TESI DOCTORAL UPF / 2013

Patterns of accumulation of persistent organic pollutants in human populations

Mercè Garí de Barbarà

upf. Universitat Pompeu Fabra Barcelona

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Ph.D. Supervisor
Dr. Joan O. Grimalt

Ph.D. Tutor
Dr. Jordi Sunyer

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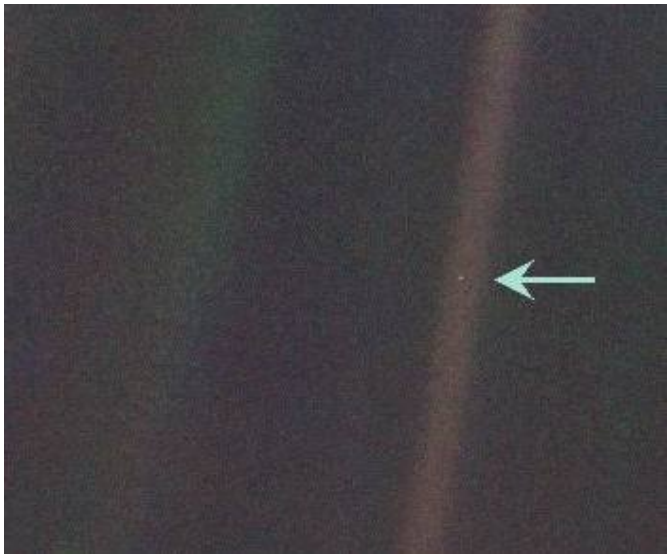
Als meus pares i germans;
a la Martina i a la Núria;
i especialment, al Xavier.

Pale blue dot

On 14 February 1990, Voyager I, after 12 years into it's journey, turned it around and about 6,000 million kilometers away gave a last look inside the solar system and captured this portrait of the Earth. In 1994, Carl Sagan shared these thoughts on the image.



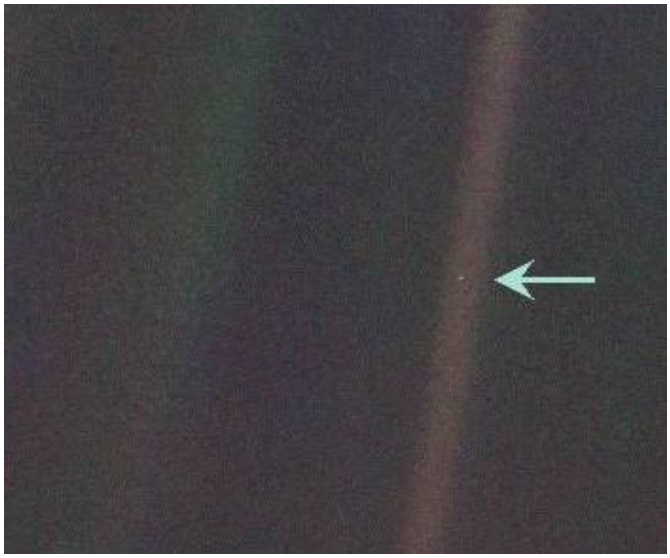
Look again at that dot. That's here. That's home. That's us. On it everyone you love, everyone you know, everyone you ever heard of, every human being who ever was, lived out their lives. The aggregate of our joy and suffering, thousands of confident religions, ideologies, and economic doctrines, every hunter and forager, every hero and coward, every creator and destroyer of civilization, every king and peasant, every young couple in love, every mother and father, hopeful child, inventor and explorer, every teacher of morals, every corrupt politician, every "superstar", every "supreme leader", every saint and sinner in the history of our species lived there –on a mote of dust suspended in a sunbeam.

The Earth is a very small stage in a vast cosmic arena. Think of the rivers of blood spilled by all those generals and emperors so that in glory and triumph they could become the momentary masters of a fraction of a dot. Think of the endless cruelties visited by the inhabitants of one corner of this pixel on the scarcely distinguishable inhabitants of some other corner. How frequent their misunderstandings, how eager they are to kill one another, how fervent their hatreds. Our posturings, our imagined self-importance, the delusion that we have some privileged position in the Universe, are challenged by this point of pale light. Our planet is a lonely speck in the great enveloping cosmic dark. In our obscurity, in all this vastness, there is no hint that help will come from elsewhere to save us from ourselves.

The Earth is the only world known, so far, to harbor life. There is nowhere else, at least in the near future, to which our species could migrate. Visit, yes. Settle, not yet. Like it or not, for the moment, the Earth is where we make our stand. It has been said that astronomy is a humbling and character-building experience. There is perhaps no better demonstration of the folly of human conceits than this distant image of our tiny world. To me, it underscores our responsibility to deal more kindly with one another, and to preserve and cherish the pale blue dot, the only home we've ever known.

Pàl·lid punt blau

El 14 de febrer de 1990, la sonda Voyager I, després de viatjar durant 12 anys, va fer una darrera mirada cap a l'interior del sistema solar i a uns 6.000 milions de quilòmetres va fer una fotografia de la Terra. El 1994, Carl Sagan compartia aquestes reflexions sobre la imatge.



Mira un altre cop a aquest punt. És aquí. És casa. És nosaltres. En ell tothom que estimes, tothom que coneixes, tota la gent de qui has sentit parlar, cada ésser humà que ha existit, hi ha viscut la seva vida. Tota la joia i el sofriment, milers de religions, ideologies i doctrines econòmiques, cada caçador i recol·lector, cada heroi i covard, cada creador i destructor de civilització, cada rei i serf, cada parella jove enamorada, cada mare i pare, infant esperançat, inventor i explorador, cada mestre de moral, cada polític corrupte, cada superestrella, cada líder suprem, cada sant i pecador en la història de la nostra espècie ha viscut aquí –en un tros de pols suspès en un raig de sol.

La Terra és un lloc petit en la vasta arena còsmica. Pensa en els rius de sang vessats per tots els generals i emperadors que, en glòria i triomf, han pogut esdevenir momentàniament senyors d'una fració d'un punt. Pensa en les llargues crueltats sofertes pels habitants d'una cantonada d'aquest píxel, escassament distingibles dels habitants de qualsevol altra cantonada, quants desacords i incomprensions entre ells, com han estat disposats a matar-se els uns als altres, que fervents els seus odis. La nostra actitud, la nostra imaginada importància, la desil·lusió de què tenim una posició privilegiada a l'univers, tenen un repte davant d'aquest punt de pàl·lida llum. El nostre planeta és un punt solitari en un gran envoltori de foscor còsmica. En la nostra obscuredat, en tota la seva amplitud, no hi ha cap prova de que l'ajut vindrà d'algun altre lloc per a salvar-nos de nosaltres mateixos.

La Terra és l'únic món conegut fins ara que té vida. No hi ha enlloc més, almenys en el futur proper, cap on les nostres espècies puguin migrar. Visitar, sí. Assentar-s'hi, encara no. Ens agradi o no, de moment la Terra és allà on podem viure. S'ha dit que l'astronomia és una experiència que fa ser més humil i canvia el caràcter. No hi ha potser millor demostració de la follia de la vanitat humana que aquesta imatge distant del nostre minúscul món. Per a mi, reforça la nostra responsabilitat en tractar-nos una mica millor entre nosaltres, i en preservar tendrament aquest pàl·lid punt blau, l'única llar que hem conegut.

Agraïments

Aquests anys de recerca doctoral han estat els més intensos i enriquidors que he viscut fins ara, tant a nivell acadèmic i intel·lectual, com a nivell personal i familiar. He viscut moltes experiències i canvis, he après infinites coses i he conegut diverses persones que m'han acompanyat al llarg d'aquest camí. No tot han estat flors i violes. Hi ha hagut temporades difícils. Alguna *porta* es va tancar, envoltant-me d'angoixa i d'incertesa, tot i que al meu voltant sempre hi van haver persones fidels que van trobar una altra sortida... Vull agrair a totes aquestes persones que han estat al meu costat durant tots aquests anys.

En primer lloc, un agraïment ben sincer al Joan Grimalt. Gràcies per oferir-me de fer el doctorat amb un projecte tan estimulant; per poder incorporar-me al teu grup de recerca i deixar-me treballar lliurement; per recolzar-me fins al final, quan ja ningú hi veia esperança, i trobar una sortida a la *porta* tancada; per permetre'm d'anar a cursos i congressos, i fins i tot treballar des d'Essex... I gràcies sobretot, Joan, per la teva flexibilitat alhora de combinar la recerca amb la meva maternitat. Potser precisament per això, aquest últim període ha estat el meé fructífer de tots.

En segon lloc, un agraïment per l'Esther Vizcaino. Què hagués estat la vida al laboratori sense una companya com tu? Ens ho hem passat bé; ens hem ajudat; hem compartit moltes hores, moltes pipetes, moltes mostres; ens hem interpel·lat, per aprendre i millorar; hem fet dos trasllats de laboratori; hem rigut i hem plorat; i han estat molts anys, colze a colze, treballant plegades. Gràcies, Esther, per fer-me la vida al laboratori tan agradable i tan estimulant, aportant-me el teu estil divertit i desenfrenat, però alhora responsable, en la tasca de la recerca.

Juntament amb l'Esther, un agraïment a la Marta Fort, amb la que també hem compartit moltes hores al laboratori de salut, moltes xerreres i ploreres. Gràcies, Marta, per la teva disponibilitat constant per ajudar i facilitar la vida al laboratori, i sobretot, per la teva alegria contagiosa.

Voldria també agrair totes aquelles persones que han fet una estada al laboratori de salut i m'han ajudat a analitzar les mostres: la Tami, l'Esther, l'Anna May i l'Ana Sesé. Gràcies per permetre'm d'ensenyar-vos el funcionament del laboratori i l'anàlisi de les mostres, i alhora, per la vostra ajuda i dedicació en el meu projecte doctoral.

Agrair també als altres científics i companys del grup, als que encara hi són i els que ja han marxat: la Pilar, el Jordi, el Daniel, l'Eva, el Roberto, la Carme, la Mireia, la Carolina, el Roger, la Nelia, la Berit, l'Anton, el Sam, la Lourdes, el David, la Belen, el Barend, l'Oscar, la Marta Fontal i la Marta Casado, l'Esther Marco, l'Anuar, el Bibi, el Guillermo, i molts d'altres que hi han anat passant...

També voldria fer un agraïment als tècnics dels equips de gasos i masses: la Patrícia, la Roser, la Dori, la Maria i la Imma. Gràcies per la vostra dedicació i ajudar-me en tot el procés d'injecció de les mostres. Sense vosaltres, les més de 1000 mostres que he analitzat haguessin quedat oblidades en un vial...

També un recordatori especial per als investigadors i doctorands d'altres grups, així com pel personal del centre i d'informàtica. Han estat molts anys i ja m'hi sento de la casa.

I sortint de l'àmbit de la recerca, voldria tenir presents als amics de sempre (del *pas*, d'*aprofundiment*, del *casal* i molts d'altres), perquè m'heu acompanyat al llarg d'aquests anys i sempre heu estat atents i encuriosits pel meu doctorat. Potser últimament us he tingut una mica abandonats, però en tots aquests anys hem viscut plegats molts canvis personals, casaments i naixements, alegries i angoixes, i moltes inquietuds vitals.

I ja acabant, vull agrair a la meva família el vostre suport en tots aquests anys: la tieta, el Carli i la Susana, la Judith, l'Elena i la Maria, la Imma, el Xesco i resta de família; així com tots els meus nebodets i nebodetes, perquè m'heu alegrat la vida: Elenita, Ana, Maria, Pablo, Daniel, Ignasi, Marta i Alex. També un agraïment a la Maria i al Josep, per la vostra comprensió i ajuda, sobretot en aquests últims mesos; i evidentment, gràcies, Òscar, per la teva disponibilitat d'última hora en dissenyar-me la portada, refer algunes imatges del manuscrit i altres retocs d'aquesta tesi.

Els agraïments més profunds, però, van dirigits als meus pares i germans. Ja des de ben petita m'heu guiat i inspirat a seguir un camí responsable, de treball i d'esforç personal; m'heu potenciat les meves qualitats humanes, artístiques i intel·lectuals; heu vetllat pel meu desenvolupament en tots els àmbits que una persona pot desitjar. Mai m'ha faltat de res, tot m'ho heu donat, però alhora m'heu ensenyat a apreciar les coses i donar-los el valor que tenen.

Gracias, Elen, por abrirme los ojos a la vida y hacerme dar cuenta de lo que realmente es importante en cada momento.

Gracias, Tono y Javi, por llevarme con vosotros por todo el mundo, y también ofrecerme el mundo entero a mi pequeño ser. Siempre estáis atentos a mis cosas, por pequeñas que sean, y me siento acompañada en mi camino vital. Gracias también, Tono, por siempre animarme a dar un paso más en todo lo que hago, y más específicamente, por ayudarme con mis artículos, con el inglés, y últimamente, en la revisión final del manuscrito de esta tesis. Gracias, de verdad.

Y gracias, papa y mama, por vuestro apoyo constante, por acompañarme, por cuidarme, por comprenderme, por estar siempre a mi lado. Siempre.

Vull també indicar que aquest últim any i mig de tesi ha estat potser el més intens de tots, precisament per haver combinat la recerca amb la tasca de ser mare. Totes dues, activitats ben aclaparadores, però alhora ben estimulants. Gràcies, Martina, per acompanyar-me de ben aprop en tot aquest últim procés, per mostrar-me que tot es pot fer, sense perdre mai de vista l'horitzó més important. Tot plegat, però, ha estat massa intens: donar el pit i integrar els PBDEs; canviar els bolquers tot pensant en com refer un gràfic; aprofitar les teves migdiades (implorant que fossin llargues) per llegir articles, o escriure'ls, o fer estadística, o seguir-ne aprenent... Puc estar orgullosa perquè he estat amb tu tots i cadascun dels dies de la teva vida, acaronant-te, cuidant-te, dedicant-me a tu; però tampoc he oblidat la meva recerca, i potser fins i tot la he valorat més i m'hi he volgut dedicar intensament, també. Gràcies, Martina bonica, per il·luminar-me els meus dies i la meva vida. Ara ja no concebo la vida sense tu. I aviat arribarà la teva germana, la Núria, les perletes més importants del meu cor. I encara que no hagis nascut, Núria, vull que sàpigues que has estat la flameta que m'ha encoratjat a acabar aquesta etapa doctoral i obrir-me a un futur nou, desconegut, incert, però alhora ben estimulant.

I per últim, però el més important, un agraïment molt especial pel Xavier. Ell va aparèixer al principi de tot, quan jo començava al laboratori a analitzar les mostres. Recordo que ens trucàvem al matí, aprofitant els meus 10 minuts (eterns) de centrífuga (i que llavors em passaven volant!); a les tardes, en sortir del laboratori, sempre em demanaves què havia fet (i sempre et deia el mateix: mostres, mostres i més mostres); m'acompanyaves els caps de setmana al captura, i més tard al nici, i m'ajudaves tapant i destapant vials i vials de mostres (tot i que també t'entretenies amb el vòrtex i altres *joguines* del laboratori...), i després, com s'havia fet tan tard, ens anàvem a fer la nostra *tantarantanada*...

Més endavant, em vas encoratjar a endinsar-me en el programa R per fer l'estadística, i fins i tot em vas *enviar* a Tartu (Estònia), amb alguns dels millors professors d'epidemiologia i R, per tal d'aprendre'l bé. Després ja vindrien els cursos d'estiu a Essex, ben estimulants intel·lectualment. Com no recordar la taula doble de les *Houses*, ordinador contra ordinador, discutint anàlisis estadístiques, models, resultats...? I no només R i estadística, sinó que amb tu va arribar el *linux*, amb la *gentoo* i l'*awesome* (heredar el teu portàtil va anar realment molt bé per fer el pas definitiu); el *dvorak*, el *pentadactil* i el *gedit*; el *latex* i el *beamer*; el *jabref* i moltes d'altres eines que m'han ajudat i facilitat molt a l'hora de treballar la tesi.

Sense tu, Xavier, aquest doctorat hagués estat completament diferent. M'has ajudat constantment, dia i nit, caps de setmana i vacances, amb paciència infinita, amb tot l'amor que ningú pot somniar, amb un somriure a la boca fins i tot quan els meus nervis no em deixaven entendre res...

Gràcies, Xavier, per tot el que m'has ensenyat; he après molt al teu costat i tot això ho he pogut aplicar a la meva tesi. Gràcies per acompanyar-me tan fidelment en aquest camí, per il·luminar-me i engrescar-me, per ajudar-me tant, per fer-te present en els moments difícils, i perquè m'ho he passat molt bé al teu costat. Ara repasso el nostre passat i sembla que només haguem compartit ciència; i així ha estat: no recordo una excursió en bicicleta, o caminant pels Pirineus, o un passeig al pantà de Vallvidrera, o un viatge post-congrés, o un diumenge de pluja al matí, que no hi haguem parlat, de ciència.

I més enllà d'això: Gràcies, Xavier, per omplir el meu cor, pel teu amor fidel i pacient, per la teva silenciosa presència, constant, en el caminar de la meva vida.

Mercè, febrer de 2013

Preface

Since the Industrial Revolution, the scientific and technological progress has turned into a rapid improvement in the well being of a large part of the human population, as a result of the increase in the production of food, provision of healthcare and comfort. Although many people still live in poverty and scarcity due to the unfair distribution of wealth and resources, science and technology are widely perceived as drivers for bettering the human condition.

However, such industrialisation has been creating **major environmental problems**. The immense demand and use of non-renewable energy (such as coal, firewood and oil) are driving ecosystem degradation and climate change at unprecedented scale. Furthermore, the human extraction, use, transformation and distribution of countless chemical products often disrupt soil, water and air resources. The extent of these environmental issues is shown by the fact that such energy and chemical products often affect sites and populations well beyond their place of production or usage, since the Earth surface and atmosphere are inter-connected. In addition, the chemical industry has increased the manufacture of a wide range of compounds, with various utilities, most of which are found to be toxic to either humans or wildlife, or both.

The notion of **persistent organic pollutants**, commonly known as POPs, refers precisely to those man-made chemical compounds, of organic nature, that are usually produced for agricultural and industrial applications, and that, once released, are persistent in the environment. They are transported long distances through the natural cycles, reaching regions where they have never been used or produced. POPs are thus toxic compounds with widespread environmental impact. In addition, and due to their ability to bioaccumulate in the organisms and even to biomagnify through the food web, they pose an important health

threat to both wildlife and people. **Human exposure to POPs**, even at apparently insignificant levels, can lead to serious health problems, including cancer. Therefore, pollution from POP compounds in the general population is a relevant public health issue, requiring careful consideration and action in public policy from environmental monitoring to health care practice, and from research to chemical and industrial regulation.

In this sense, a specific international agreement on POPs, the *Stockholm Convention on Persistent Organic Pollutants*, was adopted in 2001. However, its scope is still limited whereas the concerns around POPs and other pollutants keep growing incessantly as the chemical industry expands. Research on the environmental-health linkages of POPs is thus important to better understand the risks and to inform public policies and regulation –internationally and nationally.

The research that composes this **Ph.D. dissertation** explores the levels and patterns of accumulation of a wide range of POPs in the human population. It assesses two major families of POPs, namely organochlorine compounds (OCs) and polybrominated diphenyl ethers (PBDEs), and focuses in Catalonia, a country in South-West Europe that boasts thriving agricultural and industrial sectors. The research is mostly derived from an extensive health survey of the general population, commissioned by the Government of Catalonia in 2002, which included serum samples from around 1,000 individuals.

The dissertation consists of a **compendium of research and publications** on the linkages between chemical products, the environment and human health. It comprises a methodological innovation (on how to better detect OC levels), core quantitative and analytical research (on the patterns of accumulation of POPs in the general population of Catalonia) and complementary assessments (on POPs in a town that hosts a chemical factory; and on mercury –which is a

non-organic persistent pollutant– in the Menorca island) to provide a comprehensive and comparative insight on the effects and behaviour of POPs in the human bodies and in the human health.

The dissertation is structured into **six chapters and a research addendum**. It comprises 5 major research articles (as lead author) and 3 supplementary publications (as co-author). **Chapter 1** introduces the context of the research, including an overview of the POPs, the profile of Catalonia and the scope of the research itself. **Chapter 2** presents the main goal of the Ph.D. research and the six specific objectives that have been addressed. **Chapter 3** describes the methodology employed along the different assessments, including the study design, the ways to determine OCs and PBDEs, and the statistical analysis. The core chapter, the Results (**Chapter 4**), is a compendium of four articles, some already published while others in their way to publication, as follows:

- a methodological innovation to better detect and quantify OC levels in human serum;
- an analysis of sex-related divergence in the accumulation of POPs (both OCs and PBDEs), taking a special account of age and body mass index, in the population of Catalonia, with a comparative framework with other population health surveys (such as a major health study in the USA);
- an in-depth assessment of PBDEs in the population of Catalonia, which reveals an unexpected pattern of accumulation of this type of POP, namely an inverse age-dependence trend; and
- a specific scrutiny of the spatial influence of OCs, based on the case of the township of Flix, in southern Catalonia, which hosts a chemical factory that used to release OCs until the 1990s.

The Results chapter also includes the synthesis of a number of supplementary research, co-authored and already published, that complements the above insights on the accumulation of OCs (these publications are attached in the annexes for further detail, if necessary).

Chapters 5 and 6 contain the Discussion and Conclusions, respectively, of the mentioned research work. They coalesce all the results and analyses, comparing them, and end up providing a genuine understanding of the interface between chemical pollutants and human metabolism.

This doctoral dissertation also includes, as an **addendum**, a complementary research with a fifth article that focuses on the levels of mercury, a non-organic persistent pollutant, in the infant population of Menorca, a Mediterranean island some 200 km off Catalonia's coast. Although differing from the main research, this chapter provides a complementary insight on the accumulation of toxic pollutants in humans and illustrates the importance of interdependent research on the wide range of chemical pollutants that relentlessly and increasingly affect human societies. This last chapter is presented with the same structure as the entire manuscript, and was conducted during the same period of the core research, exposing the interest of the researcher in conducting parallel and comparative studies to confirm facts or potentially disclose new trends.

In essence, this research comprises four major articles, one complementary research paper and three supplementary studies (as co-author) that navigate a diversity of pollutants, research methodologies and geographic conditions to explore the linkages between environmental pollution and human health.

The research has been conducted at the **Institute of Environmental Assessment and Water Research** (IDÆA), in Barcelona, which belongs to the Spanish Council for Scientific Research (Consejo Superior de Investigaciones Científicas – CSIC). It simultaneously falls under the **Ph.D. Programme on Biomedicine**

of the Department of Experimental and Health Sciences at the **Universitat Pompeu Fabra** (UPF), in Barcelona. The research has been financed by the JAE pre-doctoral programme as well as other support grants from the CSIC.

This type of research, at the crossroads of environmental chemistry and public health, has potentially multiple effects. It not only serves to increase global scientific knowledge, but also to raise social awareness on environmental and health matters, as well as to inform public policies, regulation and environmental monitoring. Curiously, it also illustrates how science can expose the effects of the scientific progress itself, providing new knowledge that can serve to correct its own path.

Abstract

The present research assesses the extent of several persistent organic pollutants (POPs), specifically organochlorine compounds (OCs) and polybrominated diphenyl ethers (PBDEs), in humans. It focuses on the population of Catalonia, a Mediterranean country with active agricultural and industrial sectors, in South-West Europe, building from a public health survey conducted in 2002. A chemical analysis of a wide range of POPs in serum samples of almost 1,000 individuals from the general population is thoroughly conducted, using gas chromatography and mass spectrometry. A customised methodology to better detect OC levels is also designed for, and employed in the research. The POP concentrations are then contrasted with a set of socio-demographic factors.

The most remarkable result is an unexpected and divergent pattern of accumulation of POPs in relation to age, sex and body mass index (BMI). Certain OCs accumulate more in women through age than in men, such as hexaclorobenzene and β-hexachlorocyclohexane. The accumulation of precisely these compounds, moreover, is proportionately higher with increases in BMI among men, but not in women. Conversely, the accumulation pattern in PBDEs follows an inverse age-dependence trend, implying that younger individuals (age >30 years) have higher levels than adults, irrespectively of sex and BMI, and this is evident in the major congeners (*e.g.* BDE-47, BDE-99) and even in the decabromodiphenyl ether (BDE-209).

In essence, age, sex and body fat constitute key factors to understand the accumulation patterns of POPs in humans. The research argues that human metabolism, which precisely varies across age, sex and body fat, plays a key role in the way POPs behave inside human bodies (*i.e.* the pace of accumulation and excretion). These patterns are partly related to physical-chemical properties of POPs, namely their semi–volatile nature.

The accumulation patterns of POPs along with the human metabolic dynamics deserve careful attention in public health and environmental regulation, in view of their ability to permeate the human body, mostly through the food chain and respiration. Despite most of the studied compounds are nowadays banned or strictly controlled internationally, this research asserts the need to expand the monitoring of all types of POPs and similar chemical compounds since their effects in human health are often unexpected, even unusual and persistent through time. Multidisciplinary research on this matter is required, beyond its scientific value, to inform public policy.

Resum

Aquest estudi avalua l'abast de diversos contaminants orgànics persistents (COPs), específicament els compostos organoclorats (OCs) i els polibromodifenil èters (PBDEs), en humans. La recerca se centra en la població de Catalunya, un país mediterrani del sud-est d'Europa amb uns sectors agrícola i industrial actius, i es basa en una enquesta de salut pública de l'any 2002. S'ha realitzat una anàlisi química d'un ampli ventall de COPs en mostres de sèrum de gairebé 1000 individus de la població general, emprant cromatografia de gasos i espectometria de masses. A més, s'ha dissenyat una metodologia específica per a millorar la detecció dels nivells d'OCs, per aplicar-la a la recerca. Les concentracions de COPs han estat contrastades amb una sèrie de factors sòcio-demogràfics.

El resultat més destacable és un inesperat patró divergent en l'acumulació dels COPs en relació a l'edat, el sexe i l'index de massa corporal (IMC). Determinats OCs s'acumulen més en dones que en homes a través de l'edat, com l'hexaclorbenzè i el β-hexaclorciclohexà. L'acumulació d'aquests mateixos compostos, a més, és proporcionalment major a mida que augmenta l'IMC en homes, però no en dones. Així mateix, el patró d'acumulació de PBDEs segueix una tendència inversa amb l'edat, de manera que individus més joves ($>$ 30 anys) presenten nivells més elevats que els adults, independentment del sexe i l'IMC. Això és evident especialment en els principals congèners (BDE-47, BDE-99) i fins i tot en el decabromodifenil èter (BDE-209).

Fonamentalment, l'edat, el sexe i el greix corporal constitueixen els factors clau per tal d'entendre els patrons d'acumulació de COPs en humans. Aquesta recerca argumenta que el metabolisme humà, que varia precisament amb l'edat, el sexe i el greix corporal, juga un paper essencial en la manera com els COPs es comporten dins el cos humà (el ritme d'acumulació i excreció). Aquests patrons estan parcialment relacionats amb les propietats físico-químiques dels COPs, específicament la seva natura semi-volàtil.

Els patrons d'acumulació dels COPs, juntament amb la dinàmica del metabolisme humà, requereixen una atenció especial en l'àmbit de la salut pública i de la regulació ambiental, d'acord amb la seva facilitat d'incorporar-se en el cos humà, sobretot a través de l'alimentació i la respiració. Tot i que bona part dels compostos estudiats avui en dia estat prohibits o romanen sota un estricte control internacional, aquest estudi confirma la necessitat d'ampliar la monitorització de tots els tipus de COPs i compostos químics similars ja que els seus efectes en el cos humà són sovint inesperats, i fins i tot, inusuals i persistents en el temps. Cal una recerca multidisciplinària en aquesta qüestió, més enllà del seu valor científic, per informar també en l'àmbit de la política pública.

Contents

Abbreviations and acronyms

AMAP	Arctic Monitoring and Assessment Program
ANOVA	Analysis of Variance
ATSDR	Agency for Toxic Substances and Disease Registry
BDE	Brominated diphenyl ether
BMI	Body mass index
CI	Confidence Interval
CRM	Certified Reference Material
DDD	Dichlorodiphenyldichloroethane – a metabolite of DDT
DDE	Dichlorodiphenyldichloroethylene – a metabolite of DDT
DDT	Dichlorodiphenyltrichloroethane
EBC	Exhaled Breath Condensate
ECD	Electron Capture Detector
EPA	Environmental Protection Agency (U.S.)
ESCA	Enquesta de Salut de Catalunya [Health Survey of Catalonia]
GC	Gas Chromatography
GM	Geometric Mean
GMP	Global Monitoring Plan
GerES	German Environmental Survey
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
IAEA	International Atomic Energy Agency

IARC	International Agency for Research on Cancer
IDESCAT	Institut d'Estadistica de Catalunya [Statistical Institute of Catalonia]
INMA	Infancia y Medio Ambiente [Childhood and Environment]
IUPAC	International Union of Pure and Applied Chemistry
LD	Limit of Detection
LQ	Limit of Quantification
MS	Mass Spectrometry
MSCA	McCarthy Scales on Children's Abilities
NHANES	National Health and Nutrition Examination Survey
NICI	Negative Ion Chemical Ionization
OC	Organochlorine
PBDE	Polybrominated diphenyl ether
PBT	Persistent Bioaccumulative Toxic
PeCB	Pentachlorobenzene
PCB	Polychlorinated biphenyl
PCDF	Polychlorinated dibenzofuran
POP	Persistent Organic Pollutant
PTM	Proficiency Testing Material
rpm	Revolutions per minute
RT	Retention Time
SD	Standard Deviation
S/N	Signal/Noise ratio
SIM	Selected Ion Monitoring
TBB	Tetrabromobenzene
TC	Total Cholesterol
TG	Triglyceride
THg	Total Mercury
TL	Total Lipids
UNEP	United Nations Environment Programme
UNESCO	United Nations Educational, Scientific and Cultural Organization
WHO	World Health Organization

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1 Introduction

The chemical industry produces compounds and sub-products that, despite they are intended for some human use, have impacts in the environment, and affect the health of wildlife and humans. The concept of persistent organic pollutants (POPs) refers to a wide array of such components, with common features and a major impact on the environment and human health alike. Understanding and addressing the environmental and health impacts of chemical products, notably POPs, have not been an easy path.

This path started symbolically in 1962, when the biologist Rachel Carson from the USA published a famous book, *Silent Spring*, that raised concerns on the environmental impacts of synthetic pesticides, notably DDT (a type of POP) (Carson, 1962) (Figure 1.1). Her controversial work led to the birth of the environmental movement and to important policy changes in the USA, such a ban on DDTs and other pesticides and the creation of the U.S. Environmental Protection Agency (EPA, 2013). This dynamic soon expanded into other countries and to global discussions. Yet not until 2001 the United Nations managed to host a specific agreement on the matter, the *Stockholm Convention on Persistent Organic Pollutants*, which recognised the adverse environmental and human-health effects of such compounds, proposed measure controls and started to ban or restrict the production and use of several of them (Stockholm Convention on

POPs, a). The list of banned POPs increases through time, as research demonstrates the environmental and health impacts of each of them.

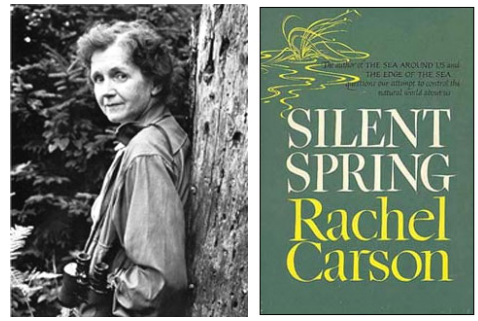

Figure 1.1: Picture of Rachel Carson (left) and title page of her book *Silent Spring* (right).

The current work examines the concentrations of a number of persistent organic pollutants, which have been chemically produced for agricultural and industrial purposes, in a representative sample of the population of Catalonia, a small agricultural-cum-industrial country in South-western Europe. It aims at enhancing the public knowledge on the human-health dimensions of POPs, using data from a significant population universe (notably the case of Catalonia, a country of 32,000 km^2, 7.5 million inhabitants and one of the Spanish regions) and contrasting it with a few specific population cases.

POPs are distributed all over the world and are also found in human tissues and fluids. This represents a public health concern since most of these compounds have toxicity and cause health problems.

There is a twofold merit of studying the presence of these compounds in human populations, as follows:

- First, humans are continuously exposed to POPs throughout life, even under low concentrations, which may result in a negative impact on the health. Further, the exposition to this kind of compounds starts before

birth since the foetus is already exposed to them through the placenta, and the newborn remains exposed through breastfeeding during the first months of life.

- Secondly, POPs interfere with the metabolism of animals, creating new effects and changing their chemical performance. In fact, the particular physical-chemical properties of POPs means they change behaviour in new conditions whereas no biological or biochemical mechanisms can treat and cleanse them adequately.

This chapter starts introducing the persistent organic pollutants that are later analysed, including organochlorine and organobromine compounds. Then explains how POPs are incorporated into the human body and hence the importance of monitoring their levels in human populations. It finally describes the geographical focus of this research, namely the region and population of Catalonia.

1.1 Persistent organic pollutants

Persistent organic pollutants (POPs) are man-made chemical substances, with an organic structure, that are usually produced for agricultural and industrial applications. They have in common three major characteristics, explained next: persistence in the environment; lipophilic behaviour; and significant toxicity to life forms.

POPs are very persistent in the environment because of their highly resistance to chemical, physical and biological degradation. They therefore remain intact for long periods of time. POPs are also semi-volatile compounds, a characteristic that favours their long-range transport. They are transported through

the atmosphere globally, being volatilised in warm areas and condensed in colder regions. This process, known as the global distillation effect (Figure 1.2), has allowed these pollutants to become widely distributed throughout the planet's surface (Simonich and Hites, 1995), including regions where they have never been used or produced, like Polar regions and high-mountain areas (Wania and Mackay, 1993; AMAP, 1998; Grimalt *et al.*, 2001).

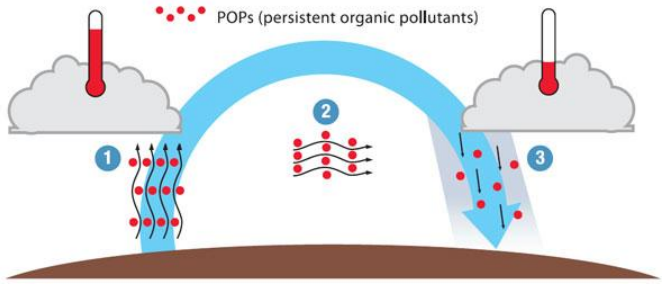

Figure 1.2: Long-range transport of POPs: (1) In warm temperatures POPs evaporate. (2) POPs move in air by winds to colder places such as the Poles and mountain tops. (3) In cold temperatures POPs condense and fall to earth.

