01-0.07)
Cockle	0.02 ± 0.00	0.1 ± 0.1	0.03 ± 0.02
	(0.02-0.03)	(< 0.1-0.3)	(0.01-0.07)
Wedge shell	0.03 ± 0.00	0.1 ± 0.1	0.04 ± 0.00
	(0.03-0.04)	(< 0.1-0.2)	(0.03-0.05)
European razor clam	0.04 ± 0.00	0.2 ± 0.1	0.04 ± 0.02
	(0.04-0.06)	(<0.1-0.3)	(0.02-0.08)
Mussel	0.02 ± 0.00	0.2 ± 0.1	0.13 ± 0.07
	(0.02-0.03)	(< 0.1-0.6)	(< 0.01-0.35)
Portuguese oyster	0.04 ± 0.00	0.1 ± 0.1	0.37 ± 0.34
	(0.04-0.05)	(< 0.1-0.2)	(0.06-1.09)
Giant cupped oyster	0.04 ± 0.00	0.1 ± 0.0	0.22 ± 0.06
	(0.04-0.04)	(0.1-0.1)	(0.18-0.26)

The highest concentration of Cd was obtained in Sado Estuary, in Portuguese oyster samples, exceeding the maximum legal limit of 1.0 mg kg^{-1} (EU 2001). Previous results obtained by Blasco and others (1999) showed values in the range found in this work. Studies performed in *Crassostrea gigas* presented different levels (Hunter and others 1995; Shulkin and others 2003). The content of Cd in some of the mussel samples reached 0.35 mg kg^{-1} which is comparable with values obtained by Mason and Wright (1999), Chase and others (2001), Zauke and others (2003), but lower than that found by Liang and others (2004). The concentrations in the other studied species were much lower than 1.0 mg kg^{-1}.

Conclusions

The levels of Hg, Pb and Cd in whole body tissues of bivalves have been found to be generally low. However, high concentrations of Pb and Cd occurred in bivalves from two areas, Tagus estuary and Sado estuary.

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Paper IX

Levels of Toxic Metals in Canned Seafood

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ABSTRACT. The production of canned fish has decreased in recent years; however, several European countries still consume large amounts of these products. It is well known that such products may contain some toxic metals such as mercury, cadmium and lead. In order to determine the level of these three elements in canned seafood, several products were analyzed (more than 1800 samples). The mean total mercury level was 0.12 ± 0.14 mg/kg wet weight, with the highest content found in canned tuna. However, such values did not exceed the proposed limit for tuna by EU (1.0 mg/kg). All analyzed samples showed lead levels lower than the indicated limits. On the other hand, a few samples (less than 2%) of canned tuna and squid exceeded the limit values proposed for cadmium, 0.1 and 1.0 mg/kg, respectively. Taking into account the way in which these products are consumed, it can be concluded that canned seafood products do not represent a risk in terms of human diet.

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Journal of Aquatic Food Product Technology, Vol. 13(3) 2004
http://www.haworthpress.com/web/JAFPT

Digital Object Identifier: 10.1300/J030v13n03_11

KEYWORDS. Canned seafood products, total mercury, cadmium, lead, legal limits

INTRODUCTION

Fish and seafood can be an important part of a balanced diet for humans; however, some fish accumulate contaminants present in the aquatic environment including mercury (Hg), cadmium (Cd) and lead (Pb), which are tolerated only at very low levels (Voegborlo et al., 1999). Although toxic metal content in the oceans is very low, higher contents are found in localized areas, like waters polluted by chemical and geochemical processes. Mercury accumulates up the food chain; therefore, high levels can be found in predatory species caught in areas with a high content of this metal. As a result, the consumption of fish becomes the primary pathway by which humans are exposed to mercury (Wheeler, 1996) and other metals. Actually, there are limits for these three metals, defined by European Community Regulation 466/2001 (EU, 2001) as amended by European Community Regulation 221/2002 (EU, 2002). The Food and Drug Administration (FDA) also has the responsibility to safeguard human health through regulation of foods and drugs. This agency suggests maximum tolerable daily intake for cadmium (55 μg/person/day) (FDA, 1993a) and lead (75 μg/person/day) (FDA, 1993b). World Health Organization (WHO) established a provisional tolerable weekly intake (PTWI) of 5 μg/kg body weight (bw)/week for total mercury (WHO, 1972), of which no more than two thirds (3.3 μg/kg bw/week) should be from methylmercury (WHO, 1989; WHO, 2000).

Canned fish is a product that still records a reasonable demand in several European countries, namely in Mediterranean ones, and frequently the possibility that such seafood products show high levels of toxic metals is debated. Several studies were performed along this line many years ago, principally in canned tuna (Chow et al., 1974; Sapunar and Jusic, 1979; Duve, 1981; Schindler, 1985; Hardisson et al., 1986; Romieu et al., 1994; Gajewska et al., 1995; Voegborlo et al., 1999), but there is scarcity of information for other canned seafood products.