POPs are also highly lipophilic compounds, with a great capacity to bioaccumulate in fatty tissues of living organisms and biomagnify up along the food chain. The highest concentrations are found in the organisms at the top of the food web, particularly humans (Figure 1.3).

Finally, many POPs exhibit significant toxicity potential, posing a threat to the human and wildlife health. Human exposure to POPs can lead to serious adverse health effects, including certain cancers, birth defects, dysfunctional immune and reproductive systems, greater susceptibility to disease and even diminished intelligence, among others (Porta *et al.*, 1999; Longnecker *et al.*, 2001; Ribas-Fitó *et al.*, 2002; Howsam *et al.*, 2004; Yáñez *et al.*, 2004; Turyk *et al.*, 2007).

For all the aforementioned characteristics, POPs are also often referred as Persistent Bioaccumulative Toxics (PBTs). In fact, POPs are not just found in several environmental compartments, such as soils and waters, but also in wildlife and inside human tissues and fluids.

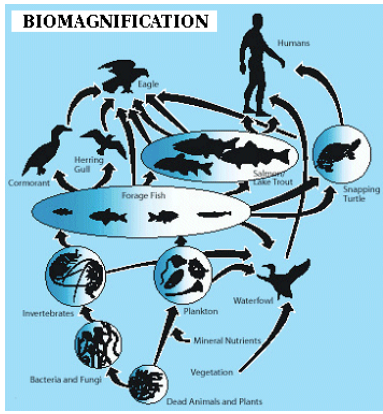

Figure 1.3: Scheme of the biomagnification process.

Given that POPs are widely distributed in geographic terms and actually represent a global problem, the United Nations called in 1995 for a global action to be taken on POPs. Accordingly, the *Stockholm Convention on Persistent Organic Pollutants* was adopted, in 2001, with the aim of eliminating or reducing the production and use of twelve initially selected POPs (Stockholm Convention on POPs, b). With time, further POPs have been added to the list of banned or restricted compounds (Stockholm Convention on POPs, c). Yet, despite the use and production of many POPs is nowadays in the process of being banned, their present use in closed installations, inadequate storage or the existing waste-dumps still constitute potential sources for these pollutants to entering the environment.

The POPs assessed in the present research are representative of the different

pollutant classes and origins, and include both legacy (old) and emergent (new) compounds. The next sections describe the studied POPs, which include both organochlorine (OC) and organobromine (OB) compounds.

Organochlorine compounds

Organochlorine compounds encompass a wide range of chemicals with different structures and purposes. This study focuses on two major sub-groups:

- Pesticides and their degradation products, such as pentachlorobenzene (PeCB), hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs) and DDT and their metabolites. Some of them are also by-products in the industrial production of other chemicals.
- Compounds that are synthetically manufactured for its insulating properties, such as polychlorobiphenyls (PCBs).

The two OC types have in common similar physical-chemical properties, despite their different origins and uses. The number of chlorine atoms and their position in the chemical structure determine their persistence and toxicity. Higher chlorine substitutions make the molecule more soluble in lipids and less in water, as well as more resistant to degradation.

With the publication of the book *Silent Spring* by Rachel Carson in 1962, public awareness about the environmental impacts of pesticides increased. The book suggested that DDT and other pesticides may have adverse effects on wildlife, particularly birds. Consequently, during the 1970s, western countries started to ban the manufacture and use of these compounds. And later in the 2000s, as

mentioned before, the *Stockholm Convention on POPs* recognized all these organochlorine compounds as causing adverse effects on humans and the ecosystems (initially DDT, HCB and PCBs in 2001, and then PeCB and HCHs in 2009).

The following subsections describe the OC compounds that have been analysed in this work.

Pentachlorobenzene and hexachlorobenzene

Pentachlorobenzene (PeCB) and hexachlorobenzene (HCB) belong to a group of chlorobenzenes. They are formed by a benzene ring attached to five and six chlorine atoms, respectively (Figure 1.4).

Figure 1.4: Chemical structures of pentachlorobenzene (left) and hexachlorobenzene (right).

In the past, PeCB was used as a fungicide, as a flame retardant agent, in dyestuff carriers and in combination with polychlorinated biphenyls in dielectric fluids. It can also be used as an intermediate in the production of other compounds. PeCB is also present as a chemical impurity in solvents or pesticides, and emitted to the environment resulting from the incineration of household waste, among others. It is moderately toxic to humans and very toxic to aquatic organisms.

PeCB production ceased some decades ago in the main producer countries, since efficient and cost-effective alternatives are available. It was added to the *Stockholm Convention on POPs* in 2009, in annexes A (to eliminate both the production and use) and C (to reduce the unintentional releases) (Stockholm Convention on POPs, c).

HCB is a potent fungicide that was first used in 1945 for seed treatment. It is also a by-product of the manufacture of certain industrial chemicals and a known impurity of some pesticide formulations. HCB is chemically very stable and resistant to degradation.

Industrial production of HCB began in the early 1930s and ceased during the 1970s in most Western countries. In Spain, however, its use continued until 1986. Nowadays this compound is banned, but it is still emitted to the environment as a by-product. HCB was included initially in 2001 in the *Stockholm Convention on POPs* in annexes A (to eliminate both the production and use) and C (to reduce the unintentional releases) (Stockholm Convention on POPs, b).

The most serious episode involving the effects of HCB on human health is related to the ingestion of treated seed grain with this product in the eastern region of Turkey, in Kurdistan, between 1954 and 1959. The seeds were to be sown in, but there was a period of great famine in the area, and they were eaten directly. The mass poisoning by consumption of HCB treated seeds generated a large number of deaths and the appearance of a variety of symptoms including photosensitive skin lesions, hyper pigmentation, hirsutism, colic and severe weakness. Several thousand of people developed a metabolic disorder called porphyrinuria. Pregnant women who ingested contaminated seeds passed the HCB to their children by placental transfer, and later through maternal breastfeeding (Peters, 1976). Children born to these women developed a condition called

pink sore (pembe yara), with a reported mortality rate of approximately 95%. Some studies have recently shown that exposure to moderate doses of HCB has a negative effect on reproduction (Ribas-Fitó *et al.*, 2002). Moreover, a chronic exposure to this compound has also been related with thyroid cancer (Grimalt *et al.*, 1994).

Hexachlorocyclohexane

Hexachlorocyclohexane (HCH) is a synthetic chemical that consist of eight isomers (Safe, 1994) (Figure 1.5). The isomers are very different between them, as well as their physical-chemical properties, depending on the position of hydrogen and chlorine atoms in the chemical structure.

HCHs are produced commercially by photochemical chlorination of benzene. Only α-HCH, β-HCH, δ-HCH and γ-HCH isomers' are of commercial significance (Figure 1.5). The technical mixture of HCH consists of approximately 65% α-HCH, 8% β-HCH, 13% γ-HCH, 8% δ-HCH and 3% ϵ-HCH (Kutz *et al.*, 1991).

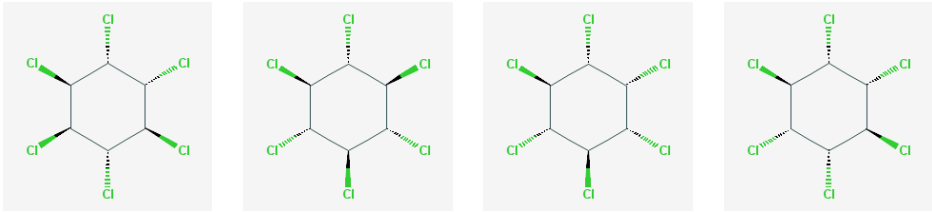

Figure 1.5: Chemical structures of four isomers of hexachlorocyclohexanes: α-HCH, β-HCH, δ-HCH and γ-HCH.

HCHs were synthesized for the first time in 1825, but their insecticide properties were not identified until 1942 (Willett *et al.*, 1998). The main use of

HCH has been as a broad-spectrum insecticide for seed and soil treatment, foliar applications, tree and wood treatment and as an agent against the parasites in both pharmaceutical and veterinary products.

The gamma isomer, called lindane, is the active ingredient of many soaps and shampoos to treat lice and scabies. It is metabolised and rapidly excreted by the organisms due to its high solubility in water. On the other hand, β-HCH is the most stable isomer due to its molecular structure. It tend to be accumulated into the organisms and some studies suggest that it may act as an estrogenic compound (Walker *et al.*, 1999).

All isomers of HCH are toxic to mammals. The *International Agency for Research on Cancer* (IARC) has classified the γ-HCH, together with the technical mixture of HCHs, in *Group 2B* as a probable human carcinogen (IARC, 1987). Chronic exposure to this compound has been associated with adverse effects in humans, affecting the entire central nervous system (Willett *et al.*, 1998). In addition, this compound can produce alterations in the reproductive, immunologic and endocrine systems (Ribas-Fitó *et al.*, 2003a; Alvarez-Pedrerol *et al.*, 2008; Willett *et al.*, 1998).

Lindane was banned or highly restricted in many countries due to its high toxicity and persistence in soils. In 2009, certain HCH isomers (namely alpha, beta and gamma) have been included in the *Stockholm Convention on POPs* (Stockholm Convention on POPs, c).

DDT and their metabolites

DDT (dichlorodiphenyltrichloroethane) is a powerful insecticide. It was first synthesized in 1874, but its insecticide properties were not discovered until the 1930s. DDT was widely used during World War II to protect people from diseases such as malaria, typhus and dengue, among other insect-borne diseases.

After the war, DDT continued to be used to control disease vectors, and it was also used extensively in agriculture.

The technical mixture of DDT contains three isomers of the molecule in the following composition: 4,4'-DDT in 85% (it is the most active isomer), 2,4'-DDT in 15% and 2,2'-DDT at trace levels.

DDT is slowly degraded to DDD (dichlorodiphenyldichloroethane) and DDE (dichlorodiphenyldichloroethylene), which are also very persistent and have similar physical-chemical and toxicological properties to the original product (Figure 1.6). The degradation products are also included in the technical mixture of DDT, although at low concentrations.

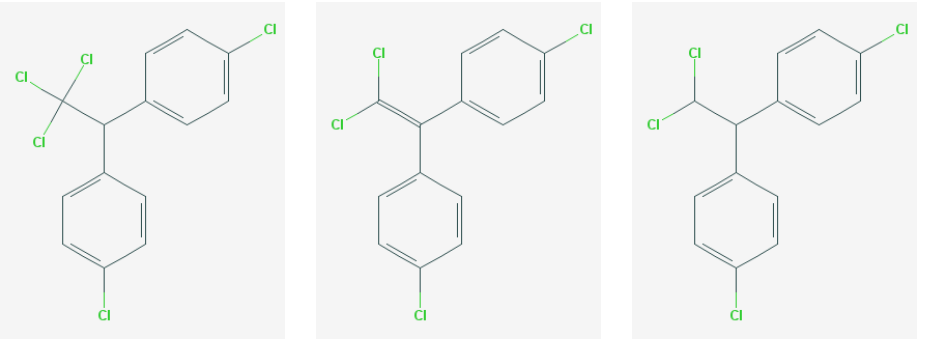

Figure 1.6: Chemical structures of 4,4'-DDT, 4,4'-DDE and 4,4'-DDD.

DDT is metabolised to DDE in aerobic biodegradation (a process called deshidrochlorination). This reaction takes place mainly in living organisms and it is catalysed by the deshidrochlorinase enzyme. DDT can also be metabolised to DDE or DDD (in this case, under anaerobic conditions) in soils and sediments.

The best known toxic effect of DDT is egg-shell thinning among birds, resulting in loss of embryos and the reduction of bird populations (Figure 1.7).

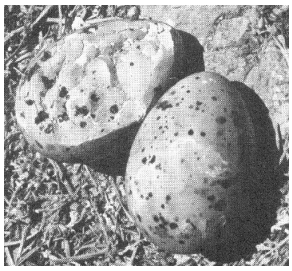

Figure 1.7: Effects of DDT on eggshells.

Rachel Carson was the first who published the toxic effects of DDT on wildlife, in the book *Silent Spring* (Carson, 1962). Its impact led to DDT bans in many countries during the decade of 1970s. In Spain, DDT was widely used since the mid 1950s until the 1970s, when its use was restricted. In 2001, DDT was listed in the *Stockholm Convention* under annex B, to restrict its production and use, with acceptable purpose for disease vector control (Stockholm Convention on POPs, b). Therefore, DDT continues to be applied in several countries to fight against malaria vector, to prevent its spread (Figure 1.8).

Figure 1.8: Indoor DDT spraying for mosquito control in malaria regions.

Long-term exposures to DDT have been associated with chronic health effects, including reproductive disorders, neurotoxicity, immunotoxicity and me-

tabolic disorders (Ribas-Fitó *et al.*, 2006; Alvarez-Pedrerol *et al.*, 2008). In addition, some studies have found a relationship between exposure to 4,4'-DDT and 4,4'-DDE and *K-ras* gene mutation in patients with exocrine pancreatic cancer (Porta *et al.*, 1999).

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are a group of 209 individual compounds, known as congeners, which are formed by chlorination of ten possible positions of a biphenyl structure (Figure 1.9). The congeners' name specifies the total number and position of chlorine atoms in the chemical structure, based on the IUPAC system.

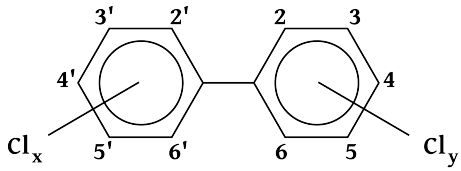

Figure 1.9: Structure of polychlorinated biphenyl (PCB) and numbering system.

The degree of chlorination as well as the position of chlorine atoms in the biphenyl molecule determine the physical-chemical properties and other specific settings. For instance, if ortho positions (positions 2 and 6) have no chlorine atoms, the molecule is kept in a coplanar structure and called non-ortho substituted or coplanar PCB. If the molecule has an ortho position in one of the phenyl rings, the PCB is called mono-ortho substituted. The rest of structures are considered as non-coplanar PCBs. Both coplanar and mono-ortho substituted PCBs can adopt a planar chemical structure similar to dioxins, which allows the molecule to rotate freely. This is of major environmental and analytical importance because these PCBs present higher toxicity.

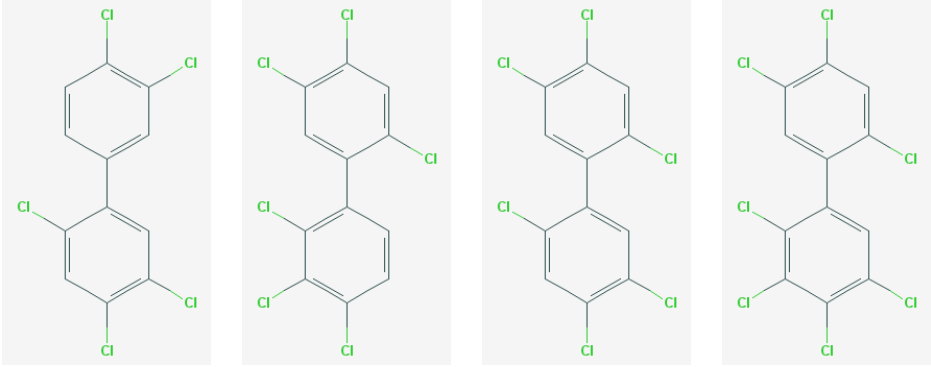

Figure 1.10: Chemical structures of PCB-118 (mono-ortho substituted), PCB-138, PCB-153 and PCB-180.

The PCB congeners' studied in this research are the followings: 28, 52, 101, 118, 138, 153 and 180 (Figure 1.10). These are the most abundant ones in environmental and human matrices.

PCBs were first synthesized in Germany in 1881, but its industrial production did not start until the 1930s. The use of these compounds in industrial and commercial applications quickly spread due to their properties, which include highly chemical stability, non-flammability, low electrical conductivity and insulating properties, high resistance to acids and oxidation, and low solubility in water, among others. All these properties have made them highly adaptable to various uses, such as heat exchangers, transformers and capacitors in electrical systems, as plasticizers in paints and plastics, in pigments and dyes and in many other industrial and commercial applications (Hutzinger *et al.*, 1974).

Their input to the environment was often related to diffusion into the atmosphere or seepage to groundwater after application. The maximum production of these compounds took place in the late 1970s, but soon after they were

found to be dangerous environmental pollutants. PCBs have been included in the *Stockholm Convention on POPs* in 2001 (Stockholm Convention on POPs, b).

Two cases of contamination by PCBs and other chemical compounds such as polychlorinated dibenzofurans (PCDF) occurred in Japan and Taiwan, due to consumption of contaminated oil. The Japanese incident, occurred in Kyushu in 1968, produced a strange disease, called *Yusho*, characterized by acne-like eruptions (chloracne), hyperpigmentation of the skin and eye discharge (Figure 1.11). More than 1,800 individuals were affected by the *Yusho* disease, as well as other adverse effects in liver and nervous systems. Children prenatally exposed to PCBs or through breastfeeding showed delayed development and mental retardation, as well as an abnormal pigmentation of the skin. These children were called *cola-colored* because of this skin hyperpigmentation (Figure 1.11) (Harada, 1976). The Taiwanese incident occurred in 1979 also produced changes in neurobehavioural development in children, as well as chloracne and hyperpigmentation.

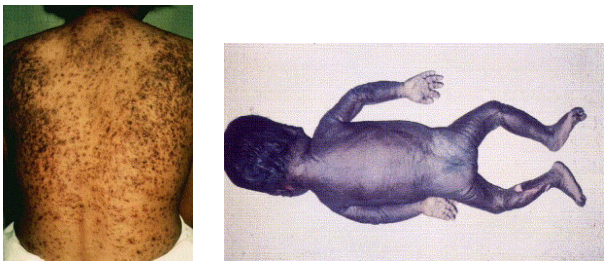

Figure 1.11: Pictures of *Yusho* disease: chloracne (left) and hyperpigmentation (right).

PCBs are toxic to fish, killing them at high doses and causing spawning failures at low doses. Children of mothers who ate large amounts of contaminated fish from Lake Michigan, in United States, showed poorer short-term memory function. PCBs are classified as probable human carcinogens (*Group 2A*) by the

IARC (IARC, 1987). A study have found that mono-ortho substituted PCBs may contribute to the colorectal cancer development (Grimalt *et al.*, 1994). In addition, PCBs are found to act as endocrine disruptors, causing alterations in the nervous and reproductive systems, liver disorders, immunotoxicity and alterations of thyroid function, since they have a structure similar to thyroxine (T4 thyroid hormone).

Organobromine compounds

Among the organic compounds with bromine substitutes, this research has focused on polybrominated diphenyl ethers (PBDEs). These compounds are described next.

Polybromodiphenyl ethers

Polybrominated diphenyl ethers (PBDEs) are a group of compounds comprising 209 congeners that vary by the number and position of the bromine atoms in the molecular structure. Similarly to PCBs, they are named under the IUPAC system, based on the position of the bromine atoms (Figure 1.12). They have been used as flame retardants for some decades in a wide range of products.

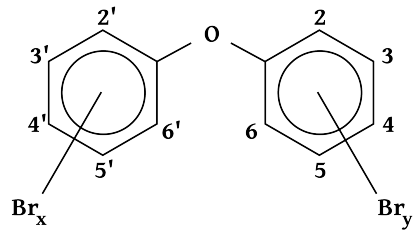

Figure 1.12: Structure of polybrominated diphenyl ether (PBDE) and numbering system.

PBDEs have been distributed in three commercial mixtures of congeners with different levels of bromination (penta-BDE, octa-BDE and deca-BDE), which are named after the dominating homologue group.

The pentabrominated formulation was the major source of BDE-47, BDE-99 and BDE-100 congeners. It was mainly employed as additive of polyurethane foams in furniture, carpets and bedding.

The octabrominated mixture was dominated by BDE-183, followed by BDE-153 and BDE-154, being used in flame-retard thermoplastics, such as high impact polystyrene.

The decabrominated product was essentially composed of decabromodiphenyl ether (BDE-209) and was used predominantly for textiles, as well as in plastics for a variety of electronic products, in particular TVs and computers. According to the PBDE global market demands in 2001, the deca-BDE formulation was the dominant one (83%), followed by penta-BDE (11%) and octa-BDE (6%) (Guardia *et al.*, 2006).

The PBDE congener's studied in this research are the followings: 17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, 190 and 209 (Figure 1.13). These are the most abundant ones in environmental and human matrices.

Public health concern has been expressed regarding the potential adverse health effects of exposure to these persistent environmental chemicals, although evidence is limited (Darnerud, 2003; Birnbaum and Staskal, 2004). Consequently, the European Union banned the pentabrominated and octabrominated formulations in 2004. Later in 2009, these two commercial mixtures were included in the *Stockholm Convention on POPs*, because there are other alternatives (Stockholm Convention on POPs, c). However, production and use of the decabrominated

Figure 1.13: Chemical structures of some PBDE congeners analysed in this work: BDE-28, BDE-47, BDE-85, BDE-99, BDE-100, BDE-153, BDE-154 and BDE-209.

formulation is still permitted, although its use for electrical and electronic applications was restricted in Europe in 2008. Nevertheless, since the only degradation pathway of PBDEs is through debromination (*i.e.* the replacement of bromine on the aromatic ring with hydrogen), higher bromodiphenyl ether congeners may be converted to lower congeners. Indeed, the decabrominated mixture has been shown to degrade to lower brominated and more toxic congeners in the environment (Ross *et al.*, 2009). Therefore, the higher congeners might be precursors to the tetra-, penta-, hexa- or hepta-BDE.

Adverse health effects of human exposure to PBDEs include endocrine disruption (Darnerud, 2003; Legler, 2008; Herbstman *et al.*, 2008; Turyk *et al.*, 2008), developmental neurotoxicity (Herbstman *et al.*, 2010; Gascon *et al.*, 2011) and other detrimental effects (Main *et al.*, 2007; Akutsu *et al.*, 2008; **?**).

1.2 Human exposure to POPs

Background levels of persistent organic pollutants are routinely detected in human tissues (*e.g.* adipose matter and the liver) and fluids (*e.g.* blood and breast milk) as a consequence of the presence of these chemicals in many products used in daily life.

Human exposure to POPs may occur in a variety of ways. Diet is certainly a major route, particularly through the consumption of contaminated foodstuffs with high lipid content like fatty fish or red meat and poultry, among others (Sjödin *et al.*, 2000; Falcó *et al.*, 2004; Domingo *et al.*, 2006; Fraser *et al.*, 2009). Nevertheless, once ingested, these compounds may be partly excreted via the faeces (To-Figueras *et al.*, 2000), since they are not always fully absorbed in the intestinal tract (Kreuzer *et al.*, 1997; Moser and McLachlan, 2001).

There are other minor routes of incorporation of POPs into the human body, such as inhalation in both indoor and outdoor environments or dermal contact. Assessments of the Flix population, which is located near an electrochemical factory with high HCB airborne levels, showed the highest levels of this compound ever found in humans, suggesting it was substantially incorporated into the body through respiration (Sala *et al.*, 1999). Similarly, inhalation of dust has been shown to be a considerable pathway for PBDE exposure (Zota *et al.*, 2008). The indoor environment at both home and the workplace may be also a substantial contributor to PBDE exposure (Lorber, 2008).

POPs are stored in fatty tissues due to their lipophilicity and their high stability to chemical degradation. The observed accumulation of these compounds in human tissues suggests that humans have an inefficient metabolism to excrete them, which favours a continued bioaccumulation through age.

Hepatic metabolism plays an important role in the elimination of many chemicals (Dayton *et al.*, 1983). Metabolic biotransformation may allow the release of POPs and their metabolites into bile or plasma, allowing then their elimination by urine or faeces (Moser and McLachlan, 2001; Jandacek and Tso, 2007). For instance, HCB metabolites have been found in urine and faeces, suggesting an enzymatic conversion of this compound in the liver (To-Figueras *et al.*, 1997, 2000).

There is a greater evidence of excretion of POPs in women, which is associated with their reproductive activities. For instance, during gestation, these compounds are transferred from the mother to the foetus through the placenta (Sala *et al.*, 2001) and, once the child is born, she continues transferring and somehow excreting these compounds through breastfeeding (Ribas-Fitó *et al.*, 2003b). Therefore, pregnancy, child delivery and lactation represent an excretion means for women.

Aside from the aforementioned daily life exposure pathways, humans can be exposed to POPs through their occupation or by accident. This acute exposure can be associated with a wide range of adverse health effects, including illness and death. Notwithstanding, the long-term implications of low level exposure is not fully known and deserves more attention in general population studies.

1.3 Biomonitoring POPs in human populations

Biomonitoring is the measurement of body burdens of toxic pollutants in biological matrices (*e.g.* blood and breast milk). Often, biomonitoring studies reporting POP concentrations have been performed mainly on certain groups particularly vulnerable to these compounds, such as children and their mothers,

due to their capacity to transferring them through placenta and maternal breast-feeding. Individuals who are particularly highly exposed to these compounds because of their occupation or residence in contaminated areas have also been studied (*e.g.* the aforementioned study on the inhabitants and workers in the electrochemical factory from Flix township, in south Catalonia).

However, the adverse health effects of these compounds in humans are not confined to these collectives. Individuals from the general population can also be affected by the chronic exposure to POPs, even to low exposure levels.

Nations of the world adopted in 2001 the *Stockholm Convention on Persistent Organic Pollutants* in order to control, monitor and regulate POPs (Stockholm Convention on POPs, a). The *Global Monitoring Plan* (GMP) of this convention enables the collection of comparable monitoring data from all regions of the world to assess the effectiveness of the *Stockholm Convention* in minimizing human and environmental exposure to POPs (Global Monitoring Plan, 2009). The objective of human monitoring within the GMP is to identify temporal and spatial trends in levels of POPs in humans (*e.g.* to know whether the levels of these compounds are increasing or decreasing over time). The GMP looks at background levels of POPs at locations not influenced by local sources, and uses human breast milk and maternal blood from the general population.

Apart from the GMP, other general populations have been monitored by their Governments to assess the baseline levels of POPs. For instance, the *National Health and Nutrition Examination Survey* (NHANES, 2004) carried out in the U.S. and the *German Environmental Survey* (GerES, 2006) from Germany are good examples of POP monitoring in general populations. Nevertheless, in many countries, including Spain, the information on the concentrations of these pollutants in the general population is still very scarce, and there are no regular studies with representative population (Porta *et al.*, 2008). In fact, there is one

study conducted in a representative sample of a general population in a Spanish autonomous community (*e.g.* Canary Islands) (Zumbado *et al.*, 2005). However, this report only focused on DDT levels and their metabolites and did not comprise other pollutants.

The research presented in this Ph.D. examines the levels of certain POPs in the general adult population of Catalonia with the aim to contribute with new data. The following section describes this population.

1.4 The population of Catalonia

The core research has been conducted on the population of Catalonia, a country of 32,000 km^2 and some 7.5 million inhabitants, located in South-western Europe, on the shores of the Mediterranean Sea (Figure 1.14).

Figure 1.14: Geographical location of Catalonia, in the Western Mediterranean area (left) and zoom of its topography (right).

Catalonia has a varied and compartmentalised topography, with ridges that follow the coastline, depressions in the interior, peaks that reach 3,000 meters of heath in the Pyrenees Mountains in the north and, 250 km further south, a sedimented delta from one of the main rivers of the Iberian Peninsula, the Ebro river.

Municipalities are the basic entity of territorial organization and administration. Currently, there are 947 municipalities in Catalonia. Most people (about 95%) are concentrated in some 300 municipalities with over than 2,000 inhabitants, which are considered, therefore, urban population. The metropolitan area of Barcelona concentrates about half of the population of Catalonia, with around 4.5 million people. Rural areas are sparsely populated.

In 2002, when the study was carried out, Catalonia had a resident population of 6.5 million inhabitants. The population growth has been continuous, largely due to migration processes, initially from other Spanish regions, and in recent years, worldwide. At that time, slightly over 60% of individuals were born in Catalonia itself, 20% were born in other Spanish regions and 15% were of foreign origin. The impact of migration has been particularly important in the metropolitan area of Barcelona.

Catalonia has a notable agricultural and cattle tradition, and to a lesser extent, also fisheries. The local gastronomy is based on the Mediterranean diet, which is rich in a variety of products including cereals, tubers, vegetables, fruits and typical crops products, such as vineyards and olive groves, among others.

Catalonia is also an advanced economy, mainly thanks to its location on the peninsula and in the Mediterranean. This strategic position has been always a gateway to Southern Europe and has ensured that, historically, Catalonia became the leading industry of Spain. The industrial activity grew especially around the conurbation of Barcelona, although numerous industrial parks have

also been developed around the country. The most important industrial sectors in Catalonia are specially chemical, food, energy, metal and transport equipment.

Among the chemical industries, there is one electrochemical factory that produced organochlorine compounds (*e.g.* HCB, DDT and PCBs) for decades. The factory is located in the Flix township, in a rural area 140 km far from Barcelona and about 55-75 km from Tarragona and Lleida, which are the closest urban areas (Figure 1.15).

On the other hand, tourism has grown in the past decades to become one of the most important economic sectors in Catalonia.

Overall, the economic growth of recent decades has noticeably increased the quality of life of Catalan citizens. In this sense, and in order to biomonitoring the levels of POPs in this population, the Catalan Government allowed the use of the Catalan Health Survey (ESCA) conducted in 2002, as well as the Health Examination Survey and blood samples to perform the analysis of POPs.

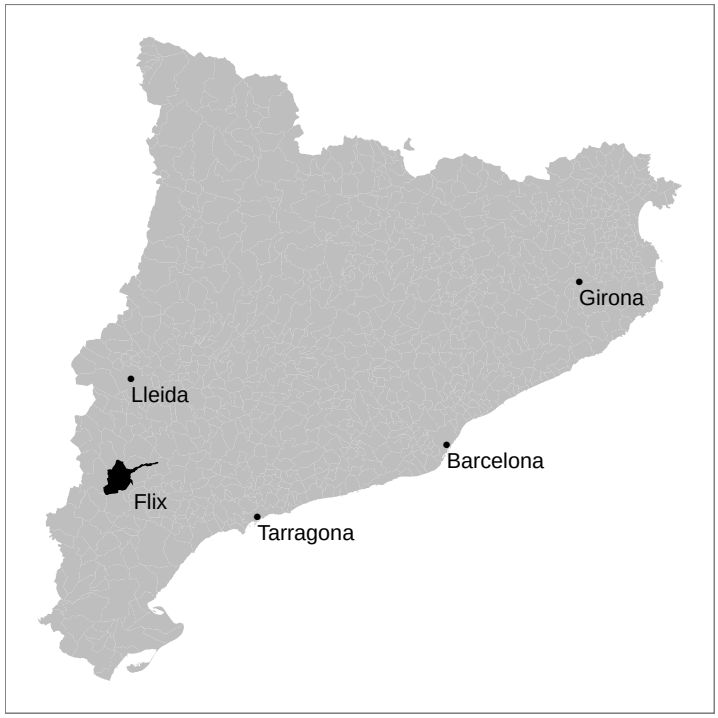

Figure 1.15: Geographical location of Flix, a municipality in south Catalonia that host an electrochemical plant.

2 Goal and Objectives

Goal

The main objective of this doctoral research is to examine the levels and patterns of accumulation of certain POPs in a general population against a set of socio-demographic factors, with a special focus on age, sex and the body fat.

Specific objectives

The dissertation blends various inter-related research lines, with the following specific objectives:

1. development of an accurate method for the calculation of the limits of detection and quantification in the analysis of organochlorine compounds (OCs) in human serum samples, which is then applied in the assessment of OCs in the population of Catalonia;
2. assessment of the distribution of organohalogenated OC and PBDE compounds in the population of Catalonia, on the basis of an analysis of almost 1,000 human serum samples;

3. comparative analysis of the levels of OCs and PBDEs between Catalonia and other researched populations worldwide;

4. investigation on the patterns of accumulation of certain POPs in relation to a set of socio-demographic factors, notably age, sex and the body mass index, using the case of Catalonia;

5. discussion and interpretation of the underlying mechanisms that potentially explain the accumulation and excretion of POPs in different human populations; and

6. verification of the supposed influence of location in the levels of OCs, on the basis of a geography-based assessment around Flix, a town in southern Catalonia that hosts a chemical factory that used to produce or emit OCs in substantial levels.

3 Methodology

3.1 Study design

The core of the present research is based on a public health survey conducted by the Government of Catalonia to its general population in 2002. It included a health exam and a blood testing (human serum) for about 1,000 individuals of the adult population of Catalonia (which represents a robust sample), as well as broad socio-demographic information such as age and social class, among other data.

Health Survey

The Department of Health of the Government of Catalonia[1] conducts periodically a health interview survey, called ESCA (Enquesta de Salut de Catalunya, Catalan Health Survey). This survey monitors, among others, the health status and use of health services among the citizens of Catalonia (ESCA, 2002).

The ESCA-2002 was conducted between October 2001 and May 2002 on a random and representative sample of the non-institutionalized resident population of Catalonia. A total of 8,400 individuals were selected from a sample design conducted in two stages:

[1] Departament de Salut de la Generalitat de Catalunya

- In the first sampling stage, 122 municipalities were selected from the eight health regions of Catalonia, according to their population size. In the case of Barcelona –the capital city with over 2 million inhabitants– the sample design was based on the districts of the city. Figure 3.1 shows the health regions and the selected municipalities.

- At the second sampling stage, a random sample from the census was used to select individuals, using proportional probabilities and based on the size of municipalities.

The health survey questionnaire was aimed to acquire a closer understanding of the health status as perceived by the individuals, of the use of health services, as well as of other determinants on habits and lifestyles (*e.g.* smoking habits, alcohol consumption, physical activity, diet and occupational risks) (ESCA, 2002).

Health Examination

A health examination was also conducted in the same year, on those individuals between 18 and 74 years of age who expressed availability to participate. A sub-sample of 2,100 individuals were extracted from the original ESCA-2002 to perform the health examination, which included a physical exam, a supplementary interview and the collection of urine and blood samples (ESCA, 2002).

Questionnaires and study variables

The participants were asked to answer several questions through both the Health Survey and the Health Examination interviews (ESCA, 2002; Juncà *et al.*, 2003). Face to face interviews were conducted at home by trained personnel. These

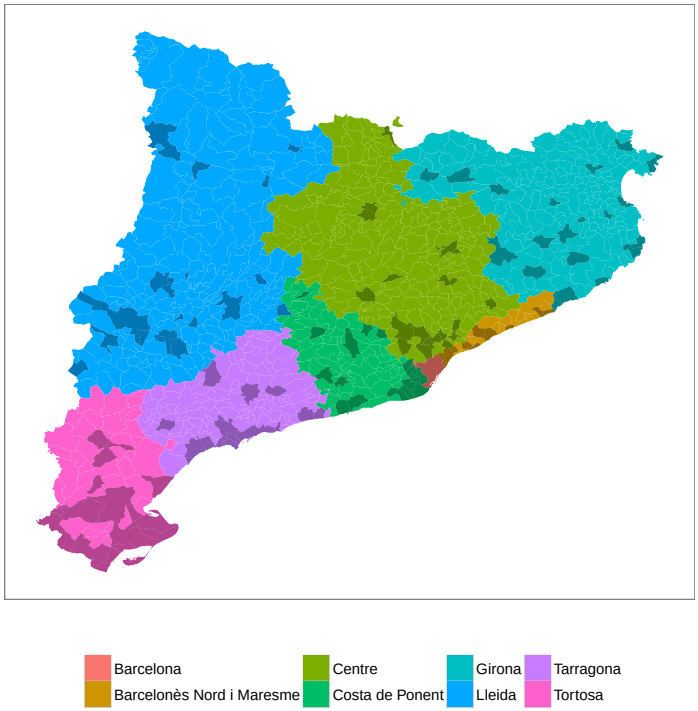

Figure 3.1: Map showing the eight health regions of Catalonia and the 122 selected municipalities, from the first sampling stage.

questionnaires provided information on socio-demographic variables such as age, sex, place of birth, parity in women, educational level, smoking habits and alcohol consumption, among others.

The variable *Age* was grouped in the same four categories previously used for calculating sample weights in the health examination, as follows: 18-29 years; 30-44 years; 45-59 years; and 60-74 years. Sometimes a cut-off age of 30 years was used to distinguish between young individuals (<30 years) and adults (>30

years).

The variable *Place of birth* comprised three categories: individuals who were born in Catalonia; individuals born outside Catalonia but within the Spanish state; and individuals born abroad. Occasionally, the first two categories were joined. Unfortunately, the database did not register the place of birth, although the questionnaires had asked it.

The variable *Parity* comprised four categories: women without offspring; 1 child; 2 children; and women with 3 or more children.

The variable *Educational level* comprised five categories: individuals without formal education (WFE); Primary school; Secondary school (first stage); Secondary school (second stage); and individuals with university degree.

The social class was estimated through the household occupation status, based on the Spanish Occupational Classification (SEE–SEMFYC, 2000).

Finally, trained nurses recorded the weight and height of individuals. Body mass index (BMI) was then calculated as the measured weight (kg) divided by the square of the measured height (m). The variable *BMI* was also grouped by categories, as follows: normal weight (<25 kg/m^2); overweight (25-30 kg/m^2); and obesity (>30 kg/m^2).

Serum samples

Blood samples were collected from blood vein using the vacuum extraction technique, between 9 and 11 am. The participants remained fasting at least 12 hours prior to the blood extraction. The tubes were labelled with the individual reference respondent to the ESCA, kept refrigerated, and then were delivered to the correspondent regional medical laboratory. There the tubes were centrifuged

and separated into aliquots, duly labelled and frozen. Subsequently, the samples were transported to a central laboratory.

One aliquot was used for the determination of various biochemical parameters, such as total cholesterol, triglyceride, HDL cholesterol and LDL cholesterol. Other biochemical parameters, such as insulin, iron or ferritin, were also determined.

The aliquots which had information on lipid concentrations (mainly cholesterol and triglyceride) together with at least 1 ml of serum were transported to the Institute of Environmental Assessment and Water Research (IDÆA) of the Spanish Council for Scientific Research (CSIC), in Barcelona city, in 2006, to undergo detailed chemical analyses by the author. These aliquots comprised a total of 919 serum samples, which constitute the basis for this research.

Subset of samples for POP analysis

The total of 919 samples underwent analysis of organochlorine compounds whereas 731 samples were used for the analysis of PBDEs. The smaller number of samples in the latter case was due to unforeseen technical complications in the analysis of PBDEs in 188 samples. In any case, these missing samples were random and hence do not affect the value and representativity of the results (Table 3.2).

Table 3.1 shows the number of individuals and municipalities included in the Health Survey (ESCA-2002), as well as in each subset of samples (OCs and PBDEs), by each health region and the totality of Catalonia.

Of the 122 municipalities selected in the Health Survey, the subset of samples with OC and PBDE analyses comprised 94 and 86 municipalities, respectively. Figure 3.2 shows the municipalities which had serum samples with OC and PBDE measured levels.

	Health Survey		OC subset		PBDE subset	
Health Region	N	Municip.	N	Municip.	N	Municip.
Barcelona	1400	1	147	1	146	1
Barcelonès Nord i Maresme	1047	13	80	10	72	10
Centre	1204	22	186	21	150	21
Costa de Ponent	1101	20	66	10	37	10
Girona	1001	20	62	11	25	6
Lleida	899	17	167	17	163	17
Tarragona	950	15	81	13	71	13
Tortosa	798	14	130	11	67	8
Catalunya	8400	122	919	94	731	86

Table 3.1: Sample design of the Catalan Health Survey (ESCA-2002) and the two subsets with OC and PBDE analysis, in relation to municipalities (*Municip.*) and individuals selected from each of the 8 health regions (*N*).

Socio-demographic determinants of the participants included in the study (in both OC and PBDE subsets) are shown in Table 3.2.

In general, similar proportions are found in both subset of samples, which indicates that there are no differences between them. There is a higher proportion of women (57% in the OC subset and 56% in the PBDE subset). The mean age of participants is 45 years (standard deviation = 15), ranging between 18 and 74 years. About 70% of the total population was born in Catalonia, 25-26% were of other Spanish origin and only 3% were born abroad. The body mass index (BMI) encompasses a large spectrum of cases, from extreme underweight (16.9 kg/m^2) to notable obesity (52.7 kg/m^2). Overweight affected 37-38% of the sample whereas obesity was found in 19-20%. Concerning parity, 27-28% of women had no descendants, 17-18% had one child and 55% were multiparous. Roughly one third of the studied individuals had a primary school degree, 46% had secondary degree and about 12% had a university degree. Almost half of

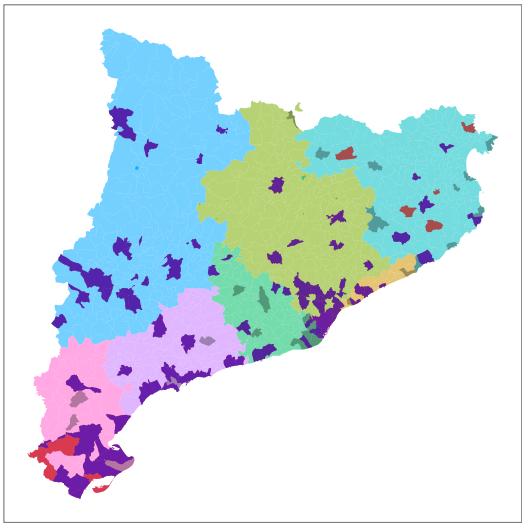

Figure 3.2: Map showing the municipalities included in the subset of samples with measured levels of OCs (n=919) and PBDEs (n=731). Blue colour indicates the municipalities with both OC and PBDE measured levels; Red colour indicates the municipalities with only OC measured levels; Grey colour indicates the municipalities without POP measurements included in the ESCA survey. The 8 health regions are also shown in the map with different colours.

the study population belonged to the most and medium affluent social classes (classes I to III), whereas about 8% belonged to the least affluent class (class V).

Regarding gender, the studied men were on average 3 years older than women and tended to have a slightly higher BMI. These differences were anyway accounted for in multivariate analyses. Apart from that, no significant differences in the distribution of place of birth, educational level or social class were found between the two gender groups (Table 3.2).

3.2 Determination of OCs and PBDEs

The analytical protocol for the determination of organohalogenated compounds requires the utmost accuracy. The following sections describes the analytical protocol that was followed to detect the compounds with a maximum of efficiency and to minimise the contamination of the samples, as well as the methodology used in the preparation of the standards and the gas chromatographic and mass spectrometric techniques employed.

Material, solvents and standards

Cleaning material

Laboratory material (*e.g.* glass tubes) were initially washed with soap and then cleaned by ultrasound sonication using an alkaline detergent (Extran, AP-13) for 15 minutes. Subsequently, the material was rinsed with Milli-Q water and acetone, and then it was left to dry in the oven at 80°C. After that, all the material (including that which can not be cleaned; *e.g.* glass Pasteur pipettes) was heated overnight in a muffle furnace at 400°C.

Sodium sulphate and glass wool were cleaned in soxhlet with a solution of dichloromethane:*n*-hexane (1:1, v/v) for 24 hours. Later, they were left to dry under ultraviolet light. Sodium sulphate was additionally heated in the muffle furnace at 400°C.

Solvents

The solvents used in the analytical methodology were isooctane (SupraSolv), *n*-hexane, dichloromethane and acetone. All them were purchased from Merck (Darmstadt, Germany) for organic trace analysis.

Concentrated sulphuric acid (*conc.* H_2SO_4) and sodium sulphate were also from Merck (Darmstadt, Germany).

Glass wool was purchased at Panreac (Barcelona, Catalonia, Spain).

Standards

The analytical standards are used to measure several parameters.

The recovery standards tetrabromobenzene (TBB) and PCB-209 measured the analyte losses by sample handling during the extraction process. Both of them were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Injection standards (PCB-142, BDE-118 and $^{13}C_{12}$-BDE-209) controlled the injection in the gas chromatograph. PCB-142 was also purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and both BDE-118 and $^{13}C_{12}$-BDE-209 were purchased from Cambridge Isotope Laboratories, Inc. (CIL; Andover, MA, USA).

The mixed standard solutions for OCs and PBDEs were prepared at different concentrations to obtain the calibration straight lines. For the OC analysis, standards of hexachlorobenzene (HCB), hexachlorocyclohexanes (α-, β-, δ-, γ-HCHs), pentachlorobenzene (PeCB), 2,4'-DDT, 4,4'-DDT and their metabolites (2,4'-DDD, 4,4'-DDD, 2,4'-DDE and 4,4'-DDE) and polychlorinated biphenyls (PCB congeners' 28, 52, 101, 118, 138, 153 and 180) were all purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The *Polybrominated Diphenyl Ether Predominant Congener Mixture* for the analysis of PBDEs was purchased from Cambridge Isotope Laboratories, Inc. (CIL; Andover, MA, USA). The solution included the following PBDE congeners': 17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, 190 and 209.

Experimental method: Liquid-liquid extraction

Serum samples were thawed and shaken through a vortex to homogenize the content. One milliliter of sample was introduced into 10 ml centrifuge tubes and 25 μl of a recovery standard solution containing TBB and PCB-209 (100 ng/ml) was added. Then, 3 ml of *n*-hexane and 2 ml of *conc.* H_2SO_4 were added, mixed in a vortex (ca. 1,500 rpm, 30 s) and centrifuged (ca. 3,500 rpm, 5 min). After centrifugation, the acid was mixed with the serum, but separated from the hexane.

The supernatant *n*-hexane layer was transferred into a second centrifuge tube using a Pasteur pipette. Further, *n*-hexane (2 ml) was added to the first tube containing the H_2SO_4/sample mixture, stirred and then centrifuged. This last step was repeated again yielding a combined extract of 7 ml of *n*-hexane, to which 2 ml of *conc.* H_2SO_4 were added. Then, the suspension was mixed (vortex mixer, ca. 1,500 rpm, 60 s), centrifuged (3,500 rpm, 10 min), and the supernatant *n*-hexane was transferred through a column of glass wool and sodium sulphate to a conical bottomed, graduated tube. The sodium sulphate retains traces of sulphuric acid, which could harm the rest of the process and damage the chromatographic capillary column.

The *n*-hexane extracts collected in the conical tube were reduced to near dryness under a stream of high purity compressed nitrogen and an injection standard solution (PCB-142 in isooctane, 10 μl) was added. Then, the solutions were quantitatively transferred to GC vials using four 25 μl rinses of isooctane.

After the instrumental analysis of organochlorine compounds by GC-ECD (see next section), vial samples were re-evaporated under a nitrogen stream. Then, 20 μl of BDE-118 and 10 μl of $^{13}C_{12}$-BDE-209 were added as injection standards.

Instrumental analysis

Chromatographic conditions for the analysis of OCs

Organochlorine compound analysis was performed by gas chromatography with electron capture detection (GC-ECD, Agilent Technologies 6890N, Palo Alto, CA, USA) (Figure 3.3). The separation was achieved with a DB-5 column (lenght of 60 m, internal diameter of 0.25 mm; J&W Scientific, Folsom, CA, USA), coated with 5% diphenylpolydimethylsiloxane (0.25 μm film thickness).

Figure 3.3: Gas chromatograph coupled to a electron capture detector (GC-ECD), with automatic injector.

Helium and nitrogen were, respectively, the carrier gas (at a constant flow of 1.5 ml/min) and the make-up gas (at 60 ml/min). Injection (2 μl) was in splitless mode at 280°C. The ECD was set at 310°C. The oven temperature program started at 90°C, which was maintained for 2 minutes. Then, it ramped to 130°C at 15°C/min and to 290°C at 4°C/min, with a final holding time of 20 minutes. Total run time was 64.67 minutes.

The resulting separation of organochlorine compounds and recovery and injection standards is shown in Figure 3.4. The compounds were identified by retention time (RT).

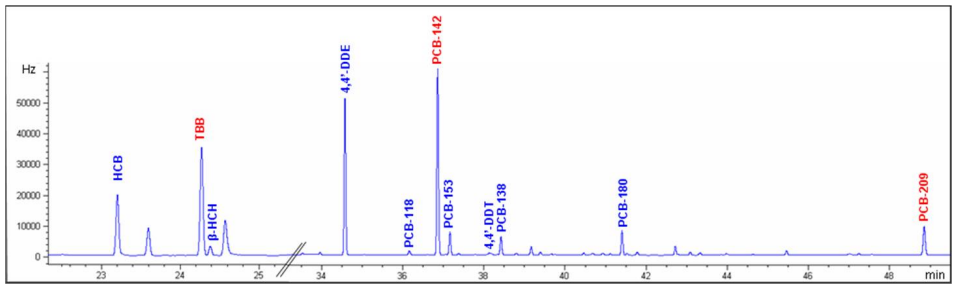

Figure 3.4: Chromatogram of a serum extract showing the peaks of major analytes (HCB, β-HCH, 4,4'-DDE, PCB-118, PCB-153, 4,4'-DDT, PCB-138 and PCB-180), the recovery standards (TBB and PCB-209) and the injection standard (PCB-142). The abscissa axis measures the eluting time (in min) and the ordinate axis shows the response (in Hz).

Chromatographic conditions for the analysis of PBDEs

Polybrominated diphenyl ethers were determined by gas chromatography (GC, Agilent Technologies 6890N) coupled to a mass spectrometer (MS, Agilent Technologies 5975N) operating in negative chemical ionization mode (NICI). The instrument was equipped with a low bleed SGE-BPX5 MS fused silica capillary column (length of 15 m, internal diameter of 0.25 mm, 0.10 μm film thickness).

Helium was used as the carrier gas whereas ammonium was the ionization gas for the MS analysis. Injection (2 μl) was performed in splitless mode. The initial oven temperature was held at 90°C, which was maintained for 1.5 minutes, followed by a first ramp up to 200°C at 20°C/min, a second ramp up to 275°C at 5°C/min and a third ramp up to 300°C at 30°C/min, with a final holding time of 10 minutes. Total run time was 38 minutes.

PBDE identification was based on retention time (RT) and mass spectral information, performed in selected ion monitoring scanning mode (SIM), using

the bromine ions m/z 79/81 for all congeners, except m/z 487/489 for BDE-209 and m/z 495/497 for $^{13}C_{12}$-BDE-209.

Quality assurance

To assess the accuracy of the analytical protocol and the methodology used in the research, some parameters were accurately revised. This quality assurance of the methodology is explained next.

Analytical blanks

A total of 94 procedural blanks were analysed for every set of 10 to 15 serum samples. Blanks were used for both the measuring of the existing contamination of the laboratory environment, including the material and solvents, and for the calculation of the limits of detection and quantification for the PBDE compounds (details are explained next). Overall, the concentrations of organohalogenated compounds found in blanks corresponded to small contamination of the analytical process and not bias the final results.

Recovery of analytical standards

As explained before, a known concentration of the recovery standards is added at the beginning of the extraction process. The calculation of the recovery function is performed not only to test the accuracy of the method, but also to correct for analyte losses.

For the analysis of OCs, mean recoveries (± SD) of TBB and PCB-209 were 100.6 ±20.5% and 94.5 ±19.7%, respectively. Relative responses of the injection standard PCB-142 were used to correct for instrumental injection variability,

and this value was also corrected by the recovery function. The use of injection standard to correct for volume allows differentiating between corrections due to analyte losses by sample handling and volume variations in the final solvent rinsings for sample introduction into the chromatographic vials. Thus the recovery standards are also corrected by the injection standard. Consequently, the % recovery observed from TBB and PCB-209 area measurements reflects the potential analyte losses independently of possible volume variations.

For the analysis of PBDEs, mean recovery (± SD) of PCB-209 was 64 ±22%. Unlike the analysis of OCs, the recovery of this standard was used directly for correction of all PBDE concentrations in each sample, independently of internal standards BDE-118 and $^{13}C_{12}$-BDE-209.

AMAP Ring Test for POPs

The analytical protocol has been validated by the analysis of Proficiency Testing Materials (PTMs) obtained through the participation in the AMAP Ring Test for Persistent Organic Pollutants in Human Serum (Centre de Toxicologie. Institut National de Santé Publique du Québec, 2012).

Since 1991, the Arctic Monitoring and Assessment Program (AMAP, 2013) has organized a comprehensive surveillance of the human maternal blood in the Arctic, based on studies with standardized protocols for collecting and analysing samples. Within the context of AMAP, the participating laboratories expressed their need to ensure comparability of the data. In 2001, an international guarantee of analytical quality control for the analysis of several analytes in blood serum was created. The program is open to all interested laboratories and IDÆA–CSIC has been participating for many years. Participants receive three runs per year, each containing three PTMs, which are analysed with the same analytical protocol used in the laboratory.

The analytical method for the determination of organohalogenated compounds in human serum samples performs satisfactorily under the AMAP standards since the results usually range within 20 % of the consensus values.

Limits of detection and quantification

Limits of detection and quantification are defined as follows:

- LIMIT OF DETECTION (LD): Value below which it is possible to find the concentration of the analyte.
- LIMIT OF QUANTIFICATION (LQ): Value below which it is possible to detect the analyte, but not quantify the exact concentration.

The compounds that are below the LD are usually specified as "not detected" (nd or <LD) and the mid-value of this limit is assigned. When the concentration of a compound is detected but under the quantification threshold (that is, between the LD and the LQ), it is considered as "not quantifiable" (nq or <LQ) due to its low concentration; then, the mid-value of the limit of detection is assigned.

For the analysis of OCs, limits of detection and quantification were calculated from the analysis of Proficiency Testing Materials and the study of the signal/noise (S/N) ratio of chromatograms obtained from GC-ECD injection, as the mean of S/N ratio plus 3 and 5 times the standard deviation, respectively. Detection limits ranged between 0.0023 ng/ml and 0.024 ng/ml, depending on the OC compound (Table 3.3).

For the analysis of PBDEs, LD and LQ were calculated from procedural blanks, as the mean of the concentrations plus 1 and 2 times the standard deviation, respectively. Detection limits ranged between 0.0018 ng/ml and 0.0089 ng/ml, depending on the BDE congener (Table 3.4).

3.3 Statistical analysis

Data analysis and corresponding graphics were performed using the statistical software R (R Development Core Team, 2012).

The analysis has included descriptive statistics and inference (mostly linear regression and non-parametric analysis, as well as hierarchical modelling).

Statistical packages

Several R packages have been used for specific analysis, as follows:

- Chemical analysis: `chemCal` (Ranke, 2012), `quantchem` (Komsta, 2012)
- Data management and analysis: `lme4` (Bates *et al.*, 2012), `arm` (Gelman and Su, 2013), `lmtest` (Zeileis and Hothorn, 2002), `gtools` (Warnes, 2012), `MASS` (Venables and Ripley, 2002), `Hmisc` (Harrell, 2012), `plyr` (Wickham, 2011), `reshape` (Wickham, 2007)
- Epidemiological analysis: `Epi` (Carstensen *et al.*, 2013), `epicalc` (Chongsuvivatwong, 2012)
- Graphical display of results, including maps: `ggplot2` (Wickham, 2009), `coefplot` (Lander, 2012), `ggmap` (Kahle and Wickham, 2012), `maps` (Becker and Wilks., 2012), `maptools` (Bivand and Lewin-Koh, 2013), `rworldmap` (South, 2012)

Lipid adjustment

Epidemiological studies usually describe the concentrations of OCs and PBDEs in human samples by normalizing them by the total lipids (TL) of each individual.

Total lipids can be estimated from the concentrations of cholesterol and triglyceride, which in this research were determined with enzymatic methods, using one of the aliquots of serum provided by the Health Examination (see section 3.1 above).

The total lipid sample was calculated using the equation 3.1, by Phillips *et al.* (1989).

$$TL = (TC * 2.27) + TG + 62.3 \tag{3.1}$$

TL: Total Lipids; TC: Total Cholesterol; TG: Triglyceride.
Concentrations are expressed in mg/dl.

The concentrations of OCs and PBDEs were individually corrected by total lipid content from equation 3.1, by dividing the crude serum concentration (in ng/ml) by TL. The concentrations were then expressed in nanograms of analyte per gram lipid (ng/g lipid).

The concentrations of OCs were only corrected by lipids in certain circumstances (*e.g.* compare the concentrations with other studies). In contrast, PBDE concentrations have been always corrected by lipids and presented in this way.

Normalizing data

The concentrations of the analysed compounds do not follow a normal distribution. Two approaches were adopted to address this: (1) the use of non-parametric techniques, and (2) the use of logarithmic transformation. Non-parametric analyses were used first, but then discarded in favour of the logarithmic transformation of the data because the latter solves the non-normality distribution in a simpler way. In addition, logarithmic transformation allows using the usual standard techniques in data analysis, such as linear regression, which ease the data analysis and interpretation of the results.

Presenting data

Following advice from statisticians on *turning tables into graphs*, the results and their substantial interpretations are generally presented using graphical figures (Gelman *et al.*, 2002).

	OC subset			PBDE subset		
	Both sexes n (%)	Men n (%)	Women n (%)	Both sexes n (%)	Men n (%)	Women n (%)
All participants	919 (100)	399 (43)	520 (57)	731 (100)	324 (44)	407 (56)
Age (years)						
18-29	173 (19)	65 (16)	108 (21)	138 (19)	55 (17)	83 (21)
30-44	272 (30)	117 (29)	155 (30)	220 (30)	97 (30)	123 (30)
45-59	287 (31)	127 (32)	160 (31)	228 (31)	101 (31)	127 (31)
60-74	187 (20)	90 (23)	97 (18)	145 (20)	71 (22)	74 (18)
Place of birth						
Catalonia	661 (72)	291 (73)	370 (71)	518 (71)	235 (73)	283 (70)
Rest of Spain	231 (25)	95 (24)	136 (26)	189 (26)	79 (24)	110 (27)
Abroad	27 (3)	13 (3)	14 (3)	24 (3)	10 (3)	14 (3)
Body mass index						
Normal weight	395 (43)	132 (33)	263 (51)	314 (43)	108 (33)	206 (50)
Overweight	344 (37)	191 (48)	153 (29)	276 (38)	155 (48)	121 (30)
Obesity	180 (20)	76 (19)	104 (20)	141 (19)	61 (19)	80 (20)
Parity						
0			144 (28)			110 (27)
1			88 (17)			72 (18)
2			184 (35)			145 (35)
≥3			104 (20)			80 (20)
Education						
WFE	146 (16)	58 (14)	88 (17)	111 (15)	44 (14)	67 (16)
Primary	242 (26)	203 (26)	139 (27)	198 (27)	82 (25)	116 (29)
Secondary (I)	227 (25)	98 (25)	129 (25)	177 (24)	83 (26)	94 (23)
Secondary (II)	192 (21)	94 (24)	98 (19)	158 (22)	76 (23)	82 (20)
University	112 (12)	46 (11)	66 (12)	87 (12)	39 (12)	48 (12)
Social class						
I (most affl.)	101 (11)	38 (10)	63 (12)	73 (10)	31 (10)	42 (10)
II	94 (10)	41 (10)	53 (10)	85 (12)	36 (11)	49 (12)
III	229 (25)	112 (28)	117 (23)	184 (25)	95 (29)	89 (22)
IV	420 (46)	177 (44)	243 (47)	328 (45)	137 (42)	191 (47)
V (less affl.)	75 (8)	31 (8)	44 (8)	61 (8)	25 (8)	36 (9)

Table 3.2: Summary of socio-demographic characteristics of the population studied for each subset of POPs, showing the number of individuals (n) and the percentages (%) in each group.

	LD	LQ
PeCB	0.014	0.041
HCB	0.011	0.033
α-HCH	0.022	0.065
β-HCH	0.020	0.060
δ-HCH	0.016	0.049
γ-HCH	0.024	0.071
2,4'-DDT	0.011	0.032
4,4'-DDT	0.017	0.050
2,4'-DDD	0.0041	0.012
4,4'-DDD	0.0079	0.024
2,4'-DDE	0.0035	0.011
4,4'-DDE	0.016	0.047
PCB-28	0.017	0.051
PCB-52	0.0027	0.0080
PCB-101	0.0023	0.0069
PCB-118	0.014	0.042
PCB-138	0.014	0.041
PCB-153	0.014	0.041
PCB-180	0.010	0.029

Table 3.3: Limits of detection (LD) and limits of quantification (LQ) for OCs (in ng/ml).

	LD	LQ
BDE-17	0.0019	0.0029
BDE-28	0.0018	0.0027
BDE-47	0.0048	0.0072
BDE-66	0.0024	0.0036
BDE-71	0.0024	0.0036
BDE-85	0.0018	0.0026
BDE-99	0.0038	0.0056
BDE-100	0.0028	0.0042
BDE-138	0.0019	0.0028
BDE-153	0.0018	0.0027
BDE-154	0.0018	0.0028
BDE-183	0.0020	0.0030
BDE-190	0.0023	0.0035
BDE-209	0.0089	0.013

Table 3.4: Limits of detection (LD) and limits of quantification (LQ) for PBDEs (in ng/ml).

4 Results

As mentioned earlier, this doctoral dissertation is a compendium of research on environmental epidemiology, focusing on two major classes of persistent organic pollutants –OCs and PBDEs– in the population of Catalonia. The results comprise the following research:

- A methodological innovation to better define the limits of detection and quantification of OCs in human serum (Article 1 – published in *Analytical and Bioanalytical Chemistry*, 2010).
- An analysis that shows sexual divergence in the accumulation patterns of POPs (Article 2 – intended for *Science*; otherwise to be submitted to *Environmental Health Perspectives*).
- An inquiry on the accumulation patterns for PBDEs, showing an inverse age-dependence trend (Article 3 – published in *Environment International*, 2013).
- An assessment on the influence of distance in the levels of accumulation of POPs, or their air-borne effects, comparing data between the general population and the population living around a POP-emitting chemical factory (Article 4 – to be submitted to *Environmental Health Perspectives* or *Environmental Pollution*).

ARTICLE 1

Use of proficiency testing materials for the calculation of detection and quantification limits in the analysis of organochlorine compounds in human serum

Mercè Garí and Joan O.Grimalt
Analytical and Bioanalytical Chemistry 397(3):1383 -1387(2010)
DOI10.1007/s00216-010-3638-3

Article 2

Sexual divergence in the accumulation of persistent organic pollutants in humans throughout life

Mercè Garí, Joan O. Grimalt and Jordi Sunyer
To be submitted

Sexual divergence in the accumulation of persistent organic pollutants in humans throughout life

Mercè Garí[*], Joan O. Grimalt[*], and Jordi Sunyer[**]

[*]Department of Environmental Chemistry. Institute of Environmental Assessment and Water Research (IDÆA-CSIC). Barcelona, Catalonia, Spain.
[**]Centre for Research in Environmental Epidemiology (CREAL). Barcelona, Catalonia, Spain.

Abstract

A broad health study of the population of Catalonia, in South-West Europe, has provided unexpected insights in the trends of human accumulation of persistent organic pollutants (POPs). There is a sexual divergence in the accumulation of HCB and β-HCH and, to a lower degree, for 4,4'-DDT, 4,4'-DDE and PCB-118, being higher in women than in men through age. This interactive effect is not observable for the other PCBs and PBDE compounds, although men show significant lower concentrations of PCB-180 and BDE-153. All these observations are further corroborated in analysis of both a highly exposed population (that of Flix, a township in Catalonia itself that hosts an industrial chemical plant) and another general population (that from the U.S., based on the national survey NHANES). In addition, increases in body mass index are also related to a higher accumulation of HCB and β-HCH, but only significant among men, revealing an additional interactive effect between sex and BMI. On the other hand, increases in BMI results in lower concentrations of precisely the compounds which were much accumulated in men: PCB-180 and BDE-153. We propose the hypothesis that such sexual difference, which is exacerbated through age, as well as the particular BMI trend, are related to the higher volatility of the concerned compounds, since a significant correlation between sexual divergence and this chemical property is clearly found.

Keywords: Organochlorine compounds; Polybromodiphenyl ethers; Human serum; Body mass index; Physical-chemical properties; Octanol-air partition coefficient; Human metabolism

Introduction

Human exposure to persistent organic pollutants (POPs) has been of increasing environmental and public health concern over the past decades, due to their ubiquitous distribution, high persistence in the environment (Simonich *et al.*, 1995) and adverse health effects (Grimalt *et al.*, 1994; Ribas-Fito *et al.*, 2001; Hertz-Picciotto *et al.*, 2005; Legler, 2008; Maervoet *et al.*, 2007). Some of them are accordingly being banned or restricted, yet they are still found in human tissues. This kind of compounds are metabolised or excreted very

slowly by the organism, as their half-lives are estimated in the range of 3 up to 15 years (Shirai and Kissel, 1996; Grandjean *et al.*, 2008; Ritter *et al.*, 2011). In this sense, the rates of intake are higher than that of their elimination processes (Yu *et al.*, 2011), although the precise accumulation and detoxification patterns in humans remain partially unknown (Kelly *et al.*, 2004; Ritter *et al.*, 2009). This research deepens in the human accumulation of these compounds, looking at differences in parameters such as sex, age and body mass index (BMI), and regarding the role of human metabolism to better understand the detoxification dynamics.

The most abundant POPs in human tissues are hexachlorobenzene (HCB), the beta-isomer of hexachlorocyclohexane (β-HCH), 4,4'-dichlorodiphenyltrichloroethane (4,4'-DDT) and its major metabolite 4,4'-dichlodiphenyldichloroethylene (4,4'-DDE), and polychlorobiphenyls (PCBs). In addition, polybromodiphenyl ethers (PBDEs) are of increasing concern since their concentrations are increasing quickly in both human and environmental samples (Hites, 2004).

POPs are principally incorporated into humans through diet (Duarte-Davidson and Jones, 1994; Domingo, 2004), but they are partly excreted in faeces (To-Figueras *et al.*, 2000), since they are not fully absorbed in the intestinal tract (Kreuzer *et al.*, 1997; Moser and McLachlan, 2001). There are minor routes of exposure, particularly inhalation. Assessments of the Flix population, which is located near an electrochemical factory with high HCB airborne concentration, showed the highest levels of such compound ever found in humans, suggesting that it was substantially incorporated into the body through respiration (Sala *et al.*, 1999b). Similarly, inhalation of dust has been shown to be a considerable pathway for PBDE exposure (Lorber, 2008).

POPs are stored in fatty tissues as consequence of their lipophilicity and high stability to chemical degradation. The observed accumulation of these compounds in human tissues suggests that humans have an inefficient metabolism to excrete them, which favours a continued bioaccumulation through age. Hepatic metabolism plays an important role in the elimination of many chemicals (Dayton *et al.*, 1983). Metabolic biotransformation may allow the release of POPs and their metabolites into bile or plasma, allowing then their elimination by urine or faeces (Moser and McLachlan, 2001; Jandacek and Tso, 2007). For instance, HCB metabolites have been found in urine and faeces, suggesting an enzymatic conversion of this compound in the liver (To-Figueras *et al.*, 1997, 2000). There is a greater evidence of excretion of OCs in women, which is associated with their reproductive activities. For instance, during gestation, OCs are transferred from the mother to the foetus through the placenta (Sala *et al.*, 2001) and, once the child is born, she continues transferring and somehow excreting these compounds through breastfeeding (Ribas-Fitó *et al.*, 2003). Therefore, pregnancy, child delivery and lactation represent an excretion means for women, decreasing their OC burden. Another detoxification pathway may be the exhalation of some OCs by the respiratory system, which this research proposes as hypothesis, considering certain physical-chemical properties of the OCs, mainly their octanol-air and octanol-water partition coefficients (Koa and Kow, respectively). In particular, OCs having low Koa values, such as HCB and β-HCH, which are the most volatile ones, could be exhaled (Carrizo *et al.*, 2006). In essence, respiration is simultaneously a possible way of incorporating HCB, as mentioned above and as suggested by the study of the Flix population (Sala *et al.*, 1999a), and a way of eliminating HCB and similar compounds with higher volatility.

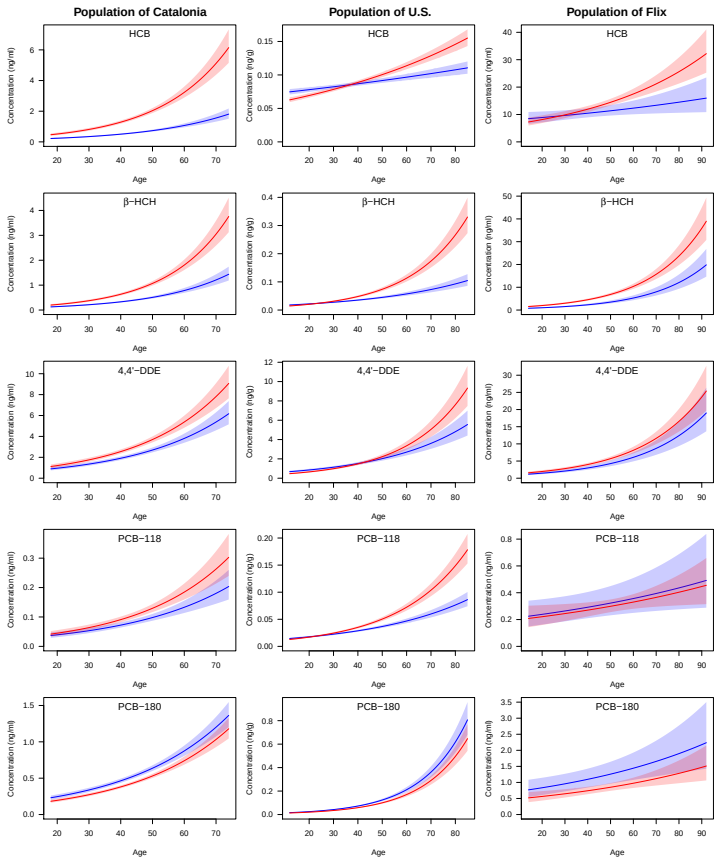

Figure 1: Comparison between the accumulation of certain OCs (HCB, β-HCH, 4,4'-DDE, PCB-118 and PCB-180) by age and sex (red colour for women and blue colour for men) in the three analysed populations: Catalonia (left panel), U.S. (middle panel) and Flix township (right panel). There is a very pronounced accumulation of certain OCs in women through age, compared to men. This is notably observed for HCB and β-HCH, as well as, to some degree, for 4,4'-DDE and PCB-118. In contrast, PCB-180 shows higher levels among men, with statistically significant results.