The purpose of the present study was to characterize the contents of total Hg, Cd and Pb in several canned seafood products commercialized in Portugal in order to evaluate the existence of contamination in these products.

MATERIALS AND METHODS

All seafood products utilized in this study were purchased from local retailers between 2000 and 2002. Canned tuna, chub mackerel, sardine, eel, lamprey, squid, octopus, blue mussels and some seafood spreads were examined.

Cans were opened and, after draining off the oil, the meat was homogenized in a food blender (Moulinette 320, Moulinex, France) and the samples quickly analyzed.

Total Hg was determined by cold vapor atomic absorption according to the procedure developed by Hatch and Ott (1968), described by Joiris et al. (1991). Accuracy was verified using the Certified Reference Material CRM-463 (tuna fish muscle: 2.85 ± 0.16 μg g^{-1} Hg dry weight) of IRMM. The mean result, for five replicates (2.87 ± 0.09 μg g^{-1} Hg dry weight) was in the range of CRM-463 with a relative error of 0.1%. Detection limit (DL) was 0.01 mg kg^{-1}. Cd and Pb were performed by flame atomic absorption, following the method described in AOAC (1990). Accuracy was examined by participating in a Food Analysis Performance Assessment Scheme organized by Central Science Laboratory (Metallic Contaminants–FAPAS, Series 7, Round 40, 2002, England). The laboratory performance is inside the satisfactory range, i.e., $|Z| \leq 2$ ($Z = -1.1$ for Pb; $Z = 0.3$ for Cd). Detection limits were 0.01 and 0.02 for Cd and Pb, respectively. Analytical data for Hg, Cd and Pb are reported as mg kg^{-1} on a wet weight basis. All data analysis was achieved using STATISTICA (Statsof, Inc., USA, 2000).

RESULTS AND DISCUSSION

Table 1 presents the results of total mercury determination in all canned seafood products analyzed. The highest values were found in canned tuna, tuna spread, eel and lamprey. However, these concentrations did not exceed the limits proposed by EU (2001 and 2002), 1.0 (tuna and eel) and 0.5 mg kg^{-1} (lamprey). The mean values obtained by other authors for canned tuna (Noirfalise and Fouassin, 1981; Gajewwska et al., 1995; Voegborlo et al., 1999) are similar to the present results, although lower than 0.7 mg kg^{-1}. In the case of eel, an interval between 0.12 and 0.19 mg kg^{-1} with a mean of 0.16 mg kg^{-1} was found. Such values are in accordance with those published by Edwards et al. (1999) (0.102-0.260 mg kg^{-1}) and by Noirfalise and Fouassin (1981) ($\leq$ 0.24 mg kg^{-1}) for the same species.

Total Hg levels detected in canned cephalopods were lower than 0.5 mg kg^{-1} (limit recommended by EU) in all samples studied, which is in agreement with values reported by other authors (Falandysz, 1989; Falandysz, 1990; Storeli and Marcotrigiano, 1999). For chub mackerel the range was between 0.01 and 0.18 mg kg^{-1} closer to those found by Storeli et al. (1998) in fish caught off the Adriatic Sea. Samples of canned sardine, sardine spread and blue mussels presented the smallest values in this work. These low levels are usual in this kind of species and are in good coherence with results obtained by Tariq et al. (1991) and I-de et al. (1994). Nevertheless, some authors

TABLE 1. Total mercury (mg/kg wet weight) in canned seafood.

Canned seafood (N = number of analyzed samples)	Mercury (mg kg^{-1} wet weight)	
	Mean ± Standard deviation	Range
Tuna (N = 425)	0.28 ± 0.18	0.08-1.00
Tuna spread (N = 60)	0.19 ± 0.09	0.09-0.31
Chub mackerel (N = 568)	0.05 ± 0.03	0.01-0.18
Sardine (N = 372)	0.03 ± 0.02	< DL*-0.08
Sardine spread (N = 98)	0.02 ± 0.01	0.01-0.03
Squid (N = 64)	0.07 ± 0.07	0.01-0.21
Octopus (N = 52)	0.14 ± 0.06	0.05-0.20
Blue mussels (N = 31)	0.03 ± 0.01	0.01-0.04
Eel (N = 6)	0.16 ± 0.05	0.12-0.19
Lamprey (N = 6–same lot)	0.34	-

*DL = Detectable Limit

(Joiris et al., 1999) found higher levels for sardine species. This can be due to the fact that these studies were performed with specimens resident in the Mediterranean, in which the process of bioaccumulation is more evident than in Atlantic species (Baldi et al., 1978; Renzoni et al., 1998).