The present study explores further these issues by examining serum-level POP concentrations in the population of Catalonia, which has been studied in the context of both a general population survey (number of analyses=919), and a specific case of the Flix township, which is situated close to an electrochemical factory that emited large amounts of HCB into the atmosphere (n=606) (Grimalt et al., 1994). The results are then compared to a major database of the general population of the U.S. (n=1961, data publicly available on the website from the NHANES study carried out in 2003-2004 (NHANES, 2004)) (Table 1 on SI).

Results and Discussion

The results show a very pronounced accumulation of certain OCs in women through age, compared to men. This is notably observed for HCB and β-HCH, resulting in a significant interactive effect between age and sex. This effect is not found in 4,4'-DDT, 4,4'-DDE and PCB-118, although women show much higher levels of these compounds than

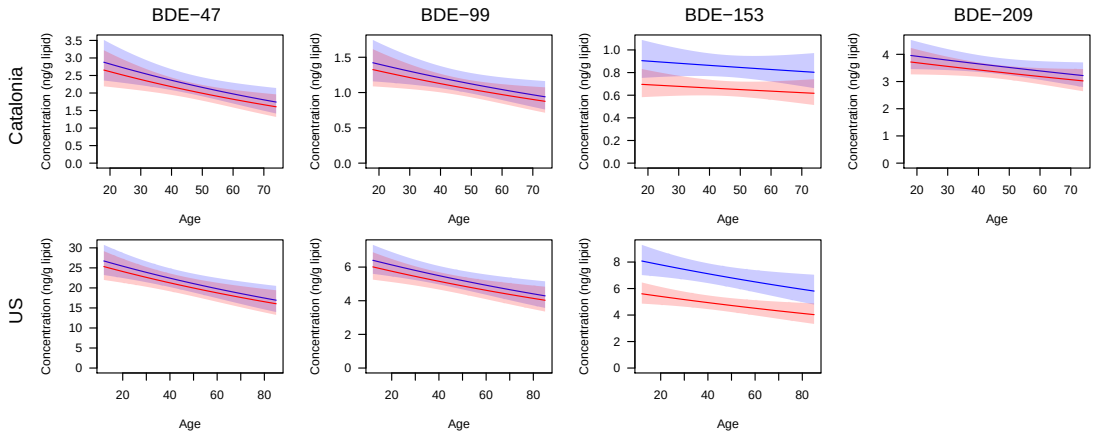

Figure 2: Comparison between the accumulation of certain PBDEs (BDE-47, BDE-99, BDE-153 and BDE-209) by age and sex (red colour for women and blue colour for men) in the population of Catalonia (top panel) and in the population of the U.S. (bottom panel). Concentrations of decabromodiphenyl ether were only available in the population of Catalonia since the NHANES study did not analysed it. Unlike OCs, among PBDEs there are no differences in the accumulation patterns regarding the interactive age and sex effect, although men have significant higher concentrations of BDE-153 than women, alike PCB-180.

men. On the other hand, the rest of PCBs and PBDE compounds do not show sexual differences, except for PCB-180 and BDE-153, which are much more accumulated in men. For comparative purposes, Figure 1 shows the patterns of accumulation of five representative OCs (HCB, β-HCH, 4,4'-DDE, PCB-118 and PCB-180) in the three analysed populations (Catalonia, Flix and the U.S.). Data for the rest of the OCs (4,4'-DDT, PCB-138 and PCB-153) are shown on Supporting Information (Figure 1 in SI). The pattern of interactive age and sex effect on OC accumulation is not only observed in the general populations of both Catalonia and the U.S., but also in the highly exposed population of the Flix township, thus indicating a trend found in both a general and a specific (i.e. chemically exposed) population. Figure 2 shows the accumulation pattern of four representative PBDEs (BDEs 47, 99, 153 and 209) in the two general populations, although the decabromodiphenyl congener was only available in the Catalan study.

An additional analysis of the body mass index (BMI) reveals an unexpected pattern of accumulation for certain OCs, found in men but not in women. Precisely HCB and β-HCH show an additional and statistically significant interactive effect between sex and BMI, resulting in a substantial difference in the accumulation of these compounds by BMI in men. Specifically, there is a higher accumulation of HCB and β-HCH in men with increasing BMI, not observed in women (Figure 3). On the other hand, precisely the compounds which are much more accumulated in men (PCB-180 and BDE-153) exhibit an inverse trend with increasing BMI. Standardized beta-coefficients from the multivariate regression models accounting for age, sex and BMI, as well as the two interactive effects (age*sex and sex*BMI), are shown in Table 2 on SI.

In summary, this research clearly reveals an interactive age and sex effect on the accumulation of certain OCs, which are accumulated much more pronouncedly in women

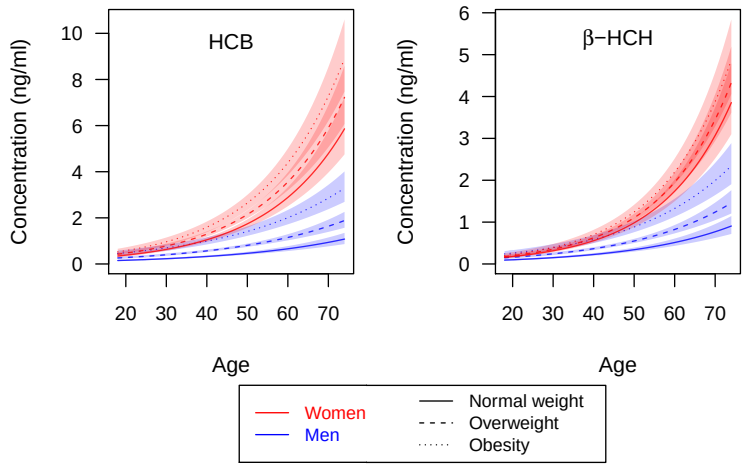

Figure 3: Comparison between the accumulation of HCB and β-HCH by age, sex and body mass index. Obese and overweight men accumulate more of these OCs than normal weight men, while the differences in BMI among women are not statistically significant. This reflects an additional interactive effect between sex and BMI.

through age. There are a few allusions to this interactive effect in the literature: Dirtu *et al.* (2006) observed a better correlation with age for females compared to males for several compounds, including HCHs, DDTs and PCB-118, Thomas *et al.* (2006) found a better adjustment with age for males for PCBs (statistically significant results) and for females for HCB, β-HCH and 4,4'-DDE; Sala *et al.* (1999a) reported that "an interaction between age and sex was found for HCB concentrations"; and finally, Patterson *et al.* (2009) also found an interaction effect between age and sex for HCB and DDE. Nevertheless, those studies have not examined in detail this effect, neither the additional BMI effect among men.

The results raise four paradoxical issues. First, women are the bearers of the higher accumulations with age, which is contrary to what may be expected, since women have additional means to excrete OCs, namely pregnancy, child delivery and lactation (Sala *et al.*, 2001; Ribas-Fitó *et al.*, 2003). However, as suggested by Verner *et al.* (2008) in a physiologically based pharmacokinetic model (PBPK) for assessing the lifetime exposure to some POPs (mainly HCB, PCB-153 and PCB-180), women blood concentrations at 55 years old could be reached despite totally different lifetime physiologic profiles. In other words, despite lactation periods and weight changes, when women are more likely to decrease their OC concentrations, the model shows that women may reach a similar concentration in a mature age. Second, the interactive effect on sex and age seems to apply only for certain OCs, but not for all. Third, the additional interactive effect on sex and BMI only appears in these OCs which also show the interactive effect on age and sex. And finally, the inverse BMI trend is only found in these compounds which are significantly much more accumulated in men (*e.g.* PCB-180 and BDE-153). To further explore these paradoxes, this study considers the potential role of the physical-chemical properties of the OCs and some features of human physiology, particularly sexual dimorphism in the cardiopulmonary system.

The OCs that clearly respond to the in-

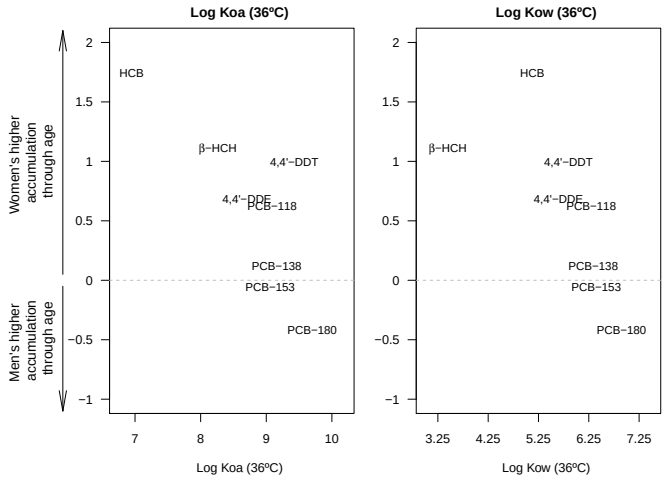

Figure 4: Relationships between octanol-air (Koa) and octanol-water (Kow) partition coefficients (in logarithmic scale) and the difference between age and sex accumulation. The OCs that clearly respond to the interactive effect are precisely the most volatile ones. The interactive age and sex effect appears to be correlated with the degree of volatility of the compounds: the women's higher accumulation is extremely pronounced in the most volatile OCs (HCB and β-HCH), it is moderately perceived in the moderately volatile OCs (4,4'-DDT, 4,4'-DDE and PCB-118), and it is absent in the least volatile OCs (PCB-138, PCB-153 and PCB-180).

teractive effects are precisely the most volatile ones. In other words, the interactive effects appear to be correlated with the degree of volatility of the compounds: the women's higher accumulation is extremely pronounced in the most volatile OCs (HCB and β-HCH), it is moderately perceived in the moderately volatile OCs (4,4'-DDT, 4,4'-DDE and PCB-118), and it is absent in the least volatile OCs (PCB-138, PCB-153 and PCB-180) and PBDE compounds. On the other hand, the men's higher accumulation with BMI is notable in the most volatile OCs (HCB and β-HCH), while it is absent in the other compounds. The relevance of volatility in the patterns of OC accumulation regarding age, sex and body mass index has never been established before and would deserve attention in similar public health studies in the future. This research proposes the hypothesis that the patterns of OC accumulation are linked to human physiology. In particular, respiration may have a higher role in the excretion of the most volatile OCs than anticipated, while this is also linked to the fact that men have more intense cardiopulmonary activity across life that allows them to excrete higher amounts of the most volatile OCs. However, obese men would have less ability to excrete these compounds due to their lower metabolic rate than men with a normal weight range, resulting in a higher accumulation almost similar to women.

To sustain these hypothesis, this study has conducted several comparisons between OC physical-chemical properties (octanol-air (Koa) and octanol-water (Kow) partition coefficients) and the age and sex variables included in the regression models (including the interactive effect). The results show a strong correlation between Koa (as indicator of OC volatility) and sex-related factors (Figure 4). This reinforces the role of the human metabolism, through respiration, in the excretion of certain OCs, the volatile ones.

The role of respiration in excreting chem-

ical compounds has been already mentioned in the literature (*i.e.* the exhalation of volatile compounds has been suggested in infants by Daston *et al.* (2004)). More specifically, Carrizo et al. proposed that the most volatile OCs, such as HCB, could also be exhaled (Carrizo *et al.*, 2006). The fact that women tend to have higher concentrations of these volatile OCs, despite they have the possibility to excreting them by reproductive factors (gestation and lactation) indicates the relevance of respiration in their excretion. In a controlled experiment in the inhalation toxicokinetics of a volatile solvent (2-propanol), sexual differences in lung metabolism were found (Ernstgård *et al.*, 2003). In fact, anatomical and physiological differences between women and men do exist. Women tend to have a minor development of the rib cage, hence having lower parameters in relation to the airways diameter, lung volume and blood/air diffusion surface in the alveoli (Chen and Kuo, 1989; Bellemare *et al.*, 2003). Moreover, women have a lower concentration of haemoglobin and minor number of erythrocytes in blood (Huxley, 2007), thus having a lower blood oxygen-carrying capacity (Wallin *et al.*, 2010). In essence, women have a lower cardiopulmonary metabolism than men, which simultaneously declines through time (Chen and Kuo, 1989). This fact may explain a lower ability of women than men to exhale the most volatile OCs by respiration, hence increasing their body burden, which is further exacerbated through age.

The interactive sex and BMI effect reinforces the role of respiration in the excretion of certain OCs since obesity is associated with reduced lung volumes and alterations in respiratory mechanics (DeLorey *et al.*, 2005; Chlif *et al.*, 2009). However, the fact that only men show increasing concentrations of volatile OCs with BMI could raise in differences in fat distributions among sexes. Women have the tendency to deposit fat in the hips and buttocks whereas men tend to deposit fat more centrally and hence to have a greatest impact on diaphragm position and lung volume regulation (Harik-Khan *et al.*, 2001). Therefore, the BMI increase among women have comparatively less relevance than in men for the accumulation of the most volatile OCs, as Figure 3 demonstrates.

Concerning the PCBs, the results also indicate that men and women show different blood congener profiles, and that this may be equally correlated to the degree of volatility of PCB compounds. With increasing the degree of chlorination (which means less volatility), the levels of a given PCB become higher in men, to the extent that men have higher concentrations of the PCB-180, which is the most chlorinated (and hence the least volatile) among the studied congeners, as shown in Figure 1. This indicates again that respiration plays a role in the excretion, but that such role is ineffective beyond certain levels of volatility. It is to be noted that the inverse BMI trend is also found in the most chlorinated PCBs (Table 2 on SI), revealing a similar pattern in BMI and sex accumulation. The majority of population-based studies report higher levels of these compounds in men, but they suggest this is due to a different occupationally exposure by sex (Wolff *et al.*, 1992). Only a recently published study conducted on elderly people from Sweden discusses the role of chlorination in the sexual differences found in PCBs: they observe higher levels of two lower chlorinated PCBs among women and higher levels of five higher chlorinated PCBs among men (Salihovic *et al.*, 2012). This study, however, did not associate the sexual difference with the BMI inverse trend. On the other hand, the fact that the only PBDE (BDE-153) which is more accumulated in men also exhibits a decreasing trend with BMI, alike PCB-180, reinforces the importance of the physical-chemical properties in the patterns of accumulation of these

compounds in humans (Figure 2 on SI shows the correlation between Koa and Kow on the BMI trend).

Considering differences in the physical-chemical properties of the congeners and the role of respiration in excreting compounds with higher volatility, both factors explain also a different accumulation by sex. In this sense, higher chlorinated congeners, such as 138, 153 and 180, are particularly persistent. They have higher half-lives and a longer retention tendency than lower chlorinated (*i.e.* more volatile) ones, such as PCB-118, hence being more difficultly metabolised or excreted by the organism (Grandjean *et al.*, 2008; Wolff *et al.*, 1992; van Larebeke *et al.*, 2001). Furthermore, some studies report human and animal sex-dependence in chemical metabolism of PCBs, attributed to the sex-specific expression or activity of certain hepatic cytochromes P450s (Mugford and Kedderis, 1998; Liu *et al.*, 2010; Roos *et al.*, 2011). The fact is not fully explained and may be due to the intervention of another, unknown factor of excretion, perhaps also related to the metabolism. Research in rats suggests that "female rats could express some protein, lipid, steroid, hormone or other type of compound, not present in males, which binds to PCB-180, reducing its effect" (Boix *et al.*, 2011). The increased accumulation of higher chlorinated PCBs in men could then be also explained by sex differences in the metabolism of these compounds.

Conclusions

In essence, this research provides a new explanation to the patterns of POP accumulation by considering the linkages between the physical-chemical properties of these compounds and the human metabolism. A careful analysis of the data shows a positive correlation between the degree of volatility of an OC, on the one hand, and the importance of the sex and age interactive effect in humans, on the other. Presumably, HCB and β-HCH, being more volatile than the other OCs, could be partly exhaled by respiration. The fact that women accumulate proportionately higher concentrations of those compounds would be explained by their lower capacity to exhale them than men, due to their lower cardiopulmonary metabolism. In conclusion, respiration represents a relevant means to excrete the most volatile OCs. Men are more able to excrete them because of their higher cardiopulmonary activity, although obesity plays a detrimental effect due to a slower metabolism. In contrast, women have proportionately less ability to eliminate volatile OCs by respiration and thus their body burden increases. For them, this effect is exacerbated through age and independent of BMI.

These new insights on the role of human metabolism in OC excretion deserve further study, addressing the accumulation of organic pollutants in humans by considering the interactive sex and age effect. This should be complemented by the potential linkages between the properties of these pollutants, such as the degree of volatility, and their excretion by respiration. In short, both physical-chemical properties and sex-dependence metabolism of OCs in humans, as well as the particular BMI trend, are relevant factors that may influence their accumulation and elimination patterns, being HCB, β-HCH, PCB-180 and BDE-153 the compounds where these differences are much more observable between men and women.

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SUPPORTING INFORMATION
Sexual divergence in the accumulation of persistent organic pollutants in humans throughout life

Materials and Methods

Population and Study Design

The study sample (n=919) is based on a public health survey (n = 8,400) that was conducted by the Government of Catalonia in 2002, including a health exam and blood test (n=2,100). Catalonia is a Mediterranean region of 32,000 km^2 in South-West Europe, with a total population of 6.5 million inhabitants as of 2002. Its major economic activities include agriculture and industry. The survey provided a valuable and representative sample of the general population in terms of age, sex and socio-demographic conditions.

Information on body mass index (BMI), cholesterol and triglyceride was obtained from the health exam. Information on the demographic variables and lifestyle, such as age, sex, parity in women and smoking habits, was obtained from face-to-face interviews that were conducted between October 2001 and April 2002. Further details of the study design are available in precedent publications (Juncà *et al.*, 2003; Porta *et al.*, 2010).

The Flix township is located in southern Catalonia and hosts a chemical factory that used to release OCs until the 1990s. In 1994, 608 individuals over than 14 years old from this high exposed population participated in the study. They were asked to answer a questionnaire with information on lifestyles, occupation and medical conditions. From the total of 608 individuals, only two did not provide information on BMI. Hence, statistical analyses are based on 606 individuals. Further information about the study design is published elsewhere (Sala *et al.*, 1999).

Concerning the U.S. study, the National Health and Nutrition Examination Survey (NHANES) conducted in 2003-2004 reported human serum levels of selected persistent organic pollutants categorized by age, sex and race/ethnicity from a statistically representative sampling of the U.S. population (Patterson *et al.*, 2009).

Table 1 on Supporting Information shows the description of the variables used for each population.

Serum Extraction and Clean-up

Blood serum analysis was performed to measure concentrations of a wide array of organochlorine compounds, such as pentachlorobenzene (PeCB), hexachlorobenzene (HCB), several isomers of hexachlorocyclohexanes (α, β, δ and γ-HCH), 2,4'-DDT and 4,4'-DDT and their metabolites, 2,4'-DDD, 4,4'-DDD, 2,4'-DDE and 4,4'-DDE, the congeners 28, 52, 101, 118, 138, 153 and 180 of polychlorobiphenyls (PCBs) and the con-

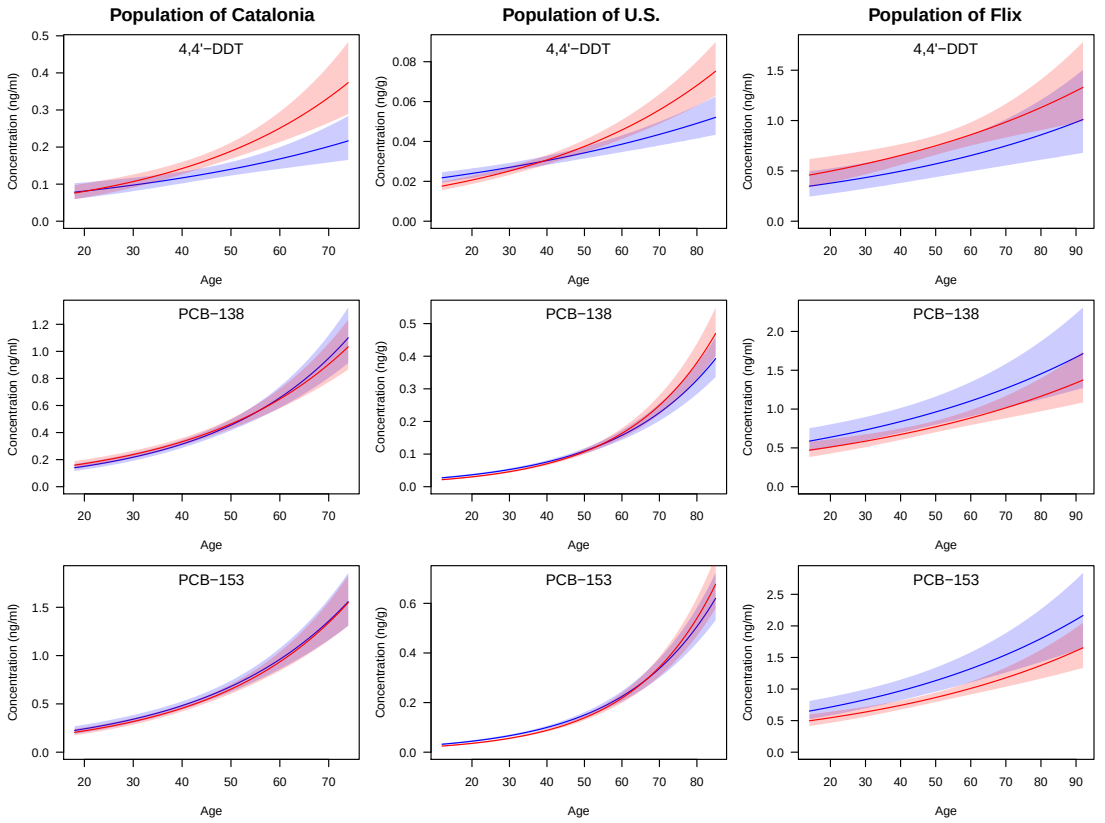

Figure 1: Comparison between the accumulation of certain OCs (4,4'-DDT, PCB-138 and PCB-153) by age and sex (red colour for women and blue colour for men) in the three analysed populations: Catalonia (left panel), U.S. (middle panel) and Flix township (right panel).

geners 17, 28, 47, 66, 71, 85, 99, 100, 138, 153, 154, 183, 190 and 209 of polybromodiphenyl ethers (PBDEs). The analysis of these organohalogenated compounds in human serum was performed according to the method described by Grimalt *et al.* (2010). Briefly, 1 ml of serum was introduced into 10 ml centrifuge tube, adding the recovery standards TBB and PCB-209, and *n*-hexane (3 ml) and *conc*-H_2SO_4 (2ml). The mixture was stirred in a vortex (ca. 1500 rpm, 30 s) and centrifuged (ca. 3500 rpm, 5 min). The supernatant *n*-hexane layer was transferred into a second centrifuge tube. Further *n*-hexane (2 ml) was added to the remaining sulphuric acid solution, stirred and centrifuged. This last step was repeated again yielding a combined extract of 7 ml of *n*-hexane, to which 2 ml of *conc*-H_2SO_4 were added, the suspension was mixed (ca 1500 rpm, 60 s), centrifuged (3500 rpm, 10 min), and the supernatant *n*-hexane was transferred to a conical bottomed, graduated tube. This *n*-hexane extract was reduced to a small volume under a stream of pure nitrogen. The extract was transferred to a gas chromatography vial using four rinses of isooctane and adding the injection standard PCB-142.

Table 1: Description of the main variables analyzed for each population.

		n	Sex (men/women)	Age[a] mean (range)	BMI[b] mean (range)	TL[c] mean (range)
Catalonia	Pesticides	919	399/520	45 (18-74)	26 (17-56)	605 (372-1334)
	PCBs	"	"	"	"	"
	PBDEs	311	131/180	44 (18-74)	26 (17-42)	602 (394-1334)
U.S.	Pesticides	903	446/457	39 (12-85)	27 (15-52)	627 (298-3772)
	PCBs	870	428/442	"	27 (15-63)	624 (325-2800)
	PBDEs	923	458/465	40 (12-85)	27 (15-52)	629 (298-3772)
Flix	Pesticides	606	249/357	49 (14-92)	25 (16-41)	–
	PCBs	"	"	"	"	–

[a] Age in years.
[b] Body Mass Index in kg/m^2.
[c] Total Lipids in g/L.

Analysis of organochlorine compounds

Instrumental analysis was performed by gas chromatography with electron capture detection (GC-ECD, Agilent Technologies 6890N) using a DB-5 column protected with a retention gap (60 m length, 0.25 mm I.D., 0.25 μm film thickness; J&W Scientific). Limits of detection and quantification (LD and LQ) were calculated according to (Garí and Grimalt, 2010). This method performed satisfactorily in repeated international intercalibration exercises within the Arctic Monitoring Assessment Program (Centre de Toxicologie. Institut National de Santé Publique du Québec, 2012).

Analysis of organobromine compounds

After OCs analysis by GC-ECD, vials samples were re-evaporated under a nitrogen stream and 20 μl of BDE 118 and 10 μl of ($^{13}C_{12}$)-BDE-209 were added as internal standards. PBDEs were determined using an Agilent 6890N GC coupled to a mass spectrometer (GC-MS) (Agilent Technologies) operating in negative ion chemical ionization (NICI) mode. The instrument was equiped with a low bleed SGE-BPX5 MS fused silica capillary column (15 m length, 0.25 mm I.D. and 0.10 μm film thickness).

Statistical analyses

Data analysis and graphics were conducted using the statistical software R (R Development Core Team, 2012).

Statistical analyses focused on the following OC compounds: HCB, β-HCH, 4,4'-DDT, 4,4'-DDE and PCB congeners 118, 138, 153 and 180. They were selected because they met quantification and detection limits in over 75% of samples (for the rest of the samples, a score of one-half the LQ and the LD was assigned). For PBDEs, the statistical analyses focused on the four most frequently detected PBDE congeners (BDEs 47, 99, 153 and 209) (Garí and Grimalt, 2013).

Serum concentrations of OCs were not always presented in a lipid-adjusted basis and, as suggested by Schisterman *et al.* (2005), multivariate regression models were then adjusted for the total lipid (TL) amount of each sample, calculated through the equation described by Phillips *et al.* (1989). The levels of OCs in the populations of Catalonia and Flix are presented in ng/ml while in the population of the U.S., in ng/g (Figure 1). In other analysis, however, the concentrations of OCs and PBDEs were lipid-adjusted and presented

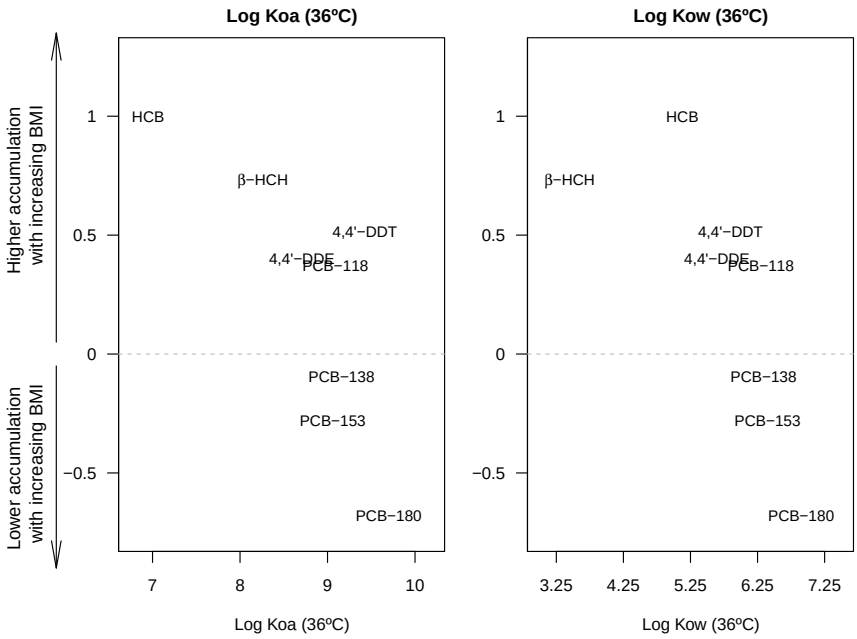

Figure 2: Relationships between octanol-air (Koa) and octanol-water (Kow) partition coefficients (in logarithmic scale) and the BMI trend. The OCs that are more accumulated with increases in BMI are precisely the most volatile ones (HCB and β-HCH), while the OCs that are less accumulated with increases in BMI are the least volatile and most chlorinated OCs (PCB-153 and PCB-180).

in ng/g lipid (Figures 2 and 3).

Concentrations of organohalogenated compounds were transformed to the natural logarithm (ln) in order to normalize the distribution of the values and then avoid violating regression assumptions of normal distribution. Results are presented in the original scale by using the inverse of the logarithm (the exponential) in the predicted values shown in figures. Within the predictor variables, BMI was centred at zero and scaled to two standard deviations, to let the intercept have a meaningful interpretation and help with the estimation of the interaction effect between age and sex described below. The age variable was settled at 0 and the reference category for sex was women.

Multivariate regression models from each population were applied to asses the effects of sex, age, and BMI in the OC and PBDE concentrations. The models included two interactions (sex*age and sex*BMI), as follows:

$$\begin{aligned} log(POP) \;=\; & \alpha + \beta_1(Sex) + \\ & \beta_2(Age) + \beta_3(BMI) + \\ & \beta_4(Sex * Age) + \\ & \beta_5(Sex * BMI) + \epsilon \end{aligned}$$

For the compounds in which the interactions were not significant, multivariate regression models did not included these two additional parameters.

For the elaboration of the Figures 1, 2 and Figure 1 on SI, BMI was established to the mean. The results do not change if the model is adjusted by other predictable variables, such as smoking status, alcohol consumption, social class, educational level and place of birth (data not shown). For some

Table 2: Comparison between the standardized β-coefficients of sex, age and BMI, as well as the two interactions (sex*age and sex*BMI), from multivariate regression models. Each compound is a different model. All the variables have been centred to zero and scaled to two standard deviations for allowing comparison between different compounds and parameters.

	Standardized β-coefficients				
	Individual terms			Interaction terms	
	Sex(□)	Age	BMI	Sex(□)*Age	Sex(□)*BMI
HCB	0.40**	0.51**	0.26**	0.17**	-0.25**
β-HCH	0.29**	0.57**	0.19**	0.18**	-0.25**
4,4'-DDT	0.10**	0.23**	0.14**		
4,4-DDE	0.16**	0.51**	0.10**		
PCB-118	0.11**	0.37**	0.095**		
PCB-138	0.024	0.50**	-0.036		
PCB-153	-0.016	0.53**	-0.078**		
PCB-180	-0.11**	0.61**	-0.18**		
BDE-47	-0.034	-0.12**	-0.021		
BDE-99	-0.029	-0.10**	-0.029		
BDE-153	-0.12**	-0.038	-0.11**		
BDE-209	-0.039	-0.079**	-0.013		

** p-value<0.05

analysis, shown in Figure 4 and in Table 2 on Supporting Information, all the variables, including the POP concentrations and the dichotomous sex one, were standardized (centred at zero and scaled to two standard deviations), in order to compare the beta coefficients between them (Gelman, 2008).

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Article 3

Inverse age-dependent accumulation of decabromodiphenyl ether and other PBDEs in serum from a general adult population

Mercè Garí and Joan O. Grimalt
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Article 4

Influence of the atmospheric emissions of organochlorine compounds from the Flix chloro-alkali plant on the population of Catalonia

Mercè Garí, Joan O. Grimalt and Jordi Sunyer
To be submitted

Influence of the atmospheric emissions of organochlorine compounds from the Flix chloro–alkali plant on the population of Catalonia

Mercè Garí[*], Joan O. Grimalt[*], and Jordi Sunyer[**]

[*]Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research (IDAEA). Spanish Council for Scientific Research (CSIC).
Jordi Girona, 18. 08034 Barcelona. Catalonia. Spain
[**]Centre for Research in Environmental Epidemiology (CREAL).
Dr Aiguader, 88. 08003 Barcelona. Catalonia. Spain

Abstract

Organochlorine compounds (OCs), including hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs), DDTs and polychlorobiphenyls (PCBs), have been studied in Catalonia, a Western Mediterranean country, by analysis of blood serum. The study examines the levels of OCs in the context of the general population of Catalonia (n=919) and the specific case of the Flix township (n=421), which is situated close to an electrochemical factory that used to produce all those compounds for decades. Comparison of the observed median concentrations from both studies shows differences possibly related to the emissions from the factory. OC concentrations within the individuals living in a first area of influence from Flix (15-25 km) are intermediate between those from Flix and the rest of the Catalan municipalities, including those located in a second area of influence of 30-40 km away from Flix, controlling by age and sex. This finding indicates a slight but noticeable influence of OC contamination from the electrochemical factory in other nearby towns. HCB shows extreme and unusually high concentrations in Flix, but its effect is quickly diffused through distance (<15 km). For the other OCs, a sustained and systematic decrease is found. The eight year time period elapsed between both studies may only explain part of the huge differences found between Flix and the first area of influence. Both analysis show high median OC levels compared to other populations worldwide, including European, American and Asian studies.

1 Introduction

Organochlorine compounds (OCs) have been used as pesticides and in industrial applications for several decades. Hexachlorobenzene (HCB), β-hexachlorocyclohexane (β-HCH), 4,4'-DDT and its major metabolite 4,4'-DDE, and polychlorinated biphenyls (PCBs) are some of the more commonly encountered organochlorine compounds (WHO, 2003). They are part of the so-called persistent organic pollutants (POPs), a widespread toxic environmental contaminants. Due to their toxicity and persistence in the environment they were highly restricted and most western countries began to ban them in the 1970s to

protect human health and the environment. In 2004, the Stockholm Convention on persistent Organic Pollutants banned twelve of these compounds, which included HCB, 4,4'-DDT and PCBs (Stockholm Convention, a). In the last years, some new pollutants have been added to the above mentioned list, such as all the HCH isomers' (Stockholm Convention, b). As a consequence of these regulatory restrictions, OC levels have diminished in biota and in human tissues, as reported in time trend studies conducted in many countries (Bignert *et al.*, 1998; Norén and Meironyté, 2000). However, since OCs can accumulate in lipids and biomagnify in the food web, they are still present in foods from animal origin, such as fish, meat and dairy products (Darnerud *et al.*, 2006; Llobet *et al.*, 2003). OCs are incorporated into humans mainly through diet, although there are other minor routes, such as inhalation, as suggested in a study conducted in the inhabitants living near an electrochemical plant (Sala *et al.*, 1999). The population of Flix, located in Catalonia (Western Mediterranean), incorporated HCB directly through the respiratory system, by airborne pollution, and had the highest HCB levels ever found in humans (Sala *et al.*, 1999). The factory has been producing volatile chlorinated solvents over the past decades, as well as other organochlorine compounds such as DDT and PCBs.

The present study is aimed to assess the influence of the emissions from the Flix chloro-alkali plant on other nearby towns of Catalonia, by comparison of OC levels in the serum samples of the Catalan individuals. The Catalan study comprises the general population of Catalonia (n=919), and the specific case of the Flix township (n=421). To our knowledge, this is the first approach that uses OC serum concentrations from human populations to assess the spatial influence of the atmospheric contamination emitted from a local environmental pollution site, such as the Flix chloro-alkali plant. Previously, the vegetation has been used as indicator of atmospheric contamination, since plants have a big potential to accumulate organic pollutants (Simonich and Hites, 1995). In addition to that, this study also compares the OC levels of Catalan individuals with other populations worldwide.

2 Materials and methods

2.1 Populations and recruitment

The present work includes the analysis of two adult populations: the general population of Catalonia and the highly-exposed one of the Flix township, in Catalonia itself. The Flix cross sectional study was carried out in 1994, based on 421 inhabitants of the village aged between 14 and 86 years old of both sexes. The study design is reported elsewhere (Sala *et al.*, 1999). The study from the general population of Catalonia was carried out in 2002, based on 919 individuals of both sexes and ages between 18 and 74 years. The study design is also reported elsewhere (Porta *et al.*, 2010).

The individuals from the general Catalan study come from 94 out of the 947 municipalities, located throughout Catalonia. Those 94 municipalities have been divided in three areas, according to their proximity to the Flix factory (Figure 1). The first area of influence includes 42 individuals from 5 municipalities located at about 15 to 25 km away from Flix. The second area includes 172 individuals from 14 municipalities located at about 30 to 40 km away from Flix. The rest of municipalities (75) are not considered to be influenced from Flix and are then named as Catalonia, with a total of the remaining 705 individuals.

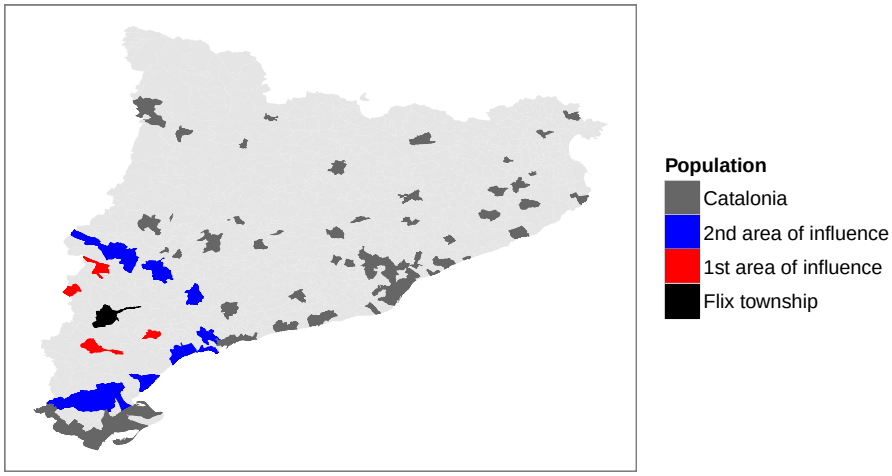

Figure 1: Map showing the areas of the population of Catalonia that have been analysed: Flix township in black, 1st area of influence from Flix in red, 2nd area of influence from Flix in blue, and the rest of the Catalan municipalities in grey.

2.2 Laboratory analytical methods

Blood serum analysis was performed to measure concentrations of a wide array of organochlorine compounds, such as pentachlorobenzene (PeCB), hexachlorobenzene (HCB), several isomers of hexachlorocyclohexanes (α, β, δ and γ-HCH), 2,4'-DDT, 4,4'-DDT and their metabolites, 2,4'-DDD, 4,4'-DDD, 2,4'-DDE and 4,4'-DDE, and the congeners 28, 52, 101, 118, 138, 153 and 180 of polychlorobiphenyls (PCBs). The analysis of OCs in human serum has been described elsewhere (Otero *et al.*, 1997; Grimalt *et al.*, 2010). Briefly, serum samples (1 ml) were introduced into 10 ml centrifuge tubs. The recovery standards TBB and PCB-209 at 100 ng/ml were added. Then, 3 ml of *n*-hexane and 2 ml of *conc* H_2SO_4 were added, mixed in a vortex (ca. 1,500 rpm, 30 s) and centrifuged (ca. 3,500 rpm, 5 min). The supernatant *n*-hexane layer was transferred into a second centrifuge tube using a Pasteur pipette. Further, *n*-hexane (2 ml) was added to the first tube containing the H_2SO_4/serum mixture, stirred and then centrifuged. This last step was repeated again yielding a combined extract of 7 ml of *n*-hexane, to which 2 ml of conc. H_2SO_4 were added, the suspension was mixed (vortex mixer, ca 1.500 rpm, 60 s), centrifuged (3.500 rpm, 10 min), and the supernatant *n*-hexane was transferred to a conical bottomed, graduated tube. These *n*-hexane extracts were reduced to near dryness under a stream of pure nitrogen. Then, the solutions were quantitatively transferred to GC vials using four 25 μl rinses of isooctane and adding the injection standard PCB-142.

Instrumental analysis was performed by gas chromatography with electron capture detection (GC-ECD, Agilent Technologies 6890N) using a DB-5 column protected with a retention gap (60 m length, 0.25 mm I.D., 0.25 μm film thickness; J&W Scientific). For the samples belonging to the Flix study, detection limits ranged between 0.05 ng/ml and 1.1 ng/ml, depending on the OC compound. The analytical performance of these samples is explained elsewhere (Sala *et al.*, 1999). For the samples of the general Catalan study, limits of detection and quantification were calculated according to Garí and Grimalt (2010). In addition to that, the laboratory is in compliance with the AMAP Ring Test Profi-

ciency Program for persistent organic pollutants in human serum (Centre de Toxicologie. Institut National de Santé Publique du Québec, 2012).

2.3 Data analysis

Data analysis and graphics have been conducted using the statistical software R (R Development Core Team, 2012). Statistical analyses are focused on the following compounds: HCB, β-HCH, 4,4'-DDT, 4,4'-DDE and PCB congeners' 118, 138, 153 and 180. These compounds have been selected because they met detection limits in over 30% of samples. Serum concentrations are expressed in crude values (ng/ml) for both studies. Moreover, for the samples obtained from the general population of Catalonia, serum concentrations are also expressed in a lipid-adjusted basis (ng/g lipid), calculated through the equation described by Phillips *et al.* (1989), based on the levels of cholesterol and triglyceride. These levels were determined enzymatically, using Txad-Pap and CIN-UV methods, respectively.

For descriptive analysis, mean, medians and selected percentiles have been used (Tables 2 and 4). For assessing the differences between Flix, 1st and 2nd areas of influence from Flix and the municipalities from the rest of Catalonia, controlling for age and sex, several multivariate regression models have been used. The proposed model was at follows:

$$log(OC) = \beta_1(\text{Area}) + \beta_2(\text{Sex}) + \beta_3(Age) + \epsilon$$

Age was settled at its mean and the reference category for sex was women. Given that there is no intercept in the equation, the meaning of is the expected value of the compound for each area: Flix, 1st area of influence, 2nd area of influence and Catalonia. Concentrations of OCs were transformed into the natural logarithm in order to normalize the distribution of the values and avoid violating regression assumptions of normal distribution. Results are presented in the original scale by using the inverse of the logarithm (the exponential) in the expected values.

3 Results and discussion

3.1 Characteristics of the studied populations

Age and sex of the participants included in the study are shown in Table 1. Of the participants, different sex ratios are found in the two populations analysed: 43% of men and 57% of women in Catalonia and 20% of men and 80% of women in Flix. This difference is explained because a high proportion of men in the studied population were current workers or ex-workers at the factory, and this research only includes those individuals who have never been working at the factory. The mean age of the Catalan individuals is 45 years (SD=15), ranging from 18 to 74 years. For the Flix study, the mean age is 46 years (SD=18), ranging from 14 to 86 years. The Flix population is slightly older than the Catalan one. For instance, the proportion of individuals in the eldest group is slightly higher in the population of Flix (27%) than in the Catalan general study (20%). This is not an effect of the sampling method but a feature of the populations (IDESCAT, 2012). For the three selected areas of the Catalan population, no significant differences in the age and sex ratios are found (data not shown).

3.2 OC detection frequencies

Hexachlorobenzene is the major congener detected (100% of the serum samples) in the Flix study, followed by 4,4'-DDE (detected in 98.7%). For the general population of Catalonia, 4,4'-DDE and PCB-180 are de-

Table 1: Characteristics of the populations studied.

	Catalonia n (%)	Flix n (%)
All participants	919 (100)	421 (100)
Sex		
Woman	520 (57)	338 (80)
Man	399 (43)	83 (20)
Age (years)		
All ages	45 (±15)[a]	46 (±18)[a]
14-29	173 (19)	82 (19)
30-44	272 (30)	119 (28)
45-59	287 (31)	105 (25)
60-92	187 (20)	115 (27)

[a] Arithmetic mean (± standard deviation).

tected in practically every sample (100% and 99.9%, respectively). Except for HCB, detection percentages for the major compounds are higher in the population of Catalonia than in Flix (data not shown). In both populations, pentachlorobenzene, α, γ and δ isomers of HCHs and the congeners 28, 52 and 101 of PCBs are found above limit of detection in less than 30% of the serum samples and they are therefore not included in the following sections. 2,4'-DDT and other metabolites rather than 4,4'-DDE (2,4'-DDD, 4,4'-DDD and 2,4'-DDE) are neither included in the analyses.

3.3 Concentrations of hexachlorobenzene

Table 2 shows the mean and selected percentiles of major organochlorine pesticides in the two populations analyzed, in order to compare them. Mean and median HCB concentrations in Flix (16.9 ng/ml and 14.4 ng/ml, respectively) were more than 10 times higher than those found in the population of Catalonia (2.0 ng/ml and 1.2 ng/ml, respectively). The maximum concentration of this compound is found in the population of Flix (222.1 ng/ml), with a high difference from the population of Catalonia (24.4 ng/ml).

Comparing to other studies worldwide (Table 3), the Flix population is the one having the highest HCB concentrations ever described in general populations, related to their exposure to high airborne levels of this compound from the chloro-alkali plant (Sala *et al.*, 1999). Apart from Flix, the high values shown in the Catalan population are consistent with the HCB levels of Spanish general populations described in previous studies, which are higher than other European countries (de Salamanca *et al.*, 1990; Jakszyn *et al.*, 2009). Jakszyn *et al.* (2009) found particularly elevated HCB levels in one of the northern Spanish regions analysed, probably due to industrial uses rather than agricultural practices. There is only one study that reports much higher levels of HCB than in Catalonia (Table 3). Petrik et al. (2006) argue that such high HCB levels in Slovakia are due to an intensive use of pesticides in agriculture in the past (Petrik *et al.*, 2006). Notwithstanding, HCB levels in Flix are the highest ever found (Table 1).

Table 2: Serum concentrations (in ng/ml) of organochlorine compounds from the populations of Flix (n=421, 1994) and Catalonia (n=919, 2002).

	Flix					Catalonia				
	Mean	P25	P50	P75	Max	Mean	P25	P50	P75	Max
HCB	16.9	8.3	14.4	21.0	222.1	2.0	0.51	1.2	2.6	24.4
β-HCH	8.7	1.5	5.1	12.1	151.2	1.2	0.29	0.67	1.5	15.1
4,4'-DDT	0.58	nd	0.13	0.90	6.5	0.32	0.087	0.18	0.35	9.4
4,4'-DDE	9.1	1.8	4.4	11.7	222.9	4.8	1.3	2.6	5.6	68.4
PCB-118	0.10	nd	nd	nq	1.1	0.18	0.061	0.13	0.24	2.5
PCB-138	0.80	0.10	0.58	1.2	4.6	0.59	0.26	0.45	0.72	11.2
PCB-153	0.89	0.21	0.63	1.3	8.2	0.83	0.36	0.63	0.98	10.0
PCB-180	1.2	0.29	0.76	1.5	12.9	0.67	0.32	0.50	0.79	10.7

P25, P50 and P75 refer to percentiles 25, 50 (median) and 75, respectively.
nd and nq are not detected and not quantifiable levels, respectively.

3.4 Concentrations of other OC pesticides

The population of Flix exhibits higher mean, median and maximum concentrations of β-HCH and 4,4'-DDE than in Catalonia (Table 2). However, for 4,4'-DDT, higher median and maximum levels are found in the population of Catalonia (0.18 ng/ml and 9.4 ng/ml, respectively) than in Flix (0.13 ng/ml and 6.5 ng/ml, respectively).

In general terms, the concentrations of these OC pesticides tend to be slightly higher than in many other previously studied populations (Table 3), such as two extensive studies from the U.S. and Canada (Patterson *et al.*, 2009; Medehouenou *et al.*, 2011); two studies from Japanese and Korean populations (Hanaoka *et al.*, 2002; Tsukino *et al.*, 2006; Kang *et al.*, 2008); a report from New Zealand (Bates *et al.*, 2004); as well as several European populations (Thomas *et al.*, 2006; Becker *et al.*, 2002; Amodio *et al.*, 2012; Glynn *et al.*, 2000, 2003; Koppen *et al.*, 2002). However, some of the aforementioned studies show higher 4,4'-DDE levels than in the population of Catalonia, *e.g.* Belgium, New Zealand, Japan and Canada (Medehouenou *et al.*, 2011; Hanaoka *et al.*, 2002; Bates *et al.*, 2004; Koppen *et al.*, 2002), which can be explained by a more intensive use of the pesticide DDT in the past. The concentrations in Catalonia are however lower than in populations that have a particularly high exposure to OC-related chemicals: for instance, a comparative study of three districts in Slovakia shows higher levels of DDTs (Petrik *et al.*, 2006); a report from Romania shows much higher concentrations of 4,4'-DDT, 4,4'-DDE and β-HCH, probably due to the reportedly excessive use of those compounds in agriculture, despite their legal prohibition (Dirtu *et al.*, 2006); as well as the population of Mexico, which used to employ HCHs and DDT until 1999 in agriculture and for combating the spread of disease-transmitting vectors in humans (Waliszewski *et al.*, 2012), and the Chinese report, which associates their high serum levels of OC pesticides with consumption of animal foods, as well as their abundant use in agriculture and for malaria control (Lee *et al.*, 2007).

Table 3: Median OC concentrations (in ng/g lipid) found in human serum from different countries.

Location	Year	n	HCB	β-HCH	4,4'-DDT	4,4'-DDE	PCB-118	PCB-138	PCB-153	PCB-180	ΣPCBs[a]	Reference
Catalonia	2002	919	201 [1.2][b]	111.6 [0.67][b]	30.1 [0.18][b]	436.3 [2.6][b]	23.0 [0.13][b]	74.1 [0.45][b]	104.3 [0.63][b]	85.3 [0.50][b]	298.5 [1.8][b]	Present study
Flix, Catalonia	1994	421	[14.4][b]	[5.1][b]	[0.13][b]	[4.4][b]	ND	[0.58][b]	[0.63][b]	[0.76][b]	[2.3][b]	Sala *et al.* (1999)
Barcelona, Catalonia	2006	231	109	64.2	22.0	219.1	13.5	48.3	68.6	63.4		Porta *et al.* (2012)
Spain	1992-1996	953	462.5	221.0	ND	857.9	34.4	104.6	182.6	119.5	449.6	Jakszyn *et al.* (2009); Agudo *et al.* (2009)
Biscay, Spain	2006	283	88.5	40.55	16.17	191.51	7.7	66.2	95.0	84.9	262.4	Zubero *et al.* (2010, 2009)
Canary Islands, Spain	1998	682	NA	NA	181[d]	262[d]	NA	NA	NA	NA	NA	Zumbado *et al.* (2005)
UK	2003	154	11	12	2.9	100	6.1	27	41	33	170	Thomas *et al.* (2006)
Germany	1998	2824	[0.40][b]	[0.16][b,d]	NA	[1.6][b,d]	NA	[0.50][b]	[0.70][b]	[0.50][b]	[1.7][b]	Becker *et al.* (2002)
Sweden	1996-1997	205	65	51	NA	497	43	101	223	152	552	Glynn *et al.* (2003)
Sweden	NR	120	61.7	41.5	16.5	586.0	37.5	134.0	295.5	206.5	700.6	Glynn *et al.* (2000)
Belgium	1999	47[f]	109.9	NA	2.6	871.3	29.2	91.8	167.6	104.1	530.2	Koppen *et al.* (2002)
Italy	2009	101	18.3	4.3	4.4	175.1	4.1	22.0	32.5	23.0	NR	Amodio *et al.* (2012)
Slovakia (BA)	2001	1038	639	44.0	33.2	1368	21.4	141[c]	232	203	743	Petrik *et al.* (2006)
Slovakia (CA)	2001	1009	690	48.6	72.9	2521	63.8	352[c]	578	526	1892	Petrik *et al.* (2006)
Czech Republic	2006	202	NA	NA	NA	NA	15	188	438	397	1027	Cerná *et al.* (2008)
Romania	2005	142	30	923	339	1975	12	38	102	107	383	Dirtu *et al.* (2006)
New Zealand	1996-1997	60[f]	ND	10.7	ND	919	ND	15.6[c]	24.6	20.0	81.9	Bates *et al.* (2004)
Japan	1999	41	[0.20][b]	[0.50][b]	[5.0][b,g]	[5.0][b,g]	NA	NA	NA	NA	NA	Hanaoka *et al.* (2002)
Japan	2000	80	ND	93.2	ND	221	10.5	16.8[c]	36.6	21.6	NR	Tsukino *et al.* (2006)
Korea	2003	87	NA	NA	NA	NA	7.6	26.7[c]	39.2	19.0	180.2	Park *et al.* (2007)
Korea	2006	40	16.7	49.0	18.6	224	6.4	14.6	20.7	13.1	104	Kang *et al.* (2008)
China	NR	250	62.7	5065.0	309.0	7635.0	ND	ND	ND	ND	0.20	Lee *et al.* (2007)
Bolivia	2010	112	22.1[e]	NA	13.0[e]	267.4[e]	NA	33.7[e]	59.0[e]	26.7[e]	NR	Arrebola *et al.* (2012)
Mexico	2010	150	NA	3100	1700	9500	NA	NA	NA	NA	NA	Waliszewski *et al.* (2012)
USA	2003	1961	14.9	ND	ND	203	5.19	15.14[c]	20.84	18.00	131.8	Patterson *et al.* (2009)
Canada	1991-2002	1979	27.8	20.8	11.3	716	23.4	42.2	73.6	60.5	289.0	Medehouenou *et al.* (2011)

NA: Not analyzed; ND: Not detected; NR: Not reported; BA: Background area; CA: Contaminated area.

[a] Sum of all congeners analysed in each study.

[b] Values expressed in ng/ml.

[c] The reported value co-eluted with other compound.

[d] Mean concentration instead of median.

[e] Geometric mean concentration instead of median.

[f] Pooled samples.

[g] The reported value is the sum of 4,4'-DDT and 4,4'-DDE.

In the Spanish context, the population from the Canary Island shows much higher levels of DDT than in Catalonia, likely in relation to an intense, greenhouse-based agricultural system that used to employ abundant chemicals (Zumbado *et al.*, 2005). The concentrations of OC pesticides in five Spanish regions studied in 1992-1996 are two fold higher than those found in Catalonia (Jakszyn *et al.*, 2009). However, the levels of these compounds in other Spanish or Catalan sites (Biscay and Barcelona, respectively) are half than those from the general population of Catalonia (Zubero *et al.*, 2010; Porta *et al.*, 2012). This is probably due to the temporal differences in the recruitment of the individuals from all these populations (2002 vs 2006). Certain temporal trend reports have observed decreases of about 30-50% in OC concentrations from the 1990s to the 2000s (Petrik *et al.*, 2006; Hagmar *et al.*, 2006). The Spanish general report (1992-96) was carried out almost 10 years before the Catalan study (2002), and the period elapsed between both studies may explain part of the differences in the concentrations of these pollutants. Furthermore, the decreasing trend has continued in the 2000s, as shown in a previous study carried out in Catalonia itself, comparing the OC concentrations between the general population (present study) and the city of Barcelona (studied in 2006) (Porta *et al.*, 2012). This study reported a decrease of about 34-56% in the 4 year-time elapsed between both studies. In this regard, the lower concentrations found in the studies from Barcelona and Biscay, both of them carried out in 2006, in relation to the Catalan population, are consistent with the decreasing trend explained before.

The higher chlorinated congeners, PCB-138, PCB-153 and PCB-180, are those most abundant in both studies, as shown in Figure 2. The figure also shows that the distribution of PCBs is dominated by the congener 153 in the population of Catalonia and the congener 180 in the population of Flix. PCB-118 is more abundant in the Catalan study than in Flix township, although it contributes less than the aforementioned congeners. Minor PCB congeners (28, 52 and 101) are contributing higher to the sum of PCBs in the population of Flix than in the Catalan general study. Conversely, PCB-118 in Flix township contributes similar to the sum of PCBs than the minor congeners.

3.5 PCB concentrations

Table 2 shows the mean and selected percentiles of the major PCB congeners'. The population of Flix exhibits higher mean and median concentrations of the higher chlorinated congeners than in the population of Catalonia. Conversely, maximum concentrations are commonly encountered in Catalonia than in Flix (*e.g.* congeners' 138 and 153). For PCB-118, however, mean and median concentrations are higher in Catalonia than in Flix, as well as the maximum levels (Table 2).

PCB levels from Flix and Catalonia are in the range of those found in other Spanish reports (Agudo *et al.*, 2009; Zubero *et al.*, 2009) and many European populations (*e.g.* Sweden, Germany, Belgium and Romania) (Becker *et al.*, 2002; Glynn *et al.*, 2000, 2003; Koppen *et al.*, 2002; Dirtu *et al.*, 2006). The concentrations are however higher than those reported in other European sites such as the UK and Italy (Becker *et al.*, 2002; Amodio *et al.*, 2012), as well as the New Zealand, Japanese and Korean populations (Tsukino *et al.*, 2006; Kang *et al.*, 2008; Bates *et al.*, 2004; Park *et al.*, 2007) and the studies conducted in different American countries, *e.g.* Bolivia, the U.S. and Canada (Patterson *et al.*, 2009; Medehouenou *et al.*, 2011; Arrebola *et al.*, 2012) (Table 3). Only the Slovak and Czech studies show much higher levels of PCBs in their inhabitants than in Catalonia or Flix,

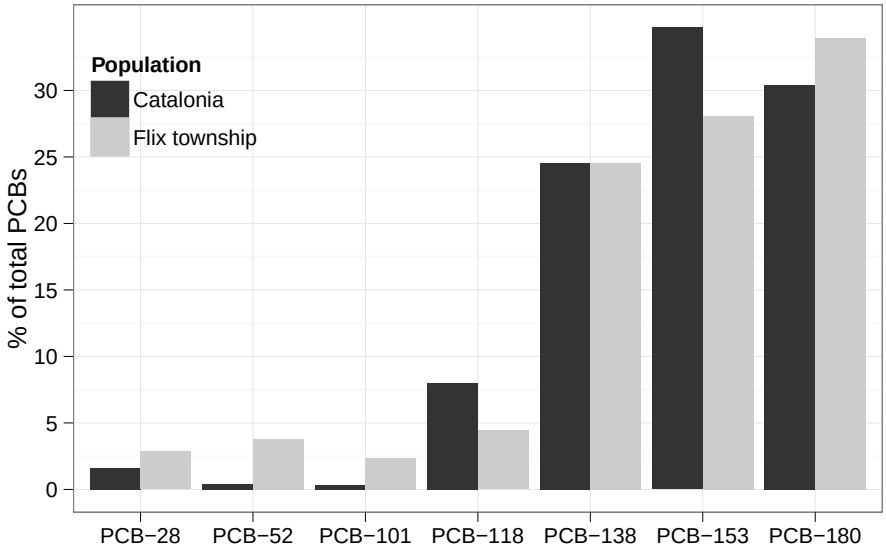

Figure 2: Contribution of each PCB congener to the total sum of PCBs in the serum samples from the populations of Catalonia (black) and Flix (grey).

due to the presence of a PCB factory in the Michalovce district in Slovakia and the intensive industrial activity, particularly in the chemical sector, in the former Czechoslovakia (Petrik *et al.*, 2006; Cerná *et al.*, 2008). In Flix, PCBs were manufactured from 1959 to 1987, when their production ended. However, the serum concentrations in their inhabitants are in the range of other populations, as mentioned before, and not reflect any particularly high exposure. On the other hand, consumption of local contaminated fish was an additional source of PCB intake in the Flix inhabitants, as reported by Sala *et al.* (1999). This could be a possible explanation for finding slightly higher levels in Flix than in Catalonia.

3.6 Influence of the Flix electrochemical factory on the Catalan population

Selected municipalities from the population of Catalonia have been grouped by the area of influence from Flix, resulting in a first area of influence located 15-25 km around from Flix and a second area located 30-40 km around from Flix (see map from Figure 1). The rest of municipalities are leaved as Catalonia.

For all the analysed compounds, the median concentrations found in the 1st area of influence from Flix are intermediate between those from the Flix township and the 2nd area of influence, as shown in Table 4. Only in the case of hexachlorobenzene, the main pollutant emitted into the atmosphere by the chloro-alkali plant, the concentrations in the Flix inhabitants are much higher than in the individuals from the rest of the Catalan areas, related to the great proximity of this population to the factory. Therefore, the individuals living in the 1st area of influence from Flix have not been directly influenced by the HCB atmospheric emissions from the factory, although their levels are slightly higher than the other Catalan areas (1.5 ng/ml vs. 1.2 ng/ml, respectively; Table 3). For the rest of compounds, the differences in the concentrations between Flix, the 1st area of influence and the rest of municipalities have a clear systematic decreasing pattern. Whereas Flix has the highest levels, the first area of influence

shows lower levels than Flix but higher than the 2nd area of influence, and finally, both the 2nd area and Catalonia have similar levels, which are the lowest ones (Table 4). Only in the case of PCB-153, median concentrations are higher in the 1st area of influence than in Flix, probably explained by the high contribution of this compound in the Catalan study (Figure 2).

Another way to present the results is shown in Figure 3, which it also takes into account age and sex to control for. As explained in the data analysis section, this figure shows the expected concentration in each area, adjusted for age and sex. The figure shows again this pattern of decreasing concentration as the distance from Flix increases. This happens for all OCs, although certain compounds show specific behaviours. As mentioned before, concentrations of HCB in Flix are so high that they do not allow to observe a clear decrease pattern within the areas of influence. This also happens but with lesser extent for β-HCH. For this compound, high differences in median concentrations are found between the selected areas: 6.5 ng/ml in Flix, 1.2 ng/ml in the 1st area of influence, and 0.61 ng/ml and 0.70 ng/ml in the 2nd area and the rest of Catalonia, respectively (Table 4). Figure 3 also shows a big difference in β-HCH levels between Flix and the 1st area of influence, after controlling for age and sex. On the other hand, the OCs that clearly show a decreasing pattern are 4,4'-DDE, PCB-153 and PCB-180. Although PCB-153 showed higher median levels in the 1st area of influence than in Flix (0.89 ng/ml vs. 0.85 ng/ml; Table 4), this concentration effect is reversed when controlling for age and sex (Figure 3). Significant differences in the concentrations between Flix and both the 2nd area of influence and the rest of Catalan municipalities are found for these compounds (4,4'-DDE, PCB-153 and PCB-180), while the concentrations in the 1st area of influence are clearly intermediate between them (Figure 3). For the other compounds (namely 4,4'-DDT, PCB-118 and PCB-138), although a decreasing pattern is also found, the concentrations in the 1st area of influence are not so clear than in the aforementioned compounds.

Those results reflect a slight but noticeable influence of OC contamination from the electrochemical factory on the population living in the municipalities located around 15-25 km away from Flix. Furthermore, at a certain point of distance, there is no effect in the variation of the concentrations in the population, as shown by the similar concentrations found in the 2nd area of influence and the rest of the Catalan municipalities.

This is the first approach that employs human serum concentrations from general populations to assess the effects of contamination from an emission point (in this case, a chloro-alkali plant). Previously, in the same Flix context, a study carried out some years ago determined the concentration of several POPs in olive tree leaves of selected sampling points around the source of emission of pollutants, in the surroundings of the chloro-alkali plant. It estimated a radial influence that varied be-

Table 4: Median concentrations (in ng/ml) of organochlorine compounds from each area. All values refer to samples with quantifiable levels.

Area	n	HCB	β-HCH	4,4'-DDT	4,4'-DDE	PCB-118	PCB-138	PCB-153	PCB-180
Flix	421	14.4	6.5	0.86	4.5	0.33	0.79	0.85	0.96
1st area	42	1.5	1.2	0.27	3.7	0.20	0.61	0.89	0.69
2nd area	172	1.2	0.61	0.17	2.4	0.17	0.43	0.60	0.47
Catalonia	705	1.2	0.70	0.22	2.7	0.17	0.47	0.64	0.50

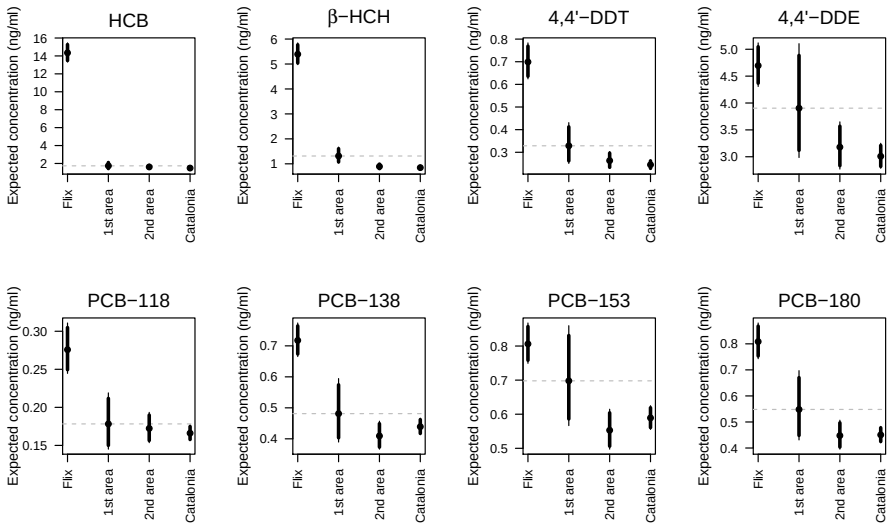

Figure 3: Expected concentrations (ng/ml) for each area of the population of Catalonia, adjusted for age and sex. Reference category is a middle-aged women. Confidence intervals at 95% and 90% in the thick and thin lines, respectively, are shown.

tween 3 and 34 km from the factory, depending on the pollutant studied (Bosch, 2009). For HCB, the radial influence from the factory was 14.3 km. This finding is consistent with our results, that suggest that HCB has a extreme effect but restricted to very close areas from the factory (less than 15 km). Therefore, we do not observe much higher HCB levels in the 1st area outside Flix.

One of the limitations of this study is related to the eight year time period elapsed between the two study recruitments'. A decreasing trend in OC serum concentrations between both studies is expected, since the use of OCs has declined as consequence of legal regulations. Studies of temporal trends of OCs in the area are very scarce. One paper reported a statistically significant reduction of 61% in the serum concentrations of HCB in women from Flix studied in 1997-99 compared to 1994 (Ribas-Fitó *et al.*, 2003). However, for the other compounds no statistically significant changes were found. Therefore, temporal differences between the studies (1994 vs. 2002) may explain part of the differences found between the concentrations in the Flix inhabitants and the individuals living in other nearby towns in Catalonia.

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Supplementary research articles

During the doctoral research, the author participated as co-author in a number of related studies, which are already published, that provide complementary information and insights to the core research project.

They all turn around the human exposure to organochlorine compounds, but assessing different geographic areas and populations, as follows: one on the population of Catalonia (using the same health survey of the core research, 2002), another on the city of Barcelona (based on a municipal health survey conducted in 2006) and another on the island of Menorca (based on infant population data from 1999).

These publications support the core research and will enhance the discussion chapter further down; they are next cited and summarised, while their full text is attached in Annexes I, II and III, respectively, at the end of the dissertation.

Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Catalonia
Environment International 36: 655–664 (2010)
Article attached in Annex I

This article highlights the socio-demographic determinants influencing the levels of organochlorine compounds in the general population of Catalonia.

Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Barcelona in 2006, and comparison with levels in 2002
Science of the Total Environment 423: 151–161 (2012)
Article attached in Annex II

This article compares the levels and trends of organochlorine compounds in the general population of Catalonia (2002) and the population of the city of Barcelona (2006), both conducted with the same chemical methodology.

An evaluation of the sexual differences in the accumulation of organochlorine compounds in children at birth and at the age of 4 years
Environmental Research 110: 244–250 (2010)
Article attached in Annex III

This article verses about the sexual differences of organochlorine compounds in the infant population of Menorca.

5 Discussion

Despite the growing concern on the health impact of POPs on humans, research on both the occurrence and the patterns of accumulation of POPs in general human populations is rather limited, and often based on small research samples. In-depth analysis on the observed data and related patterns of accumulation is usually missing. The current research precisely aims at enriching both the information and the analysis on the bioaccumulation dynamics of these compounds in an entire population (that of Catalonia), on the basis of a large experimental sample (around 1,000 well-representative samples), and carefully assessing the extent and patterns of accumulation of POPs against several sociodemographic determinants. Such research then allows exploring various hypotheses on the role of both the physical-chemical properties of pollutants and the human metabolism in explaining the accumulation patterns, hence showing how the physical-chemical environment and the biological systems interact.

The present chapter, organised into six sections, discusses the different results generated by this varied research project, comparing and contrasting them, both between them and with the existing literature, while also examining various hypotheses that may explain the encountered patterns. In essence, it comprises as follows:

- Discussion on the methodology that has been employed, and partly designed specifically for this project, to detect and quantify the studied compounds, and that has been applied to the samples of the population of Catalonia.

- Cross-examination of the concentrations of OCs and PBDEs in the studied population of Catalonia, comparing them with results from other populations worldwide and with other related research, while also looking at differences across contaminant classes, such as between legacy (old) and emergent (new) POPs.

- Discussion on the specific and unusual patterns of accumulation of POPs by age, sex and body fat, and when they are considered together, that this research has encountered.