In order to make illustrate the distribution of mercury levels of the most relevant canned seafood commercialized in Portugal, namely tuna, sardine and chub mackerel, all values are presented in the histograms (relative frequency of total mercury, in percentage) shown in Figures 1, 2 and 3. The levels, expressed in mg kg^{-1} wet weight, were clustered in several classes, indicating a range of concentrations [higher or equal to, and lower than]. Of the 425 samples of canned tuna fish analyzed (Figure 1), only one reached the EU limit (1 mg kg^{-1}) (EU, 2002). The majority of samples showed mercury levels between 0.10 and 0.40 mg kg^{-1}, being the higher relative frequency presented by class 0.20-0.30 mg kg^{-1}. Such distribution was similar to that found by several authors for this kind of product (Duve, 1981; Krueger and Kruse, 1984; Schindler, 1985; Gajewwska et al., 1995; Voegborlo et al., 1999).

All canned sardine samples (Figure 2) showed levels below the EU limit for this species (0.5 mg kg^{-1}). Major relative frequency was exhibited by class 0.02-0.04, and only 3.2% of samples had concentrations higher than 0.08 mg kg^{-1}. The distribution in canned chub mackerel (Figure 3) is similar to that found for canned sardine; however, a higher number of samples (25.4%) displayed upper levels.

The levels of cadmium are presented in Table 2. The maximum mean concentrations were found in canned molluscs (0.29 mg kg^{-1} for squid and 0.16

FIGURE 1. Distribution of mercury content (relative frequency, %) in canned tuna. (A) [0.0-0.1], (B) [0.1-0.2], (C) [0.2-0.3], (D) [0.3-0.4], (E) [0.4-0.5], (F) [0.5-0.6], (G) [0.6-0.7], (H) [0.7-0.8], (I) [0.8-0.9], (J) [0.9-1.0].

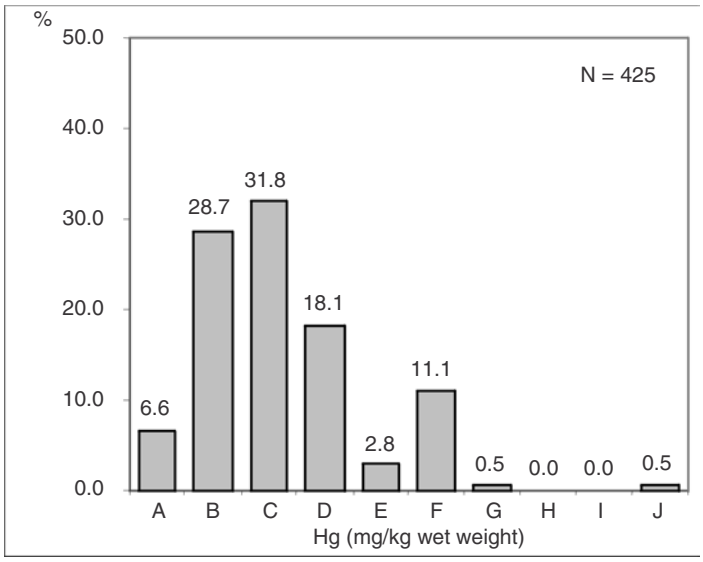

FIGURE 2. Distribution of mercury content (relative frequency, %) in canned sardine. (A) [0.00-0.02], (B) [0.02-0.04], (C) [0.04-0.06], (D) [0.06-0.08], (E) [0.08-0.10].

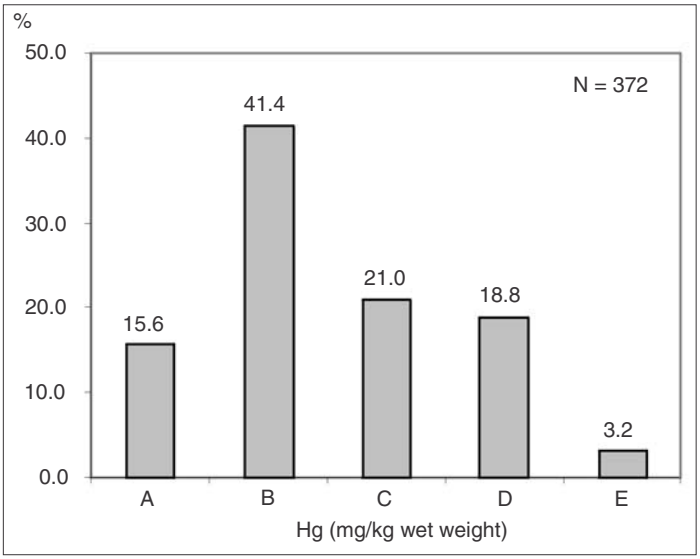

FIGURE 3. Distribution of mercury content (relative frequency, %) in canned chub mackerel. (A) [0.00-0.02], (B) [0.02-0.04], (C) [0.04-0.06], (D) [0.06-0.08], (E) [0.08-0.10], (F) [0.10-0.12], (G) [0.12-0.14], (H) [0.14-0.16], (I) [0.16-0.18], (J) [0.18-0.20].