- An inquisitive discussion on the role of the physical-chemical properties of POPs, on the one hand, and the role of the human metabolism, on the other, to explain the patterns of accumulation observed. This section explores some optional hypotheses that end up revealing the intricate and important connexions between the physical environment and the human metabolism that underlie the accumulation dynamics of pollutants.

- Discussion on results when other socio-demographic determinants (*e.g.* educational level, social class, and places of birth and residence) are considered; they overall do not seem to be relevant determinants in POP accumulation in humans.

- Final remarks, highlighting which should be the key factors to examine first to assess the accumulation of OCs and PBDEs in humans.

5.1 Detection and quantification of POPs

The detection of POPs needs accurate methodologies, including due consideration of the limits of both detection and quantification (LD and LQ, respectively), for each compound (see section 3.2 for details). Such accuracy, in measuring techniques and in analytical parameters, is indispensable to produce reliable estimations of the presence of such compounds in any studied population and hence to infer hypothesis and conclusions with confidence.

LD and LQ are important parameters for defining the accuracy of the analytical method. There are different ways to estimate them, but they are not all equally valuable. Depending on the method used, the values may vary by an order of magnitude. For this reason a robust research should first establish uniform definitions of these terms, in order to be able to compare data from different laboratories and analytical methodologies.

Traditionally, detection and quantification limits were calculated using the concentrations found in procedural blanks, but there are other methodologies for their calculation that have been applied in some studies (*e.g.* signal/noise ratio and calibration lines). The present research has actually designed a specific, tailored methodology to better assess LD and LQ and, hence, to enhance the accuracy of the results and the ability to compare them across the board.

A new methodology for the calculation of LD & LQ in OCs

The existing measurement methods of LD and LQ in OCs have room for improvement, notably to enhance accuracy, and hence the research tried successfully to develop a new, more accurate method. ARTICLE 1 in the Results chapter introduces and describes such new methodology for the calculation of LD and

LQ, specifically tailored for the analysis of OCs in human serum. It was then applied for the assessment of OCs levels in the population of Catalonia.

The developed methodology takes into account various factors, notably:

- the effects and interference of the matrix (in this study: blood serum);
- variability of the extraction procedure; and
- background noise generated by the analytical instrument (in this study, GC-ECD).

This methodology is based on the signal/noise (S/N) ratio found in the chromatograms of Proficiency Testing Materials (PTMs) from the AMAP Ring Test Programme (Centre de Toxicologie. Institut National de Santé Publique du Québec, 2012). The advantage of this approach is threefold. First, the PTMs from the AMAP Ring Test are real serum samples and therefore matrix effects and interferences can be taken into account. Secondly, being samples from an inter-calibration programme between laboratories, the variability of extraction procedures can also be taken into account. Finally, the concentration of each analyte in the PTMs is known from the Ring Test report and hence can be considered for the final calculation of the LD/LQ, thus enhancing accuracy and robustness.

As also shown in ARTICLE 1, the LD and LQ obtained from the methodology developed in this study have been compared to those calculated with other two different methodologies, namely blank samples and calibration straight lines, from the study itself. The S/N based methodology that has been designed was not only the one that provided the lowest values, but also the one with regular ones (ranging from 0.010 to 0.020 for LD and from 0.029 to 0.060 for LQ; Figure 3 in ARTICLE 1); conversely, the LD and LQ obtained from the other two

methodologies (blank samples and calibration curves) provided fluctuating values according to the compound (ranging from 0.021 to 0.058 for LD and from 0.034 to 0.129 for LQ in both other methods; Figure 3 in ARTICLE 1).

In essence, the research work conducted in ARTICLE 1 has not only broadened the array of methodologies available to estimate LD and LQ for the analysis of OCs in human serum, but also has found a more robust one. The Ph.D. endeavour thus comprises a methodological achievement.

Detection frequencies of OCs

Figure 5.1 shows the percentages of detection of the studied organochlorine compounds, based on the newly designed methodology.

Although the studied OCs were banned in Spain at the time the serum samples were taken (*i.e.* year 2002), all the individuals from the population of Catalonia show detectable levels in one or several OCs, as already discussed in SUPPLEMENTARY RESEARCH ARTICLES (Annex I; Porta *et al.* (2010)). This is due to the persistent presence of these compounds in the environment, resulting in a recurrent contamination of food products of animal origin (Darnerud *et al.*, 2006; Llobet *et al.*, 2003).

In fact, all the most common OCs were detected in over 95% of the samples. 4,4'-DDE and PCB-180 were practically present in every sample (over 99.8%), followed by HCB, β-HCH and PCB congeners' 138 and 153 (96-98%). Only 4,4'-DDT and PCB-118 showed less presence, at 84% and 80% respectively, yet they are notably frequent (Figure 5.1).

2,4'-DDT and both isomers of dichlorodiphenyldichloroethane (2,4'-DDD and 4,4'-DDD) were detected between 45% and 55% of the serum samples. For the rest of compounds, the percentages of non-detected were below 25% (PeCB;

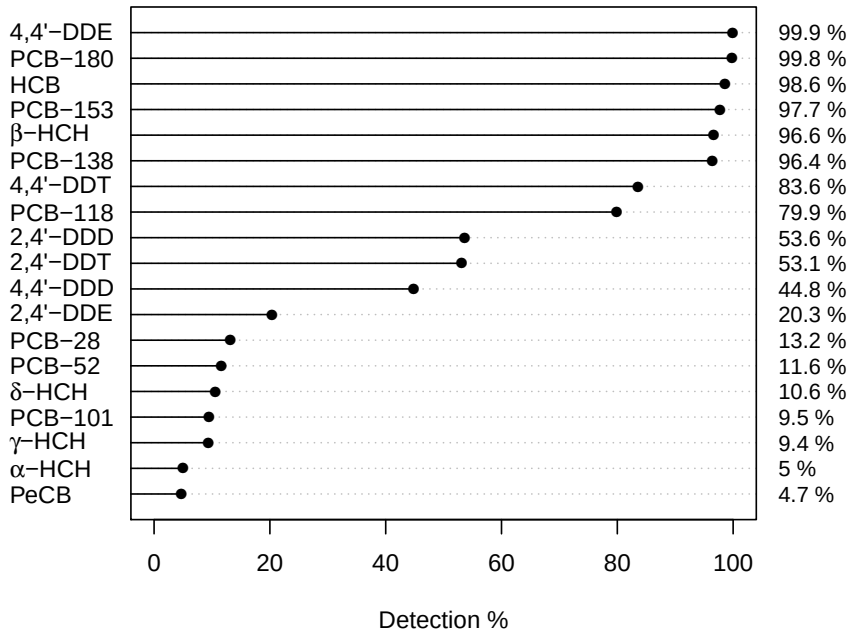

Figure 5.1: Detection levels of the OCs analysed.

the isomers α, δ and γ of HCHs; PCB congeners' 28, 52 and 101; and 2,4'-DDE).

Similar OC detection frequencies have been observed in other population studies from North America, Asia and Europe (Medehouenou *et al.*, 2011; Patterson *et al.*, 2009; Thomas *et al.*, 2006; Dirtu *et al.*, 2006; Petrik *et al.*, 2006; Becker *et al.*, 2002; Kang *et al.*, 2008; Gallo *et al.*, 2011; Jaraczewska *et al.*, 2006), as well as in several Spanish reports (Agudo *et al.*, 2009; Jakszyn *et al.*, 2009; Botella *et al.*, 2004; Zubero *et al.*, 2010). Curiously, the studies conducted in

Germany and Poland detected β-HCH in a much lower frequency (34% and 39%, respectively) (Becker *et al.*, 2002; Jaraczewska *et al.*, 2006).

Detection frequencies of PBDEs

The limits of detection and quantification of the analysed polybrominated diphenyl ethers (PBDEs) were calculated based on procedural blanks (n = 40). For PBDEs, unlike OCs, the existing methodology for the calculation of these values is well established, and robust enough, and does not require any refining or designing effort. At the IDÆA–CSIC laboratory, other studies measuring PBDEs in serum samples have also employed procedural blanks to calculate LD and LQ, with satisfactory detection frequencies (Carrizo *et al.*, 2007; Vizcaino *et al.*, 2009; Grimalt *et al.*, 2010).

Figure 5.2 shows the percentages of detection of each PBDE congener analysed.

As already presented in ARTICLE 3, the decabrominated congener is the most detected compound, in a relatively high presence (82%), followed by BDE-47 (74%) and BDE-153 (71%). Other PBDEs that have been detected between 50% and 70% of the serum samples are the congeners 28, 85, 99, 100 and 154 (Figure 5.2). The rest of PBDE congeners' analysed in this study (BDEs 17, 66, 71, 138, 183 and 190) have been detected in less than 50% of the samples (Figure 5.2).

In general, these detection frequencies are similar or even slightly lower than those found in other population studies from Europe, Asia and North America (Thomas *et al.*, 2006; Sjödin *et al.*, 2008a; Castorina *et al.*, 2011; Zhu *et al.*, 2009; Uemura *et al.*, 2010), including some Spanish reports (Gómara *et al.*, 2007; Vizcaino *et al.*, 2011a). Otherwise, congeners' 47 and 153 are always detected in

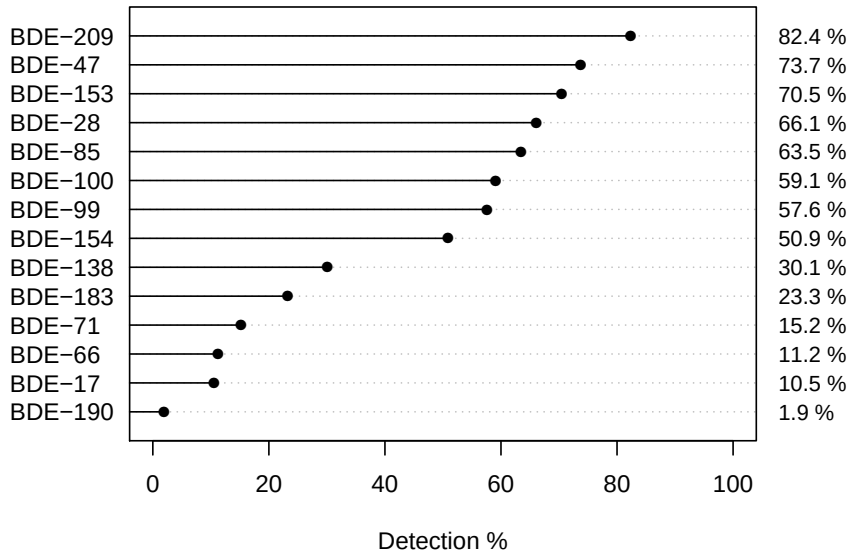

Figure 5.2: Detection levels of all the PBDE congeners' analysed.

much higher rates than BDE-209, whereas this latter compound, if analysed, is usually detected in a much lower frequency (for instance, 7% in the UK (Thomas *et al.*, 2006); 33% in Valencia (Vizcaino *et al.*, 2011a); 22% in China (Zhu *et al.*, 2009); 8% in the US (Johnson *et al.*, 2010)).

Detection percentages for PBDEs are lower than for the OCs, probably related to the lower levels of these compounds in human matrices and thus the added difficulty to detect them. In fact, an accurate analytical work was carried out by a highly sensitive system, namely the GC-MS-NICI, which allowed PBDEs to be detected in quantifiable concentrations.

The results show again the persistent presence of these compounds in the en-

vironment, similarly than for the case of OCs. In this case, however, PBDEs are not only present in foodstuffs (Schecter *et al.*, 2004, 2006), but also in household dust and other indoor environments, at both home and the workplace (Schecter *et al.*, 2005; Stapleton *et al.*, 2005; Jones-Otazo *et al.*, 2005; Webster *et al.*, 2005), and thus people are exposed to them in daily life.

5.2 Comparative analysis of POP levels in Catalonia

This research has, for the first time, assessed and described the concentrations of several persistent organic pollutants in the serum of individuals from the population of Catalonia. This deserves a careful examination, more so as the population sample was notable (*i.e.* around 1,000 samples from the general adult population, higher than most similar research). It further merits a comparative discussion with other populations across the world and with previous analogous research.

OC levels

Among the organochlorine compounds, 4,4'-DDE is the predominant one (median of 2.6 ng/ml; 436.3 ng/g lipid), followed by HCB (1.2 ng/ml; 201 ng/g lipid) and β-HCH (0.67 ng/ml; 111.6 ng/g lipid). Regarding PCBs, the most abundant congener is PCB-153 (median of 0.63 ng/ml; 104.3 ng/g lipid), followed by PCB-180 (0.50 ng/ml; 85.3 ng/g lipid) and PCB-138 (0.45 ng/ml; 74.1 ng/g lipid). 4,4'-DDT and PCB-118 are the major OCs found in less concentrations (medians of 0.18 ng/ml; 30.1 ng/g lipid for 4,4'-DDT and 0.13 ng/ml; 23.0 ng/g lipid for PCB-118). These concentrations have been presented and discussed in ARTICLE 4 in the Results chapter, as well as in SUPPLEMENTARY RESEARCH ARTICLES (Annex I; Porta *et al.* (2010)).

The compound distribution of DDTs in the Catalan population is remarkably dominated by the degradation product 4,4'-DDE (Figure 5.3). This means a lack of recent input of (or exposure to) the parent compounds, which is coherent with the ban of DDTs as agricultural pesticide in the country in the 1970s.

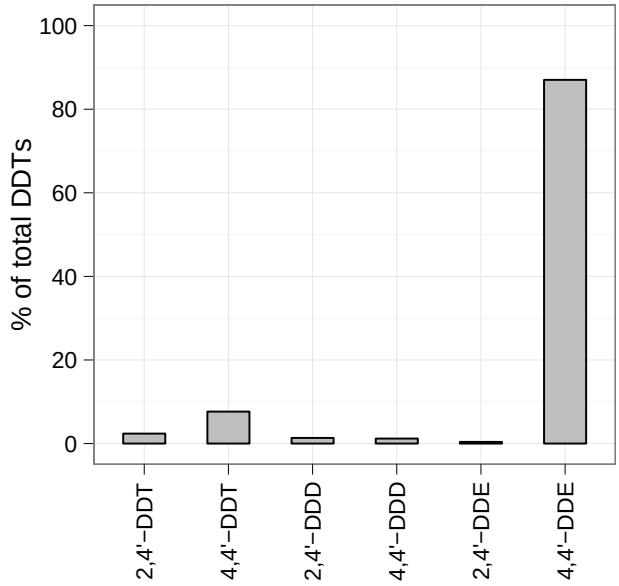

Figure 5.3: Contribution of the different isomers of DDTs to the total Sum of DDTs in the population of Catalonia.

The PCB congener profile is dominated by PCB-153, which contributes to the ΣPCBs in a percentage of 35% (Figure 5.4). Moreover, the correlation of this congener with the total amount of PCBs was also high (r=0.97), which is consistent with some other studies (Glynn *et al.*, 2000; Park *et al.*, 2007; Agudo *et al.*, 2009; Grimvall *et al.*, 1997). In fact, PCB-153 has been often used as a marker

of exposure for PCBs in monitoring studies. The other major PCB congeners (PCB-180, PCB-138 and PCB-118) contribute to ΣPCBs in less proportion (30%, 25% and 8% respectively), and the rest of PCBs (congeners 28, 52 and 101) are contributing to ΣPCBs in less than 2% (Figure 5.4).

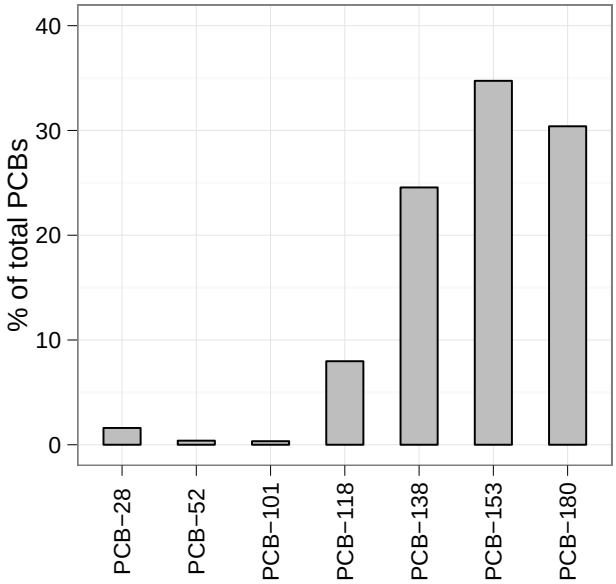

Figure 5.4: Contribution of the different individual congeners of PCBs to the total Sum of PCBs in the population of Catalonia.

In general, OC concentrations in Catalonia are slightly higher than in other populations worldwide, as already discussed in ARTICLE 4 (see Table 4). This should be put in the socio-economic context of Catalonia: a country with a strong commercial agricultural system, with a likely intensive use of organochlorine pesticides in the past. In this sense, in Slovakia, which has a solid farming

sector as Catalonia, the reportedly excessive use of pesticides in agriculture in the past also led to particularly high levels of 4,4'-DDE and HCB (Petrik *et al.*, 2006). When comparing the concentrations of 4,4'-DDT in Catalonia with reports conducted in countries with a current use of this compound to combat malaria (*e.g.* Mexico and India), the levels of Catalonia are proportionately much lower (Waliszewski *et al.*, 2012; Aulakh *et al.*, 2007).

Furthermore, differences in the levels of OCs between populations can be partly explained by differences in dietary habits. It is widely accepted that consumption of fish and other seafood are main determinants in higher levels of accumulation of organochlorine compounds, mainly PCBs (Sjödin *et al.*, 2000; McGraw and Waller, 2009). In short, higher rates of fish intake are associated with a higher body burden of OCs (Asplund *et al.*, 1994). Spain is a high fish-consuming country compared to other European countries or the United States (Welch *et al.*, 2002). In the study of Welch *et al.* (2002), Spain reported the highest intake of fish within ten European countries, for both white and fatty fish, although for very fatty fish (defined as containing more than 14% of fat) the highest consumption was reported in the coastal areas of northern Europe (Norway, Sweden and Denmark). In fact, Catalonia shows lower PCB levels than Sweden (Glynn *et al.*, 2000, 2003), likely related to this fatty-fish factor. Otherwise, data from the Slovak and Czech republics report much higher levels of PCBs because of a particularly intensive industrial activity, with poor regulation, in their original mother state of Czechoslovakia (Cerná *et al.*, 2008; Petrik *et al.*, 2006).

Another captivating finding from the present study is the comparatively high levels of HCB and β-HCH in Catalonia, as well as other Spanish populations, in relation to those found in other populations worldwide (Jakszyn *et al.*, 2009; de Salamanca *et al.*, 1990). This observation excludes the particular case of the Flix population, in Catalonia itself, which showed the highest HCB

concentrations ever found in humans due to high airborne levels of this compound from a nearby electrochemical factory (Sala *et al.*, 1999). The reason for the particularly high levels of these organochlorine pesticides in Spain is so far unknown, but has been revealed in the literature (Vizcaino *et al.*, 2010).

PBDE levels

The research project conducted represents, with n=731, the largest one on human PBDE concentrations ever performed in Europe and the second largest in the world, after the US-based NHANES study (Sjödin *et al.*, 2008a; NHANES, 2004). In addition, the research also includes the determination of decabromodiphenyl ether (BDE-209), a congener that is rarely analysed in studies conducted on general populations across the world (Hites, 2004) and that is absent in the large US-based NHANES database itself (NHANES, 2004).

In fact, the congener BDE-209 prevails among all the PBDEs in the research of the population of Catalonia (median of 3.7 ng/g lipid), representing itself alone more than a quarter of the volume of all the 14 PBDEs studied (Figure 5.5). This makes the absence of determination of BDE-209 in most environmental-health analysis across the world a surprising gap that this research compels to correct in future research designs. BDE-209 is followed by BDE-47, BDE-99 and BDE-153 (medians of 2.6 ng/g lipid, 1.2 ng/g lipid and 0.94 ng/g lipid, respectively); PBDE congener profiles (in percentages) are shown in Figure 5.5. In essence, BDE-209 accounts for 28% to total PBDEs, followed by BDE-47 and BDE-99 (18% and 10%, respectively).

The PBDE congener profile in the population of Catalonia, with a higher median concentration of BDE-209 than the usually dominant BDE-47, is unusual compared to other published data on humans (this question has been already discussed in ARTICLE 3). Usually, the pattern of distribution of PBDE

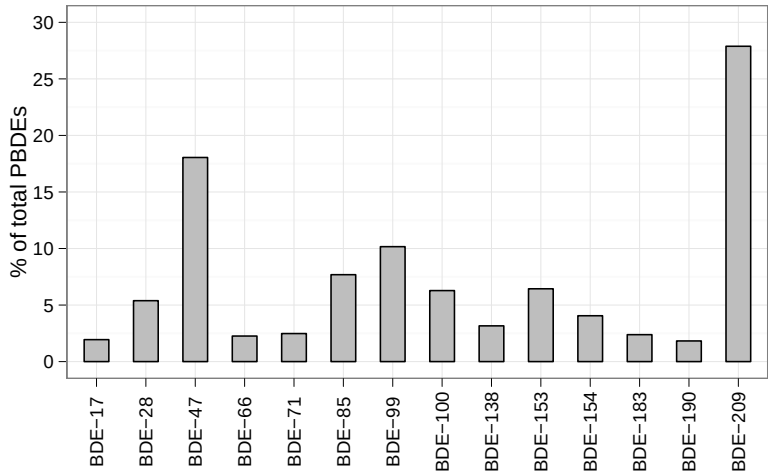

Figure 5.5: Contribution of the different individual congeners of PBDEs to the total Sum of PBDEs in the population of Catalonia.

congeners' in European and Asian studies is dominated by BDEs 47, 153 and 154 and, in lesser amounts, by BDE-99 (Vizcaino *et al.*, 2011b; Roosens *et al.*, 2010; Fängström *et al.*, 2005; Guvenius *et al.*, 2003). In North America, however, the distribution is dominated by BDEs 47 and 99, as well as BDEs 100 and 153 (Sjödin *et al.*, 2008a; Castorina *et al.*, 2011). Differences in congener profile may reflect exposure to different commercial PBDE formulations available: for instance, while Europe and Asia have mainly used octa-BDE and deca-BDE in high amounts (in which BDEs 153, 154 and 209 are the markers of these mixtures), the U.S. has used principally the penta-BDE formulation (in which BDEs 47 and 99 are the main congeners) (BSEF).

A final remark is that ΣPBDE concentrations in Catalonia rank among the highest in relation to other European and Asian countries but, as expected, they

are lower than those found in North America, as already discussed in ARTICLE 3 in the Results chapter (see Table 3).

The case of the Decabromodiphenyl congener (BDE-209)

According to the results from the present study, the most abundant BDE congener in the Catalan population is the decabromodiphenyl (BDE-209). This is a remarkable finding that demonstrates that BDE-209 is bioavailable and hence deserves due attention in health assessments.

A high contribution of BDE-209 to the total PBDEs has been already reported in previous Spanish studies conducted on breast milk and placenta samples (Gómara *et al.*, 2007, 2011). Moreover, this congener was the most predominant one in Spanish food (Gómara *et al.*, 2006), household dust (Fabrellas *et al.*, 2005), in sediment samples from several hot spots in the Spanish coast (Eljarrat *et al.*, 2005) and also in eggs from white stork colonies (Muñoz-Arnanz *et al.*, 2011), yet little attention was raised.

When the serum samples from the Catalan individuals were taken, in year 2002, the three major PBDE commercial mixtures were available in Europe and in significant volumes, especially octa- and deca-BDE formulations, so the population was widely exposed. The results from the present general-population research show such levels of exposure. Only two years later the European Union phased out the penta- and octa-BDEs due to their clear toxicity, bioaccumulation and persistence (their production also ceased in North America). Both formulations were later added to the *Stockholm Convention on Persistent Organic Pollutants*, in 2009. However, the deca-BDE formulation, which contains around 97% of BDE-209, remains legally available and continues being heavily used, more than ever. This is of concern because if in 2002 the decabromodiphenyl was the

most abundant BDE congener in the Catalan population, nowadays the serum concentrations of this compound could even be higher, in view that the other two BDE formulations have been already banned. Furthermore, studies have indicated that BDE-209 can be debrominated to form hexa- and penta-BDES, which are even more toxic (Stapleton *et al.*, 2004). Debromination of the higher congeners could have profound implications for public health and for the regulation of these compounds. The high levels of BDE-209 found in the individuals from Catalonia, coupled with its debromination potential through time, clearly suggests the need for policy and regulatory decisions to ban or phase out the major remaining PBDE commercial mixture (namely, the deca-BDE formulation).

Comparing legacy with emerging POPs

The levels of PBDEs in the population of Catalonia are approximately one to two orders of magnitude lower than the OC compounds (Figure 5.6), which is in accordance with data from other European population studies that have simultaneously investigated OC and PBDE levels in human serum or breast milk samples (*e.g.* U.K., Sweden, Poland and Spain itself) (Thomas *et al.*, 2006; Norén and Meironyté, 2000; Guvenius *et al.*, 2003; Gómara *et al.*, 2011; Jaraczewska *et al.*, 2006; Schuhmacher *et al.*, 2007, 2009). In the U.S., however, the levels of both types of POPs are in the same order of magnitude, with even comparatively higher PBDE levels (Sjödin *et al.*, 2004; She *et al.*, 2007). This is related to the high use of the three different commercial mixtures of PBDEs (penta-, octa- and deca-BDE) in North America, at 95%, 40% and 44% of the total world demand in year 2001 (BSEF 2003). In contrast, in Europe, the estimated uses of these formulations corresponded to 2% of penta-BDE, 16% of octa-BDE and 14% of deca-BDE of the total world demand (BSEF), which are considerably much lower than in North America.

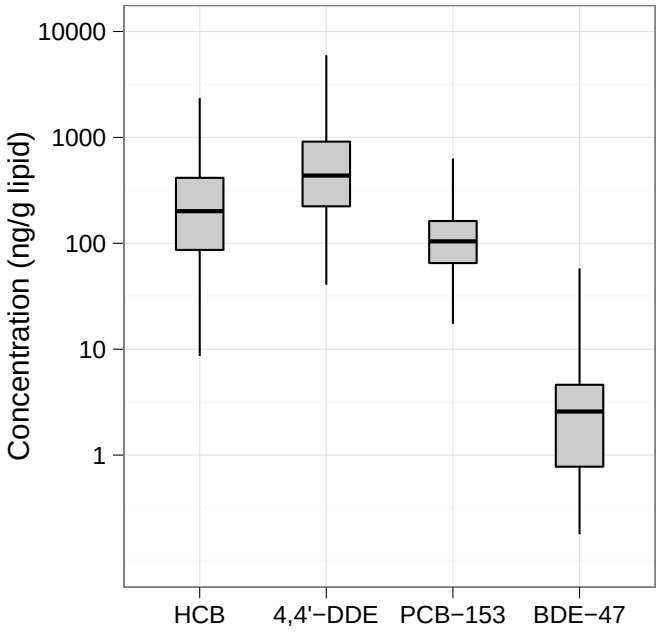

Figure 5.6: Boxplots of representative POPs: HCB, 4,4'-DDE, PCB-153 and BDE-47. Note that the y-axis is in logarithmic scale.

In any case, the differences in the concentrations between both classes of POPs are consistent with their different production and use periods. This raises the importance of distinguishing between legacy (old) and emergent (new) POPs. In Spain, as in many other Western countries, production and use of OCs started in the 1930s and lasted until the end of the 1970s, when they became to be restricted. In contrast, PBDE production was initiated in the 1970s. Consequently, human exposure to PBDEs is relatively recent compared to the legacy POPs such as PCBs and DDTs. In addition, and as a consequence of regulatory

restrictions, OC levels have diminished in biota and in human tissues (Bignert *et al.*, 1998), although the presence of OCs in food of animal origin remains a major source of human exposure (Ahlborg *et al.*, 1995).

In general, time trend studies conducted in human populations from Europe and the U.S. reflect a decreasing pattern in the concentrations of OCs since the 1970s and/or an increasing tendency for PBDEs (Thomsen *et al.*, 2002; Hagmar *et al.*, 2006; Schröter-Kermani *et al.*, 2000; Norén and Meironyté, 2000; Sjödin *et al.*, 2004; Axmon *et al.*, 2008; Hardell *et al.*, 2010). This demonstrates the positive impact of regulation, while appealing for more research and increasing controls on the remaining and the new (emergent) POPs.

Figure 5.7 shows the time trend pattern of certain organochlorine compounds and PBDEs in Sweden (extracted from Norén and Meironyté (2000)).

In Spain, studies on temporal trends of POPs are very scarce. One paper reported a statistically significant reduction of 61% in the serum concentrations of HCB in women from Flix studied in 1997-99 compared to 1994 (Ribas-Fitó *et al.*, 2003a), although for the other OC compounds the changes were not statistically significant. Jakszyn *et al.* (2009) also observed a clear decrease in serum levels of OC pesticides in different Spanish regions in a relatively short period of time (from 1992 to 1996): the decreases ranged between 13% for 4,4'-DDE to 50% for HCB. Another study reported a general decrease in PCB concentrations in breast milk samples collected between 1998 and 2007 in Catalonia (Schuhmacher *et al.*, 2009).

The most recent temporal trend study conducted in Catalonia focuses on the inhabitants of the city of Barcelona, as explained in SUPPLEMENTARY RESEARCH ARTICLES (Annex II; Porta *et al.* (2012)). Comparisons of the OC concentrations in the individuals living in Barcelona between 2002 (as extracted

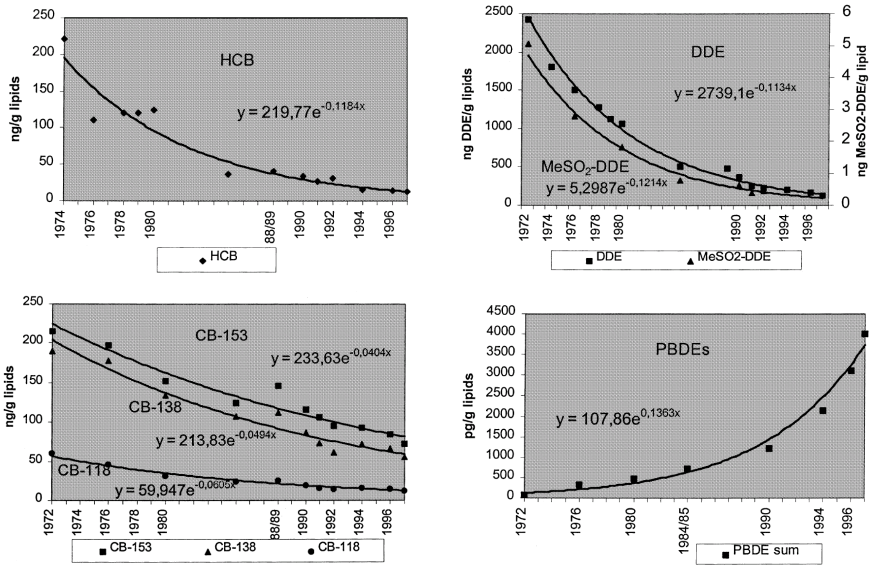

Figure 5.7: Figure showing the differences in time trend pattern of OCs and PBDEs, extracted from Norén and Meironyté (2000).

from the studied population of Catalonia) and 2006 (from a specific health analysis of the population of the city of Barcelona) show a decrease in the range of 34-56% for those compounds. This is a very significant decrease in only 4 years, demonstrating again that regulatory action can yield a positive health impact in short periods of time.

In summary, the population of Catalonia is contaminated with both classes of POPs. Although most of these compounds have been banned, they are still present in the food chain and in other environments and, thus, humans remain exposed to them. Furthermore, the still legal production and wide use of the fully brominated deca-BDE commercial mixture poses a major concern for the Catalan population since the studied individuals showed already in 2002,

a decade ago, relatively high levels of its major product, the decabromodiphenyl ether (BDE-209). Finally, inclusion of BDE-209 should be systematically incorporated in POP analysis as this represents a major population health threat, more as deca-BDE remain legal and used.

5.3 Influence of age, sex and the body mass index in the accumulation of POPs

One of the main findings in this Ph.D. research are the patterns of accumulation of POPs when age, sex and body mass index are considered, both separately and together. More specifically, the concentrations of certain organochlorine compounds are much more accumulated in women through age than in men, thus revealing an interactive effect between age and sex. Furthermore, the influence of the body mass index (BMI) in the accumulation of these compounds is proportionately higher among men than in women, thus reflecting another interactive effect (that between sex and BMI, in this case). On the other hand, PBDE compounds follow an inverse-age dependence trend, as young individuals show higher concentrations than adults (>30 years of age). These results were not so clearly revealed or demonstrated in previous research worldwide, and they are for the first time sustained by a large experimental sample and a robust analytical work. These results are presented in ARTICLE 2 (which deals on the interactive age& sex and sex& BMI effects for OCs) and ARTICLE 3 (on the inverse-age dependence in PBDEs); they are now reviewed and discussed together.

Nota bene for the clarification of terms: This research focuses on *sex* (which comprises variables that are exclusively biological) and not on *gender* (which refers to the socially-defined differences between men and women, as well as possible interactions between biological and environmental factors in humans) (Nobelius, 2004). Although any study on the environmental exposure of humans

to POPs may well consider gender differences (as they relate to diet, lifestyle or occupation), this research has focussed on sex differences to better assess the possible role of human metabolism in the accumulation of POPs, as done in section 5.4 below. Therefore the concept *sex* (or *sexual*) is used to properly capture the focus on the biological dimensions (such as metabolism) of POP accumulation.

Age divergence in POP accumulation

The research in the population of Catalonia shows a number of age-based differences in the accumulation of POPs, some of which are expected whilst others are rather unexpected.

First, the concentrations of organochlorine compounds increase with age (as indicated in the top and middle panels of Figure 5.8). This effect is logical, being already known and supported by an extensive literature that shows the positive association of the accumulation of OCs with age in very different general populations, from the U.S. to Slovakia, and from South Korea to Germany (Patterson *et al.*, 2009; Kang *et al.*, 2008; Dirtu *et al.*, 2006; Petrik *et al.*, 2006; Bates *et al.*, 2004; Becker *et al.*, 2002). The same pattern remains even in extreme conditions of exposure, such as in the highly-exposed population from the Flix township, in southern Catalonia, which hosts a chemical factory that used to produce various POPs (Sala *et al.*, 1999).

For PBDEs, in contrast, the study shows an inverse relation between age and serum concentrations (see bottom panel series in Figure 5.8). In essence, young individuals (<30 years of age) show the highest concentrations of major PBDE compounds (mainly BDEs 47, 99 and 209), in relation to adults. Otherwise, people aged over 30 years show similar PBDE concentrations irrespective of ageing.

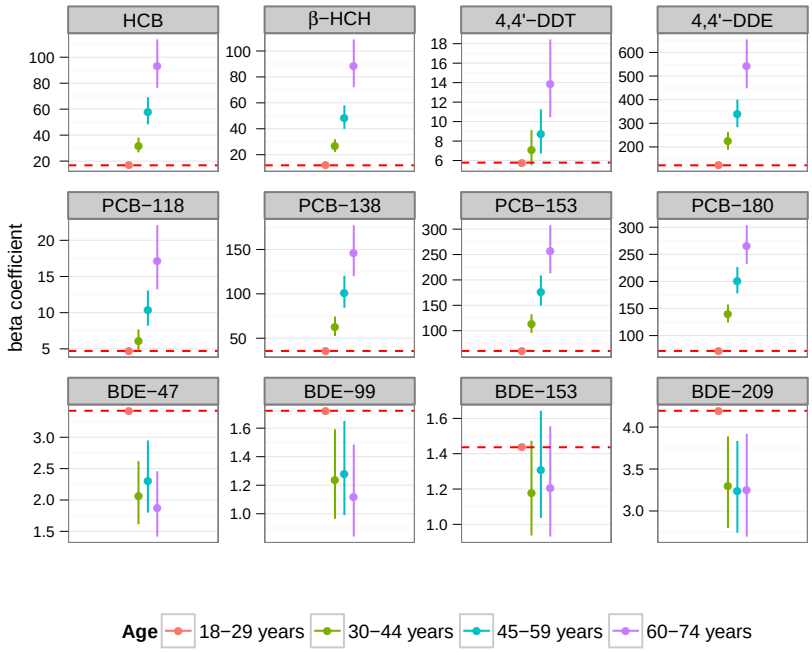

Figure 5.8: Adjusted effect of age on POP concentrations. Each panel (describing accumulation of a specific compound) results from a different multivariate regression model adjusted for sex and body mass index. The reference category is the youngest age group (18-29 years), represented by the horizontal line.

This set of results go against the logics of a higher presence of POPs with age, which is related to the persistence property of POPs, as well as the majority of the research literature. Most previous reports did not observe any change in adult PBDE concentration with age (Bradman *et al.*, 2007; Gómara *et al.*, 2007; Harrad and Porter, 2007; Julander *et al.*, 2005; Lee *et al.*, 2007). Only two major studies, conducted in the U.S. and Australia, found higher concentrations of some PBDEs (congeners 47, 99, 100 and 153) among the youngest individuals,

including infants, children and young adults (<30 years of age) (Sjödin *et al.*, 2008a; Toms *et al.*, 2008, 2009). These studies, however, did not explore their findings and therefore some hypothesis are next discussed.

Since both compound groups (OCs and PBDEs) have similar physical-chemical properties, one would predict a similar pattern of accumulation. An hypothesis to explain their divergent pattern of accumulation draws attention to their different history in terms of human manipulation: OCs began to be manufactured and used in the 1930s whereas PBDEs did not begin to be commercially distributed until the 1980s, that is, half a century later. In consequence, OCs have been polluting human populations for a much longer period of time compared to PBDEs.

When age is further disaggregated by year/period of birth, the differences in the pattern of accumulation between both types of POPs are even better revealed (Figure 5.9).

In essence, the individuals who were born in the 1930s and later (when OCs began to be manufactured and used) show much higher levels of organochlorine compounds than people born in the period 1970–1980 (when those compounds actually began to be highly restricted or banned). Therefore, young people have not only been exposed to OCs for less time, but also to lower concentrations due to recent regulatory restrictions or even prohibition in the releasing of those components. In consequence their body burden is lower in both length of time exposure and intensity (Medehouenou *et al.*, 2011; Gaffney *et al.*, 2005).

Furthermore, the timing of exposure is also a contributing dimension. In the case of PBDEs, the youngest individuals from the present study (who were born between 1972 and 1984) were particularly exposed to PBDEs during their growth period, when they were newborns and infants, whereas the rest of the population –the adults– had a PBDE-free infancy. In fact, children are more

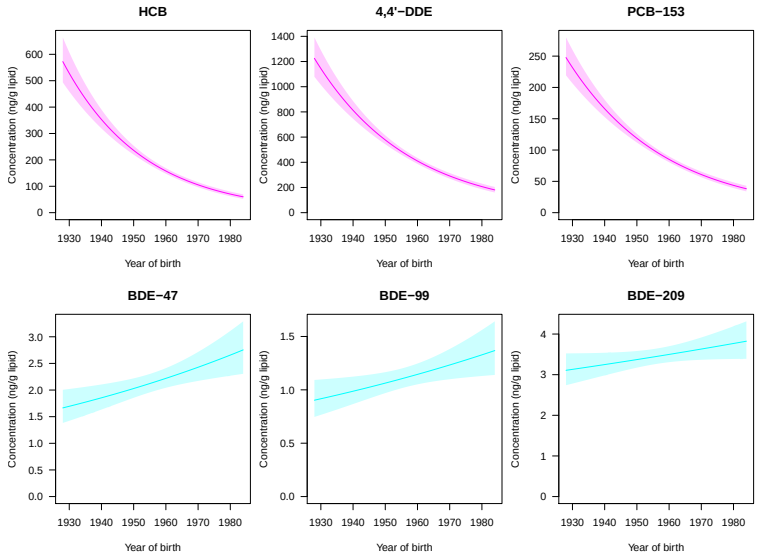

Figure 5.9: Series of graphics showing the patterns of accumulation of the two types of POPs studied (*i.e.* OCs and PBDES) by the year of birth, adjusted by sex and body mass index. The top panel shows representative OCs (HCB, 4,4'-DDE and PCB-153) whereas the three major PBDEs are shown in the bottom panel (BDEs 47, 99 and 209).

prone to incidental dust ingestion, which is a considerable pathway of PBDE exposure (Toms *et al.*, 2009); besides, children have a higher metabolic pace than adults. Therefore, POP exposure during infancy may accelerate body burden. Moreover, the mentioned young people were children precisely in the period of the highest degree in the use of materials containing PBDEs. In addition, the relatively recent history of PBDEs implies shorter time of population exposure and thus the absence of any relevant age-related difference among older people. On the other hand, the fact that BDE-209, which is the compound with the most dissimilar physical-chemical properties among those studied, also shows the same trend reinforces the hypothesis that timing of exposure is equally a

crucial factor in the accumulation patterns of POPs.

In essence, the human history of POP manipulation represents a relevant factor explaining the age-based divergence in the patterns of accumulation between OC and PBDE compounds. Nevertheless, one should not rule out the possible influence of lifestyles, dietary habits or metabolic differences between young and old individuals to explain the higher accumulation of PBDEs among the younger segment (Sjödin *et al.*, 2008b). This has not been demonstrated so far, and the present research lacked accurate data to verify that.

In summary, the pattern of accumulation of POPs is not simply related to age in a positive correlation, but rather to the time and timing of exposure. Individuals that were intensively exposed to a pollutant at infancy may actually suffer more accumulation than elder individuals. Regulatory restrictions also free population segments from exposure and ease reduction in body burdens. Therefore, environmental and public health agencies should be continuously alert to the production, crafting and release of new chemical compounds, which could suddenly pose a health toll to the population, with a disproportionate impact with lasting effects to our children (especially in view that children are particularly vulnerable to exposures of all types of pollutants).

Sex-based differences in the accumulation of POPs

The pattern of accumulation of POPs in the population of Catalonia shows differences by sex, notably for organochlorine compounds (Figure 5.10).

In particular, women show particularly higher concentrations of HCB, β-HCH, 4,4'-DDT, 4,4'-DDE and PCB-118, in all of them with statistical significant results. The concentrations of PCB congeners' 138 and 153 do not show sex differences. In contrast, men have significantly higher concentrations of PCB-180.

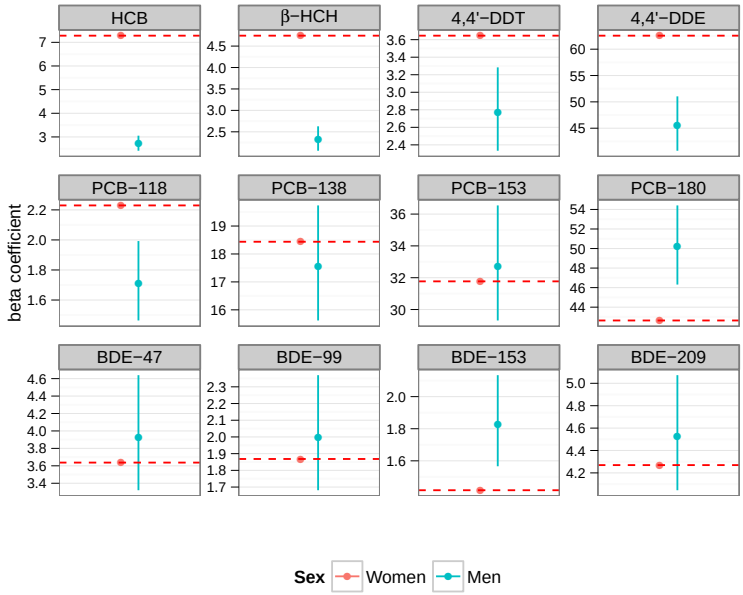

Figure 5.10: Adjusted effect of sex on POP concentrations. Each panel (referring to a single compound) is a different multivariate regression model adjusted for age and body mass index. The reference category is women, represented by the horizontal line.

Sexual differences in the accumulation of OCs have also been reported in previous, robust studies (Patterson *et al.*, 2009; Dirtu *et al.*, 2006). A recently published Canadian report, which was conducted on older individuals (aged 65 and over; n=1,979), also showed this differential sexual pattern, although in this case the PCBs 138 and 153 were also higher among women (Medehouenou *et al.*, 2011). Other studies, however, have reported opposite concentration levels (Petrik *et al.*, 2006; Gaffney *et al.*, 2005; Bates *et al.*, 2004; Cruz *et al.*, 2003) or did not show much evidence of sexual differences in OC concentrations (Thomas *et al.*, 2006; Hanaoka *et al.*, 2002; Cerná *et al.*, 2008; Park *et al.*,

2007). Nonetheless, some of these latter studies were based on reduced samples, which may partly explain their inconsistencies. It is to be noted that the present research used a population sample of more than 900 individuals, which was unusually higher than previous, similar researches, while also based on a robust analytical method with precise detection limits, which reinforces the confidence in the results obtained.

Regarding PBDEs, sexual differences are only found for BDE-153, with statistically significant higher concentrations among men (see botom panel of Figure 5.10). For the rest of PBDE congeners, women and men show similar levels (just slightly higher in men).

In general, no sexual differences in the levels of PBDEs have been found in other population studies (Anderson *et al.*, 2008; Harrad and Porter, 2007). Only two major reports have also found a statistically significant sexual difference in BDE-153 accumulation (Sjödin *et al.*, 2003; Toms *et al.*, 2009). These studies were also conducted on a large sample sizes (n=2,400 in the NHANES study from the U.S. and n=80 pooled samples from a population of 8,132 individuals in Australia) and hence the pattern could be retained.

The differences in the concentrations of certain POPs between men and women are likely to result from differences in diet, in occupational exposures or in metabolism (Robrock *et al.*, 2008; Salihovic *et al.*, 2012). In fact, dietary habits between men and women diverge more than it is generally assumed: for instance, a study conducted on an elderly population (65-79 years of age) from the United Kingdom demonstrated significant gender differences in food choices, energy and nutrient intakes, as well as plasma concentrations of cholesterol (Bates *et al.*, 1999). The dietary pattern, in terms of products and quantities, might play a significant role in the body burden of POPs. At the same time, occupational conditions may also diverge between men and women, although

increasingly less with social changes towards gender equality in the most recent decades (Takasuga *et al.*, 2004; Covaci *et al.*, 2008). Finally, human metabolism is a relevant factor to consider to explaining sex differences in POP accumulation, related to different means and capacity to excrete accumulated POPs (this is further explored in the section 5.4 later on).

In essence, sex differences in the accumulation of several POPs remains clearly observed, yet with inconclusive explanations. In order to further examine this notable sexual difference, the research also considered the interactive effects between sex and other parameters, discussed later in this section.

Body mass index and POP accumulation

The studied population of Catalonia shows different patterns of accumulation of POPs in relation to the body mass index (BMI) (Figure 5.11).

The concentrations of the pesticide-related OCs tend to increase along with an increasing BMI (top series in Figure 5.11). In short, overweight and obese individuals have significantly higher concentrations of HCB, β-HCH and 4,4'-DDT than normal weight individuals. For 4,4'-DDE, the concentrations are only significantly higher in obese individuals, while no statistically significant differences are found between normal weight and overweight individuals.

The pattern of accumulation of PCBs by BMI, however, does not show any regular pattern (middle series of Figure 5.11). Whereas the concentrations of PCB-118 in overweight and obese individuals are significantly higher than in normal weight individuals, the differences between the three BMI groups for PCB congeners 138 and 153 are not statistically significant; furthermore, the concentrations of PCB-180 in overweight and obese individuals are, in fact, much lower than in normal weight individuals, with statistically significant differences.

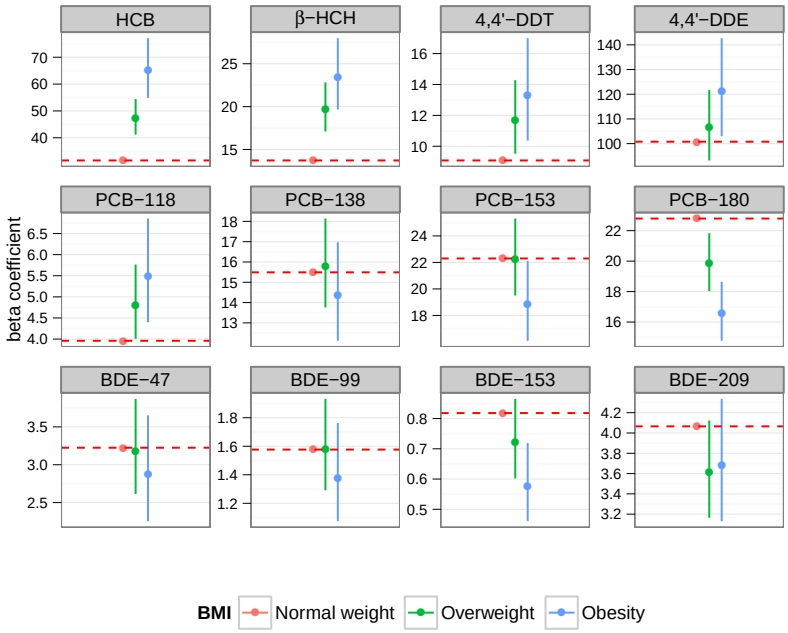

Figure 5.11: Adjusted effect of body mass index on the concentrations of OCs. Each panel (referring to a specific compound) is a different multivariate regression model adjusted for age and sex. The reference category is the normal weight status, represented by an horizontal line.

In the case of PBDEs, the concentrations between normal weight, overweight and obese individuals do not show statistically significant differences, except in the case of BDE-153 (bottom panel of Figure 5.11). For the BDE-153, the concentration follows a significant inverse relation with BMI, with lower concentrations among obese individuals in relation to overweight and normal weight individuals, alike the case of PCB-180.

The influence of body mass index in the accumulation of POPs has been extensively studied, yet with rather inconclusive insights. This research results are

consistent with such previous research. In particular, ARTICLE 3 and SUPPLEMENTARY RESEARCH ARTICLES (Annex I; Porta *et al.* (2010)) demonstrates an inverse pattern of accumulation of certain compounds (*e.g.* PCB-180, PBDEs) by BMI.

It can be noticed that the patterns of accumulation of POPs by BMI mirror those found when sex is considered as a factor, as discussed in the previous section. This research thus shows, for the first time, a similar pattern of accumulation according to BMI and sex, as follows: whereas women have higher concentrations of certain OCs (notably HCB, β-HCH, 4,4'-DDT, 4,4'-DDE and PCB-118), precisely these compounds follow an increased pattern of accumulation with increasing BMI; conversely, for the compounds that are more accumulated in men (mainly PCB-180 and BDE-153), the BMI shows an inverse trend, as the concentrations are lower among individuals with higher BMI; and, finally, for those compounds without significant sexual differences (*e.g.* PCB-138, PCB-153 or BDE congeners 47, 99 and 209), no divergent BMI tendency is observed.

Furthermore, the fact that the only brominated compound that shows a significant sexual difference also presents a significant BMI trend reinforces the hypothesis that the accumulation patterns of persistent organic pollutants are similar for BMI and sex and, hence, a likely connection exists.

These research findings therefore suggest that both BMI and sex are important and interrelated factors explaining the patterns of accumulation of POPs in humans, together with the age. The next section discusses these observations all together, simultaneously, thus introducing the interactive effects between age and sex, on the one hand, and sex and BMI, on the other.

Simultaneous consideration of age, sex and BMI as key factors in POP accumulation

The simultaneous analysis of the concentrations of POPs by age and sex results in an interactive effect between both factors in various compounds (Figure 5.12). This is a new finding of this research, unexpected according to scientific predictions. More specifically, there is a very pronounced accumulation of certain pollutants in women through age, compared to men. This is notably observed for HCB and β-HCH, as well as, to some degree, for 4,4'-DDT, 4,4'-DDE and PCB-118. The interactive age and sex effect is however absent in the other PCB congeners and also in PBDE compounds (middle and bottom series of Figure 5.12).

This interactive effect between age and sex was also found in another general population (U.S.) and in a highly-exposed one (Flix industrial township in Catalonia itself), as already shown in ARTICLE 2 within the Results chapter.

Furthermore, the research reveals that HCB and β-HCH (the two compounds with the highest interactive effect between age and sex) also exhibit an additional interactive effect between sex and BMI. More specifically, these compounds are much more accumulated with increases in body mass index in the case of men; yet not for women (Figure 5.13). This interactive sex and BMI effect is not observed in the other studied compounds, in an analogous way as no sex-and-age interaction was significantly observed.

ARTICLE 2 in the Results chapter discussed these facts and explored various hypothesis, concluding that these differential trends may probably lie in the conjunction of both the physical-chemical properties of these compounds and the dynamics of human metabolism. In fact, human metabolism remarkably differs between men and women, across age (infancy *versus* youth *versus* old people),

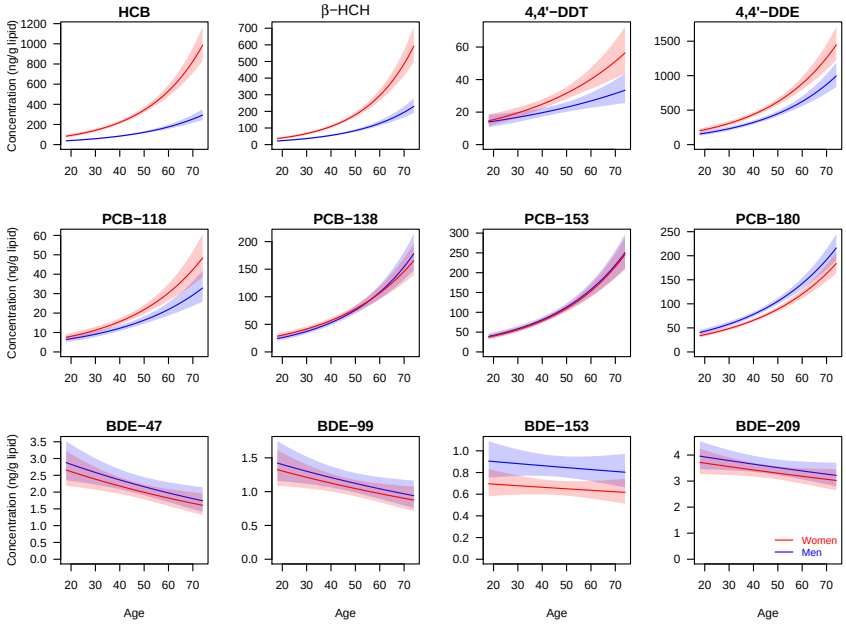

Figure 5.12: Series showing the accumulation of several POPs by age and sex. Certain compounds (notably HCB and β-HCH) show an interactive effect between both factors. Women are represented in red and men in blue.

as well as between normal weight and obese individuals, hence likely explaining the unexpected patterns of divergent accumulation of POPs when sex, age and BMI are considered, whether separately or together. The potential roles of both the physical-chemical properties of POPs and the human metabolism in the patterns of POP accumulation are discussed next.

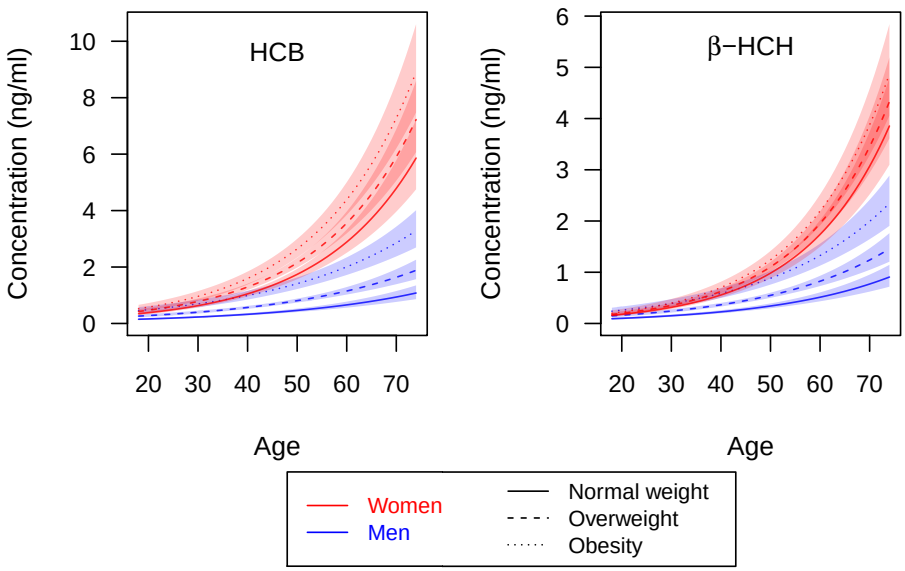

Figure 5.13: Figure showing the accumulation of HCB and β-HCH by age, sex and BMI. These compounds show two interactive effects: age*sex and sex*BMI. Women are represented in red and men, in blue. Weight representation: continuous line: normal range; dashed line: overweight; dotted line: obese.

5.4 Exploring the underlying hypotheses: from the physical-chemical properties of POPs to the dynamics of human metabolism

This research has observed a number of patterns of accumulation of POPs in a general population, based on a large sample size as well as comparative analysis, and revealed along the way some unexpected tendencies and associations between certain factors (notably age, sex and body mass index). When carefully

considering these results, a number of explanatory hypotheses were explored, which led into considering that both the physical-chemical properties of POPs and the dynamics of human metabolism lie beneath the observed facts and patterns. This section examines these working hypotheses.

The potential role of the physical-chemical properties of POPs

The analysis of the relationships between persistent organic pollutants, on one side, and the effects of age, sex and BMI, on the other, has revealed specific patterns and some unexpected trends. They seem to be partly related to the physical-chemical properties of the concerned compounds.

First, and above all, the OCs that clearly respond to the interactive effect between age and sex are precisely the most volatile ones. This interactive effect thus appears to be correlated with the degree of volatility of the compounds: the women's higher accumulation of OCs is extremely pronounced in the most volatile ones (HCB and β-HCH), it is moderately perceived in the moderately volatile OCs (4,4'-DDT, 4,4'-DDE and PCB-118), and it is absent in the least volatile OCs (PCB-138, PCB-153 and PCB-180).

Furthermore, the patterns of accumulation in PCBs by sex appear to be correlated with the degree of chlorination of the congeners (which at the same time is a physical-chemical property intrinsically related to the previous one on the volatility of the compounds, as the more chlorinated ones are the least volatile). Hence, with increasing the degree of chlorination, the levels of PCBs become higher in men, to the extent that men have higher concentrations of PCB-180, which is the most chlorinated one among the studied congeners.

A similar observation was recently published in a study conducted on elderly people from Sweden, which found higher accumulation levels in the two least

chlorinated PCBs (including the congener 118) among women and higher levels of five PCBs (including the congener 180) among men (Salihovic *et al.*, 2012).

To further examine if the physical-chemical properties of all those compounds are associated with the interactive age and sex effect, an analysis of two selected properties (Koa and Kow) against the accumulation pattern obtained from multivariate regression models are shown in Figure 5.14. They reinforce the hypothesis that the physical-chemical properties of POPs explain, albeit partly, the patterns of POP accumulation found.

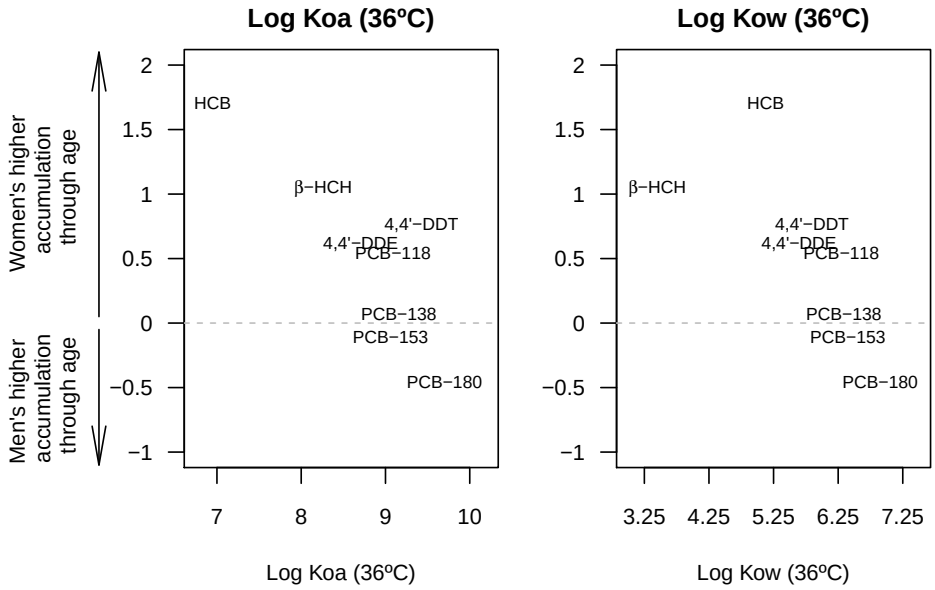

Figure 5.14: Associations between OC physical-chemical properties (Log Koa and Log Kow) and age, sex and interactive age and sex effect from multivariate regression models.

The present research went one step further, with the analysis of another factor, namely the body mass index, which reveals the same tendency found in

age/sex accumulation in relation to the degree of volatility of OCs and the degree of chlorination of PCBs. Whereas an increasing concentration trend with BMI is observed in the more volatile OCs, aside from the interactive effect between sex and BMI, a clear decreasing trend is found among the highest chlorinated PCB.

Figure 5.15 shows the relationships between BMI trends and the two physical-chemical properties previously selected (Koa and Kow), hence reinforcing the proposed hypothesis.

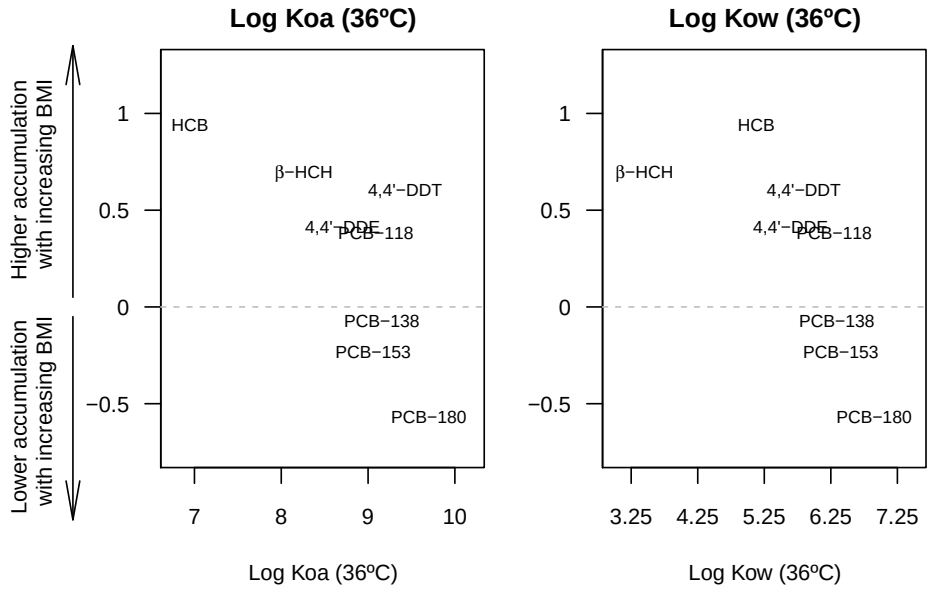

Figure 5.15: Associations between OC physical-chemical properties (Log Koa and Log Kow) and BMI from multivariate regression models.

Therefore, the physical-chemical properties of OC compounds explain, even if partly, their pattern of accumulation, which remains consistent across varia-

tions due to the interactive age and sex effect or the BMI.

The hypothesis of a role of the physical-chemical properties of POPs in their pattern of accumulation cannot be contrasted with PBDEs because their modelling explain much lower variability, specially for age and sex (next shown in Figure 5.18). However, there is one PBDE that appears to show a similar pattern of accumulation regarding sex and BMI, alike PCB-180. It is the case of the higher brominated BDE-153, which is significantly more accumulated in men and simultaneously shows a decreasing concentration trend with BMI.

In general, the results obtained and the cross-verifications reinforces the hypothesis of the role of the physical-chemical properties of OCs in their accumulation, mainly related to their volatility, but also their degree of chlorination/bromination.

In fact, the relevance of the volatility of OCs in their patterns of accumulation in humans has never been established before. It opens a new horizon of discussion, revealing the role of respiration in the absorption (*e.g.* inhalation) and elimination (*e.g.* exhalation) of these compounds in the human body. This then triggers another related hypothesis to explain the underlying factors of accumulation of POPs, namely human metabolism. In fact, the differentiate effect of the physical-chemical properties of OCs is realized through human metabolism (respiration notably).

Role of human metabolism

Another hypothesis that this research has examined to explain the underlying factors in the accumulation of POPs is the role of human metabolism. In particular, ARTICLE 2 deepens in it when trying to explain the sexual differences in the accumulation of certain OCs (the most volatile ones).

This hypothesis is related to the sexual differences in metabolism. In fact, the sex-based physiological differences in humans, particularly in the cardiopulmonary system, are well documented. Women tend to have a minor development of the rib cage, as well as lower parameters in relation to the airways diameter, lung volume and blood/air diffusion surface in the alveoli (Chen and Kuo, 1989; Bellemare *et al.*, 2003). Women also have a lower concentration of haemoglobin and a lower number of erythrocytes in blood (Huxley, 2007), thus having a lower blood oxygen-carrying capacity (Wallin *et al.*, 2010). In essence, women have a lower cardiopulmonary metabolism than men (Chen and Kuo, 1989). This fact may explain a reduced ability of women to exhale the most volatile OCs by respiration, hence increasing their body burden, which is further exacerbated through age (as their metabolism declines more rapidly with age than in men).

Furthermore, the observed fact that increases in body mass index result in higher accumulation of certain compounds, precisely the most volatile ones, reinforces the hypothesis of the role of respiration in the excretion of these pollutants since obesity is associated with reduced lung volumes and alterations in respiratory mechanics (DeLorey *et al.*, 2005; Chlif *et al.*, 2009). However, this fact is mostly observed in men, which triggers us to consider another aspect of human physiology: the differences in the fat distribution among sexes. Women have the tendency to deposit fat in the hips and buttocks, whereas men tend to deposit fat more centrally and hence men's fat deposits have a greater impact on the functioning of the diaphragm and on the regulation of lung volumes (Harik-Khan *et al.*, 2001). In this sense, BMI increases among women (*i.e.* more obesity) have comparatively less impact in their respiratory function and in their ability to excrete volatile OCs via respiration. Conversely, men's stronger pulmonary activity is impaired when BMI increases, hence limiting their advantage in the excretion of the most volatile OCs.

To further examine the proposed hypothesis that certain OCs (*i.e.* the volatile ones, such as HCB and β-HCH) are less exhaled in women, a subsidiary research line was conducted, analysing exhaled breath condensate (EBC) samples from both sexes and different ages.

Analysis of human breath samples

Human EBC samples were obtained from a study analysing the respiratory alterations of individuals who participated in the clean-up of the Galician coastal oil spill after the Prestige disaster in 2002 (Rodríguez-Trigo *et al.*, 2010). The samples were obtained by using an EcoScreen condenser, through breathing at normal frequency for 10-20 minutes. More details about the methodology are published elsewhere (Horvath *et al.*, 2005; Rodríguez-Trigo *et al.*, 2010).

In a preliminary study, 8 samples from 5 women and 3 men were selected. OC compounds in EBC samples were analysed following the same methodology as human serum samples, doing several rinses of hexane to resuspend the condensate. Two blanks were conducted in parallel to account for possible contamination: one blank was made from the plastic tube where the EBC samples had been stored and the other blank was done from the hexane solvent itself. Samples were analysed by GC-MS-NICI.

The concentrations found in human breath samples are shown in Tables 5.1 and 5.2.

Women present higher mean concentrations of HCB, β-HCH and PCB-153 compared to men, with a statistically significant difference. For the rest of compounds, however, no significant differences in mean concentrations have been found between both sexes.

The fact that women show higher levels of the most volatile OCs is contrary to the aforementioned hypothesis, since women are expected to exhale less

	Women					Men		
	1	2	3	4	5	6	7	8
HCB	0.0126	0.0054	0.0096	0.0069	0.0052	0.0042	0.0047	0.0037
β-HCH	0.0095	0.0054	0.0080	0.0041	0.0026	0.0012	0.0020	0.0021
4,4'-DDT	0.0232	0.0243	0.0154	0.0077	0.0143	0.0206	0.0000	0.0144
4,4'-DDE	0.0774	0.0261	0.0304	0.0438	0.0246	0.0408	0.0153	0.0208
PCB-118	0.0261	0.0205	0.1301	0.0072	0.0042	0.0049	0.0075	0.0050
PCB-138	0.0203	0.0160	0.0348	0.0127	0.0069	0.0071	0.0115	0.0078
PCB-153	0.0087	0.0061	0.0075	0.0061	0.0026	0.0022	0.0040	0.0028
PCB-180	0.0058	0.0050	0.0000	0.0033	0.0021	0.0033	0.0040	0.0028

Table 5.1: Concentrations of selected organochlorine compounds in EBC samples from women (n=5) and men (n=3).

HCB or β-HCH than men. However, the interpretation of these results should be done with caution since few samples were analysed. Moreover, the levels of OCs in EBC samples were extremely low and the procedure needs a more developed technique. In addition, the concentrations in blanks for some OC compounds were slightly higher than those found in EBC samples. For all the aforementioned limitations (*e.g.* few samples, low levels, possible contamination of blanks) the results are non-conclusive and this line of research was abandoned and no more EBC samples were analysed.