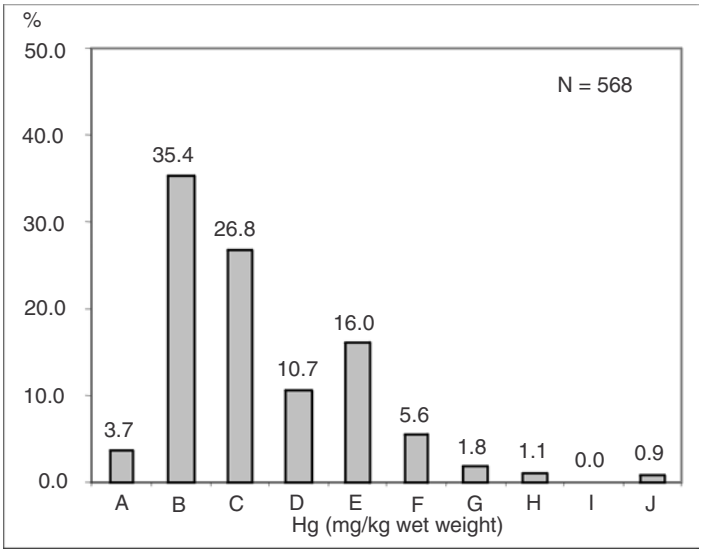

TABLE 2. Mean contents of cadmium (mg/kg wet weight) in canned seafood samples.

Canned seafood (N = number of analyzed samples)	Cadmium (mg kg^{-1} wet weight)	
	Mean ± Standard deviation	Range
Tuna (N = 139)	0.04 ± 0.03	0.01-0.16
Tuna spread (N = 50)	0.02 ± 0.01	0.02-0.01
Chub mackerel (N = 40)	0.04 ± 0.00	0.03-0.04
Sardine (N = 113)	0.02 ± 0.02	0.01-0.07
Sardine spread (N = 40)	0.01 ± 0.01	0.01-0.02
Squid (N = 54)	0.29 ± 0.42	0.04-1.10
Octopus (N = 52)	0.05 ± 0.03	0.02-0.12
Blue mussels (N = 21)	0.16 ± 0.07	0.09-0.22
Eel (N = 6)	0.01 ± 0.00	0.01-0.01

mg kg^{-1} for blue mussels) and the range was between 0.02 and 1.10 mg kg^{-1}. Cisneros et al. (1995) indicated similar mean values for canned squid. Some samples of canned squid (18.5%) exceeded the EU limit (1.0 mg kg^{-1}) proposed for this species (EU, 2001). These high levels can be attributed to the presence of visceral tissue in this canned seafood (Falandysz, 1989; I-de et al., 1994; Shulz-Schroeder and Schering, 1995). Values of Cd found by Schindler (1985) in canned mussels (0.30 mg kg^{-1}) are higher; however, the range is similar. Lowest contents were presented by canned chub mackerel, spreads and eel samples; regarding canned tuna, in spite of one sample exceeding the EU limit (0.05 mg kg^{-1}), the concentrations obtained in this work were lower than that found by Voegborlo et al. (1999).

Lead was only detected in 120 samples of 526 analyzed; however, the maximum levels obtained, 0.1 mg kg^{-1} (tuna), 0.2 mg kg^{-1} (sardine) and 0.3 mg kg^{-1} (blue mussels), were below the permitted limits (0.4, 0.4 and 1.5 mg kg^{-1}, respectively) (EU, 2002). Studies performed in earlier years (Schindler, 1984; Hardisson et al., 1985; Romieu et al., 1994) showed mean levels greater than 2 mg kg^{-1} in some cases. These Pb high concentrations can be explained by the use of lead soldered side seam in cans.

CONCLUSIONS

In conclusion, this study indicates that canned fish follows a pattern Hg > Cd > Pb while canned molluscs show a profile Cd > Hg ≈ Pb. All values were lower than those found by other authors. Although high contents of mercury in fresh tuna are a concern, the present study showed the prevalence of levels between 0.1 and 0.4 mg kg^{-1} in canned product. Such fact is probably due to the utilization of small size fish and, moreover, the seafood industry selecting fish caught in less polluted areas. In reference to concerns about cadmium levels in canned molluscs like squid, in which 18.5% of samples tested exceeded 1 mg kg^{-1}, the major problem seems to be the utilization of whole body.

Taking into account the type of consumption of these products it can be concluded that canned seafood products do not represent a risk in terms of human diet.

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