5.5 Influence of other socio-demographic factors in the accumulation of POPs

One of the specific objectives derived from this Ph.D. research is to determine if other socio-demographic characteristics (namely educational level, social class, place of birth and current place of residence) are influencing in the exposure to persistent organic pollutants in the population of Catalonia.

	Women	Men	Significance of T-test
HCB	0.0079	0.0042	p<0.05
β-HCH	0.0059	0.0018	p<0.05
4,4'-DDT	0.0170	0.0120	p=0.25
4,4'-DDE	0.040	0.0260	p=0.14
PCB-118	0.0380	0.0058	p=0.12
PCB-138	0.0180	0.0088	p=0.060
PCB-153	0.0062	0.0030	p<0.05
PCB-180	0.0032	0.0034	p=0.54

Table 5.2: Mean concentrations of selected OCs in EBC samples by sex.

ARTICLE 3, ARTICLE 4 and SUPPLEMENTARY RESEARCH ARTICLES (Annexes I and II; Porta *et al.* (2010, 2012)) have been already addressed these questions, yet some other findings have not been discussed in any publication. All these issues are globally discussed below.

Educational level and social class

Educational level and social class are two socio-economic indicators intrinsically related, as it is assumed that individuals with higher education (*e.g.* university degree) will reach a higher social class. Therefore, both variables are discussed together.

Figures 5.16 and 5.17 show the adjusted effect of the educational level and social class on the concentrations of OC pesticides and major PCB and PBDE congeners in the Catalan individuals, respectively.

On the one hand, education is related to the levels of some compounds (*e.g.* HCB, PCB-118, PCB-153, BDE-209), but with different tendencies. Whereas for the aforementioned OCs the concentrations increase significantly with the educational level (from individuals without formal education through those with a university degree), in the case of BDE-209 there is a negative association.

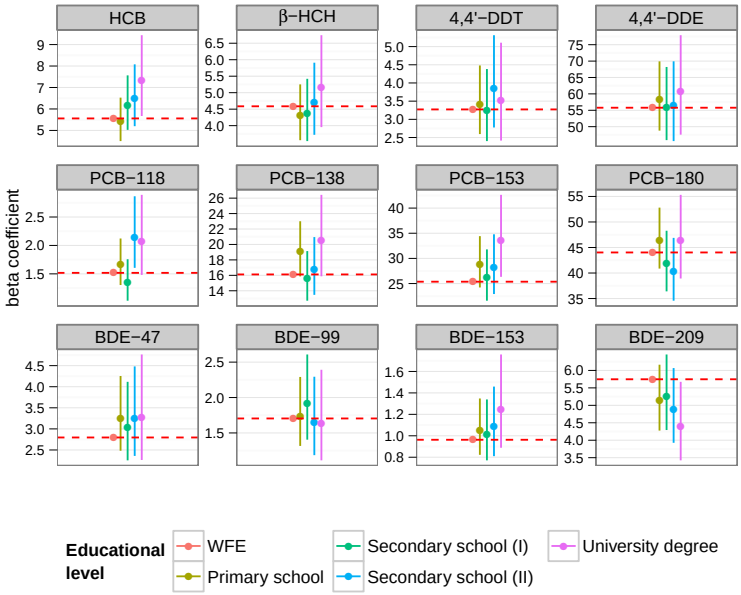

Figure 5.16: Adjusted effect of educational level on POP concentrations. Each panel (compound) is a different multivariate regression model adjusted for age, sex, body mass index and social class. The reference category is *WFE* (Without formal education), represented by the horizontal line.

Some studies have also found that individuals with a higher level of education have also higher concentrations of OCs (Vizcaino *et al.*, 2010; Vrijheid *et al.*, 2012). One possible explanation raises from dietary patterns since people with higher educational level may follow a more balanced diet with higher fish intakes (Darmon and Drewnowski, 2008). As discussed above, diet is the main route of entrance of these compounds into the human body and PCB levels are often associated with higher rates of fish consumption (Knutsen *et al.*, 2011). Unfortunately, the present study can not verify it because of the lack of dietary information of the studied individuals. Otherwise, the fact that BDE-209

follows an inverse pattern of accumulation as increasing the level of education suggests a different exposure pathway to this compound compared to OCs.

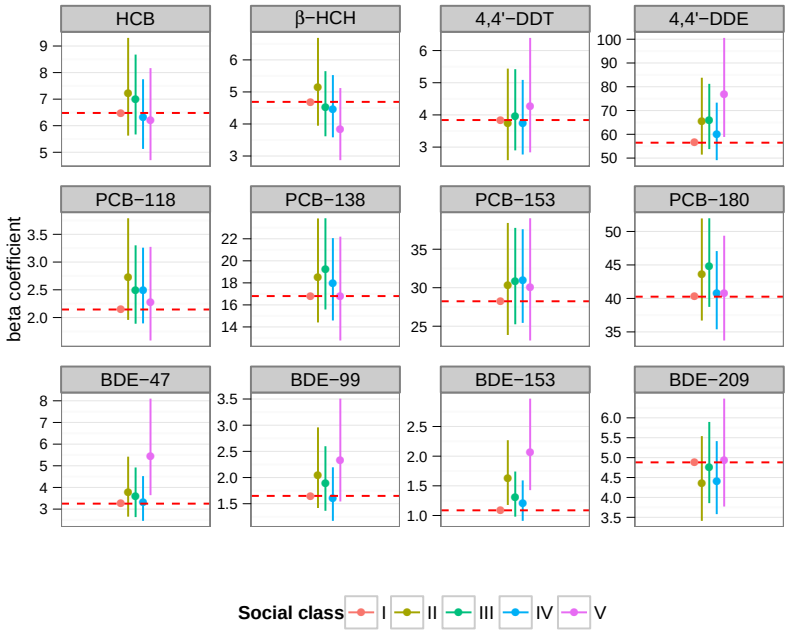

Figure 5.17: Adjusted effect of the social class on POP concentrations. Each panel (compound) is a different multivariate regression model adjusted for age, sex, body mass index and educational level. The reference category is the social class I (the most affluent one), represented by the horizontal line.

On the other hand, social class is not fully related with the levels of POPs. Only for the least affluent social class (social class V), the levels of 4,4'-DDE, BDE-47 and BDE-153 are significantly higher than for the rest of social classes.

Overall, minor and inconsistent differences in the accumulation of POPs regarding educational level and social class are found. The differences are not

systematic among the studied compounds, as both positive and negative associations with both factors are found, suggesting that each compound should be studied separately.

Moreover, these socio-demographic indicators only explain a very small part of the variability in the levels of POPs after adjusting for other covariates, as shown in Figure 5.18).

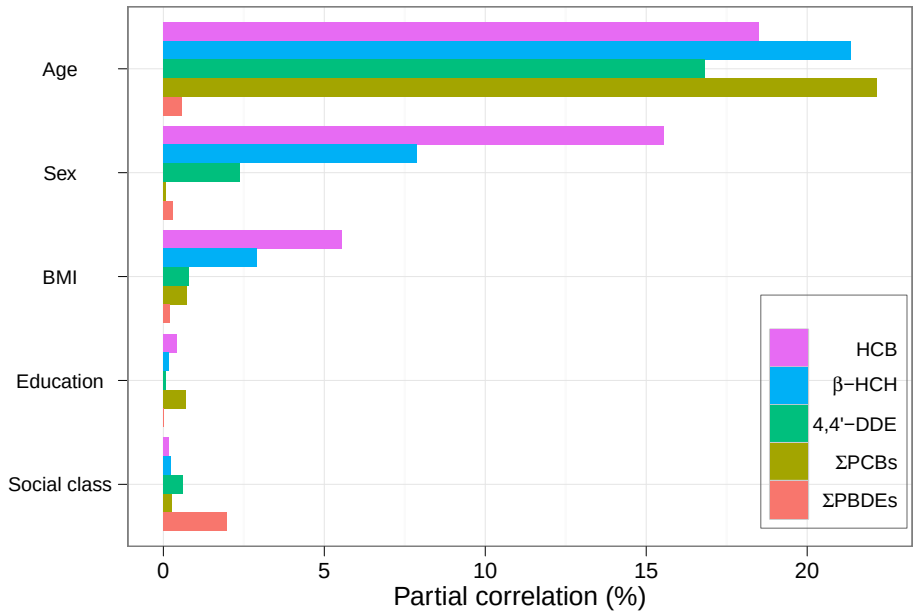

Figure 5.18: Percentage contribution to total variability in log-transformed and lipid-adjusted concentrations of HCB, β-HCH, 4,4'-DDE, ΣPCBs and ΣPBDEs, explained by several covariates in multivariate regression models.

This figure displays the partial correlations (R^2) of several variables on POP concentrations from multivariate regression models. Both educational level and social class contribute less than 1% to the total variability in the concentrations

of OCs. In the case of PBDEs, however, social class is contributing a bit more to the total variability (1.9%), yet the adjusted R^2 in this model is only of 2%, whereas for OCs this value ranges between 30% and 50%.

According to these results, other factors rather than educational level and social class (those discussed in the previous section: age, sex and BMI) are the ones that clearly contribute to explain the levels of POPs in the studied adult population.

Place of birth

Figure 5.19 shows the adjusted effect of the place of birth on the concentrations of organochlorine pesticides, ΣPCBs and ΣPBDEs in the Catalan individuals.

The results clearly show that the place of birth is a strong determinant in OC concentrations from the individuals studied. Individuals who were born abroad have much lower concentrations of HCB, β-HCH and ΣPCBs than those born in Catalonia or other Spanish regions, with statistically significant results ($p<0.05$). For 4,4'-DDT, 4,4'-DDE and ΣPBDEs, however, the differences are not significant, although there is a tendency that those people born abroad have lower levels of DDT and slightly higher levels of DDE and PBDEs.

One of the limitations of this analysis is the lack of information on the exact place of birth of the individuals (*e.g.* province for those born in other Spanish regions; country for those born abroad). This fact makes difficult to establish clear links between the place of birth and POP concentrations through past exposures (*e.g.* childhood, malaria-influence regions, agricultural or industrial environments), specially for foreigners, yet people from other Spanish origin have similar levels than Catalan-born individuals, for all the compounds (Figure 5.19).

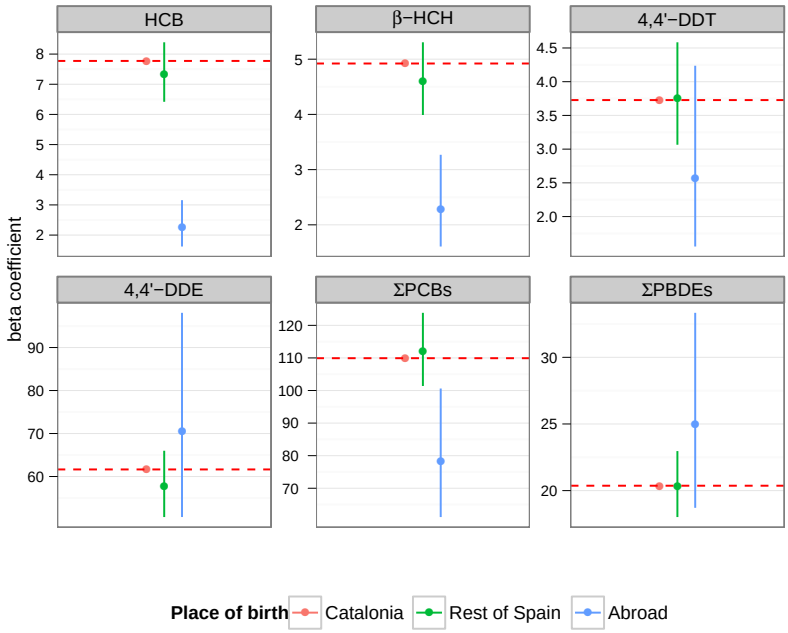

Figure 5.19: Adjusted effect of the place of birth on OC concentrations. Each panel (compound) is a different multivariate regression model adjusted for age, sex and body mass index. The reference category is the Catalan-born individuals, represented by the horizontal line.

Place of residence

The Catalan health system in year 2002 was divided into eight regions (see section 3.1 in the Methodology chapter), with the city of Barcelona being a single health region.

The analysis of POP levels from each health region, controlling by age, sex and BMI of the individuals, is shown in Figures 5.20 and 5.21. These figures

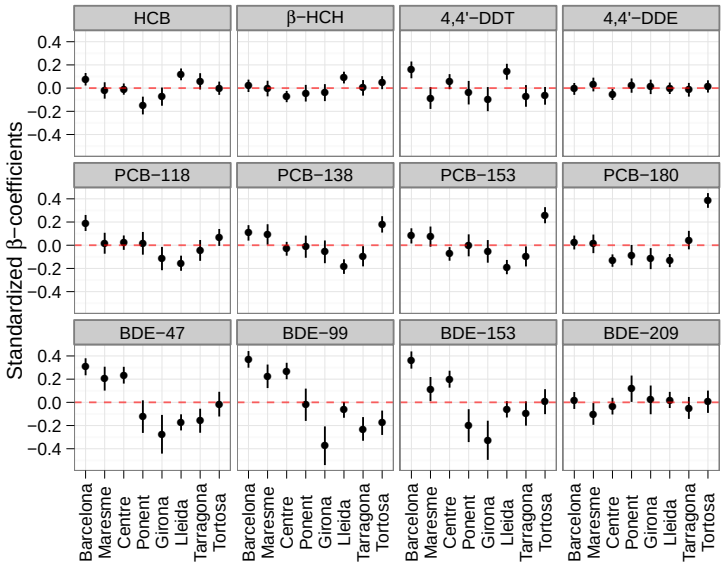

Figure 5.20: Standardized β coefficients and 95% confidence intervals (CI) from multivariate hierarchical models of POP concentrations adjusting for individual factors (age, sex and BMI) and region. β coefficients can be interpreted as standardized differences between regions and the overall mean of Catalonia, represented by the red horizontal line. Each panel is a different compound.

present the standardized residuals from the overall population of Catalonia, which allows to compare between regions and compounds.

These figures reveal a variable and not clearly uniform pattern of contamination by different pollutants regarding the health region.

By pollutant, the most variable ones through the territory are BDE congeners 47, 99 and 153 (standardized SD ranging between 0.23 to 0.27), and the least ones, 4,4'-DDE, β-HCH, BDE-209 and HCB (standardized SD ranging

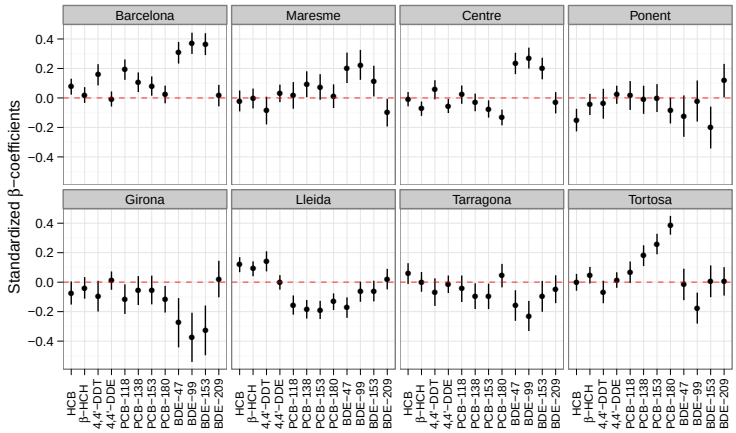

Figure 5.21: Standardized β coefficients and 95% confidence intervals (CI) from multivariate hierarchical models of POP concentrations adjusting for individual factors (age, sex and BMI) and region. β coefficients can be interpreted as standardized differences between pollutants and the overall mean of Catalonia, represented by the red horizontal line. Each panel is a different region.

between 0.04 and 0.09). Standardized SD of 4,4'-DDT and PCBs are ranging between 0.11 and 0.18, indicating that the variability of these compounds by each health region would be intermediate.

By health region, there is also a great variability. For instance, Lleida shows a higher level of exposure to HCB, β-HCH and 4,4'-DDT but a lower one to PCBs in relation to the overall population of Catalonia. In contrast, Tortosa shows the highest levels of exposure to PCBs, particularly to PCB-180. The health regions of Barcelona, Maresme and Centre would have comparatively much higher levels of major PBDE congeners (BDEs 47, 99 and 153) than the rest, but however not of BDE-209. Girona appears to be the health region with the lowest levels of exposure to POPs in relation to the rest of the Catalan territory.

Figure 5.22 shows similar information than Figures 5.20 and 5.21, but in the original scale of the compounds (ng/g lipid), hence the concentrations can be better understood.

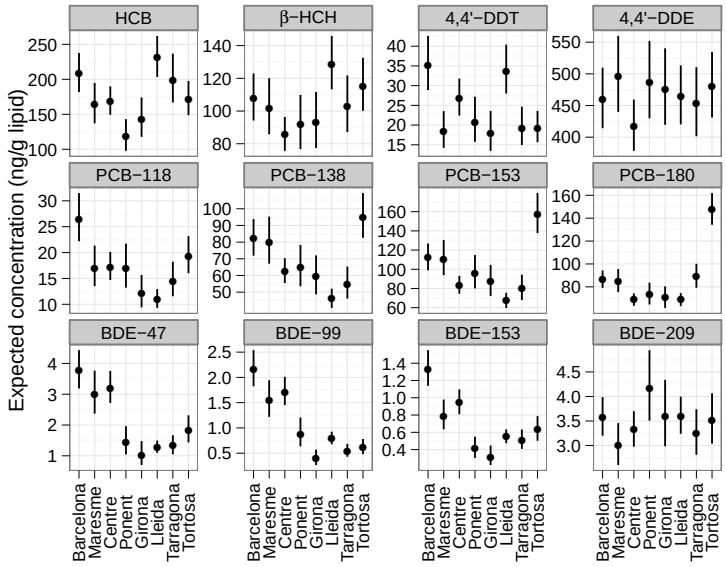

Figure 5.22: Expected concentrations (ng/g lipid) and 95% confidence intervals (CI) from multivariate hierarchical models of POP concentrations adjusting for individual factors (age, sex and BMI) and region. Each panel represents a different compound.

And finally, Figures 5.23 and 5.24 show a spatial representation of the compounds, presented in the same scale, so that comparisons between compounds and regions can be observed.

As it has already been discussed, some pollutants such as 4,4'-DDT, PCBs and BDE-47 are the most divergently distributed throughout the Catalan territory, whereas other compounds such as 4,4'-DDE and BDE-209 show more

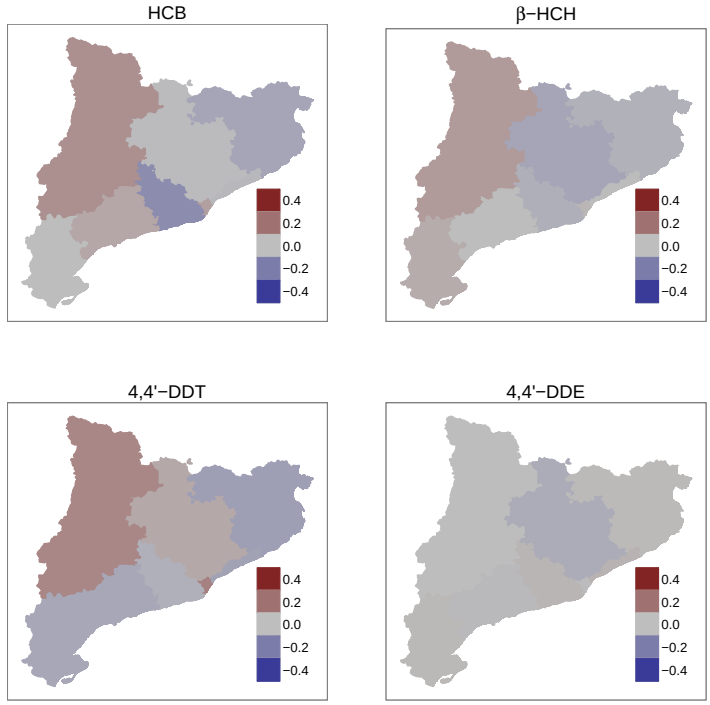

Figure 5.23: Standardized differences of the concentrations of organochlorine pesticides between regions, with respect to the overall population of Catalonia.

uniform concentrations.

This irregular pattern of contamination, as well as its low variance with respect to individual factors, evidences again that what is certainly important in determining the concentrations of POPs in humans are not only the environment but essentially individual factors such as age and sex.

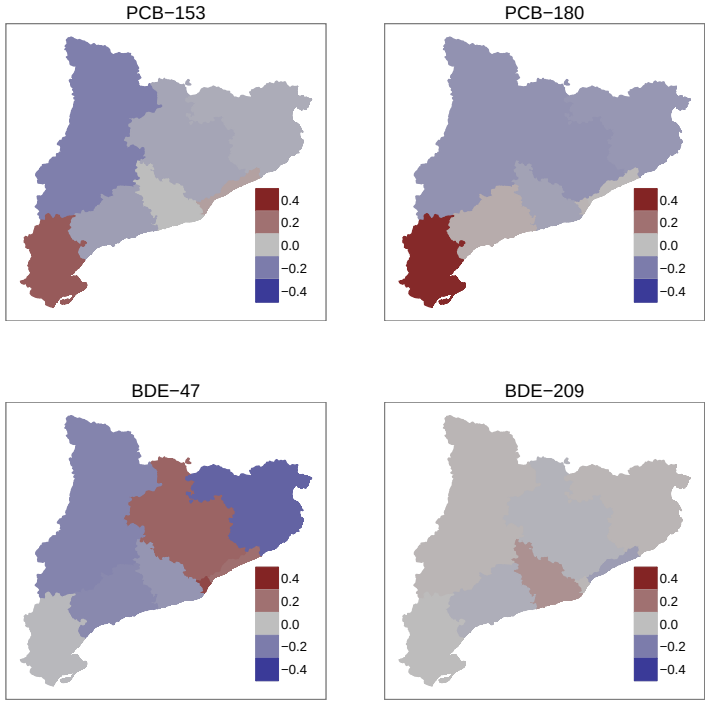

Figure 5.24: Standardized differences of the concentrations of PCBs (congeners 153 and 180) and PBDEs (congeners 47 and 209) between regions, with respect to the overall population of Catalonia

Comparison with Flix township

This part of the discussion presents a closer look of the territory, not based on health regions but in the municipalities themselves. Since there is a factory that produced OCs for decades in South Catalonia (in the municipality of Flix), a comparison of the levels of nearby municipalities with the study of the Flix population conducted in 1994 has been performed and presented in ARTICLE 4 in the Results chapter.

Slightly higher concentrations of major OCs have been found in the studied municipalities located closest to Flix (around 15-25 km), while similar levels have been found in a second area of influence (around 30-40 km from Flix) and the rest of the studied Catalan municipalities. The results show that there is certain influence of the electrochemical factory in the nearby municipalities, but as it has been evidenced before, the geographical dispersion is quite restricted because at a certain point of distance, the concentrations with the rest of Catalonia are similar.

5.6 Final remarks

One of the crucial challenges in environmental epidemiology lies in identifying and characterising the main determinants of individual exposure to persistent organic pollutants. This can then serve to understand the underlying dynamics, to reveal public health issues and to identify needs in policy, regulatory and monitoring measures to better safeguard the life and health of humans and wildlife.

This research aligns with this multidimensional nature of environmental epidemiology. The relevance and credibility of such type of research lies in its technical and analytical methods. It is first essential to acquire a robust measure of

the exposure levels, notably in terms of accuracy and reliability; otherwise the results can be biased and the effects, under-estimated. Moreover, research samples of large size and representing general (or target) population are desirable, accompanied with detailed information of the studied individuals on physical features (such as age, sex or body mass index), socio-demographic characteristics (such as educational level and social class), and environmental factors (such as proximity to a pollution source or site). Results and parameters are then carefully assessed, statistically verified, and contrasted with the existing literature. Hypotheses are then formulated, analysed, and either adopted, refined or rejected. Conclusions and recommendations are formulated, and new research questions are proposed for possible follow up by other scientific teams.

This research has circumscribed in this approach. It has developed a methodology to better detect the persistent organic pollutants, so results are robust and reliable. It has studied the degree of exposure of a large sample of individuals from a general population to different compound families, namely organochlorine pesticides, PCBs and PBDEs. Supplementary research has been also conducted to better sustain the analysis and the formulation of hypotheses. The research has simultaneously assessed different individual, socio-demographic and environmental factors that may influence the accumulation of these pollutants. Therefore, this research has included analytical work, statistical analysis, identification of patterns and trends, comparisons with existing literature and discussion of a number of hypothesis.

When coming back to the mentioned essential challenge of environmental epidemiology, this research has overall tried to respond to the following question:

Which are the main and most useful and informative determinants to predict the POP concentrations of an average individual in the population of Catalonia?

Clearly, the age of an individual is, on a general basis, the single most important factor, followed by sex and, at a minor degree, the body mass index. Other socio-demographic or environmental factors explain in much less proportion the variations, and thus determines little the concentrations of POPs to be found in an individual. These trends remain even in individuals living or working in a highly-exposed location, such as Flix township. Indeed, levels are extremely higher in this latter case, but both the age trend and the sexual differences are maintained, which reinforces the hypothesis that there are underlying factors catalysing the trend – we explored the role of the physical-chemical properties of the different POPs and the dynamics of human metabolism, and their interrelation.

6 Conclusions

This research on environmental epidemiology has mainly explored and analysed the patterns of accumulation of certain chemical products, namely POPs, in human bodies, examining the determinants and assessing a number of hypothesis to explain the underlying dynamics. The variety of research lines that have been undertaken, which include a new methodological design, core research on the patterns of accumulation of OCs and PBDEs, and supplementary assessments, provide a comprehensive and comparative insight on the effects and performance of pollutants inside human bodies, and hence on the scope of impact of pollutants in the human health. A number of hypotheses were formulated to explain both the patterns of accumulation encountered and the unexpected findings. A synthesis of the research accomplishments is next compiled.

On the detection of persistent organic pollutants. A new methodology for the calculation of the limits of detection and quantification of OCs in human serum has been specifically designed. It clearly enhances the reliability of data compared to previous, similar research. After using the designed methodology, all the studied individuals of the population Catalonia show detectable levels of persistent organic pollutants, for at least one compound. This denotes

the impact of these compounds, and their persistent presence in the environment, continuously affecting human populations, even if most of them have been already banned or restricted for a number of years. The condition of Catalonia as a country with important agricultural and industrial sectors not only explains these findings but also highlights the need for continued action on both environmental-health monitoring and industrial regulation to prevent such health impacts in the future.

On the levels of POPs in the population of Catalonia. The concentrations of organochlorine compounds (OCs) in the population of Catalonia are equivalent to those described for other populations worldwide. The compound 4,4'-DDE (which is a degraded metabolite from DDT) is the most detected and abundant, as well as the dominant among the total of DDTs, which is coherent with the fact that DDT as agricultural pesticide was banned in the 1970s and hence one just encounters the resulting metabolite. The compounds HCB and β-HCH are found in relatively high concentrations, which is consistent with findings in other Spanish regions and locations. Furthermore, there are high levels of PCBs, which are dominated by the congener 153. All this is partly explained by the reportedly high fish intake in Spain. Regarding the concentrations of PBDEs, Catalonia ranks among the highest in the world, with the exception of North America and highly polluted sites. This finding is probably due to the important plastic and textile industry in the country (which are heavy users of PBDEs). The decabromodiphenyl ether (BDE-209) is the most abundant congener, which represents a public health concern since this compound is still legally available and can be further debrominated into even more toxic forms.

On the history of POPs and their differentiated levels and trends of

accumulation. The research has shown that the history of a POP influences their levels of accumulation at present time. *Legacy* POPs, called as such because they were produced and used in earlier periods, have been impacting people for longer time, yet they tend to be among the banned or restricted ones, so their influence may have been reduced in the recent decades. In the case of the population of Catalonia, the levels of OCs (considered as *legacy* POPs) are approximately one to two orders of magnitude higher than for PBDEs. This reflects their different history and period of production and use: the human exposure to PBDEs is relatively recent compared to the legacy POPs, as OCs are. At the same time, OC levels show a time-based decreasing trend, which indicates that their regulatory restrictions are enforced and yielding public health benefits.

On the predictive role of age, sex and BMI in the accumulation patterns of POPs, along with unexpected trends and divergences. The research confirms that the concentrations of OCs increase with age, as older people have been exposed to them at higher doses before they were banned or regulated, and during a longer period of time, thus increasing their overall body burden. In contrast, the correlation between age and PBDE levels follows an inverse trend, explained by the fact that young people have been exposed to them during their infancy, when metabolism is active and exposure sources such as dust are more prevalent. In essence, age is a strong determinant in their levels of accumulation of OCs and PBDEs, yet in opposite senses. In addition, the research reveals divergent patterns in the accumulation of POPs when body mass index and sex are considered. In this sense, the compounds that are more accumulated in women show an increase pattern of accumulation with increasing BMI of the individual (*i.e.* HCB, β-HCH, 4,4'-DDT, 4,4'-DDE, PCB-118). Conversely, for the compounds that are more accumulated in men, the BMI shows an inverse trend, as the concentrations are lower among the individuals with higher BMI (*i.e.* PCB-

180, BDE-153). The research thus reveals an interrelated pattern between age and BMI to explain differentiated trends in the accumulation of POPs.

On the non-relevance of socio-demographic determinants in POP accumulation. The research shows that socio-demographic determinants, such as educational level, social class, and places of birth and residence, do not seem to be relevant determinants of POP accumulation in humans. This concurs with previous research.

On the interactive effects in certain OCs. Certain OCs are much more accumulated in women through age than in men, revealing an interactive effect between age and sex. Furthermore, the influence of the body mass index in the accumulation of these compounds is proportionately higher among men than in women, thus reflecting another interactive effect: sex and body mass index. These two interactive effects are observed for HCB and β-HCH, which are precisely the most volatile OCs. This supports the examination of the hypothesis that both the physical-chemical properties of POPs and the human metabolism are underlying factors to explain the patterns of accumulation encountered by the present research and others.

Final and overall hypothesis: On the mutually-related role of both the physical-chemical properties of POPs and human metabolism in the patterns of accumulation of POPs. The findings of the research, notably the patterns of accumulation revealed, some of which are rather unexpected or unusual, led to the examination of some related hypothesis, suggesting that both the physical-chemical properties of POPs and the human metabolism play a role in the accumulation levels and trends of POPs, and that they are mutually related. In this sense, the degree of volatility of OCs clearly determine their level

of accumulation in human bodies, hence suggesting the role of respiration in the absorption (inhalation) and elimination (exhalation) of these compounds in the human body. Furthermore, the lower cardiopulmonary metabolism of women in relation to men may explain a reduced ability of women to exhale de most volatile OCs by respiration, hence increasing their body burden, which is then exacerbated through age. On the other hand, men's stronger pulmonary activity could be impaired when their BMI increases because their fat deposits are distributed in the abdominal area, thus limiting the respiratory function as a means to expel volatile OCs.

ADDENDUM

COMPLEMENTARY RESEARCH ON THE ACCUMULATION OF MERCURY IN THE INFANT POPULATION OF MENORCA

Introduction

Children's vulnerability to pollutants

Children are particularly vulnerable to the exposure to pollutants, suffering heavier and more enduring health impacts (Goldman and Koduru, 2000), because their immunological system and the internal mechanisms of chemical decontamination are not completely developed, but just partly functional (Olsen, 2000). Their reduced body sizes and growth pace makes exposure more impacting. In fact, it is estimated that 40% of diseases in children under 5 years of age is attributable to environmental pollution factors (Smith *et al.*, 1999). Accordingly, the physical, social and intellectual development of children, from their conception (including fetal stages) to the end of the adolescence, requires a safe environment. However, humans live in constant contact with environmental pollutants.

Exposure to persistent pollutants such as organochlorine compounds and mercury have been related to prematurity, delays in intrauterine and postnatal growth, and alterations in the child's neurodevelopment and conduct (Ribas-Fitó *et al.*, 2002, 2003a; Forns *et al.*, 2012). These pollutants are easily incorporated through placenta (in fetuses), through breastfeeding (in early infants) and through the diet (along childhood). Mercury is of particular concern in the health of children.

During the doctoral research, the author had the opportunity to assess the accumulation of mercury in the infant population of Menorca Island, which is located some 250 km east of Catalonia. This allowed to further examine the influence of diverse socio-demographic and dietary factors, along with maternal-child linkages, in the accumulation of a major persistent pollutant, as well as to compare the patterns of accumulation across the organic-nonorganic border, since mercury is a non-organic substance (it is actually a constitutive chemical element). It therefore provides a complementary insight on the environmental epidemiology matter of this doctoral research. It represents the first assessment of accumulation of mercury in the population of Menorca with a reliable sample (n>300). This chapter includes a publication (the fifth article of this Ph.D. dissertation, which is under review), attached in the Results section.

Mercury, a highly toxic, non-organic persistent pollutant

Among pollutants, mercury is probably an emblematic case due to its extensive use, from historical times, and its demonstrated toxicity. Mercury is a well known metal, used since ancient times. It has been found in Egyptian tombs from around 1,500 BC and ancient Greeks, Egyptians and Romans used it in ointments and cosmetics. Nowadays, mercury is used in many technical devices such as thermometers, in lighting, in scientific research applications, in amalgam

materials (*e.g.* dental implants), as well as in gold mining and the chlor-alkali industry. Mines in the Mediterranean basin (namely Almadén in Spain, Monte Amiata in Italy and Idrija in Slovenia) dominated the world mercury production until the end of the 19th century, when new deposits started to be found.

Mercury is released into the environment by both natural and anthropogenic processes. Natural processes mainly encompass volcanic activities and weathering of rock and ores, while mercury releases by anthropogenic sources are primarily due to fossil fuel combustion, mining, smelting and solid waste incineration (ATSDR, 1999). Although mercury is naturally present at very low concentrations, which have remained relatively constant through time, human activities have resulted in a rapid increase in the release of mercury to the environment and in the concentrations found in many locations and external layers.

Mercury and almost all mercury compounds are toxic to organisms, whether aquatic and terrestrial, and can be actually dangerous at very low levels. Moreover, in aquatic ecosystems, mercury can be converted to methylmercury, a highly toxic, organic compound. This process, coupled with the fact that mercury bioccumulates in the living organisms and biomagnifies up along the food chain, results in higher concentrations of this compound among predatory fish species. That makes the consumption of fish and other seafood, particularly those who are high on the food chain, such as swordfish and tuna, the main route of incorporation of mercury in human populations (Myers *et al.*, 2003).

History has unfortunately shown in many times the direct impact of mercury pollution on human health, particularly in children. In 1955, in the Minamata Bay, Japan, an accidental emission of methylmercury to the marine water caused many people to be heavily exposed to this compound from the consumption of contaminated marine animals (Harada, 1976). As a consequence, many cases of neurological disease were diagnosed in the adult population, yet worst

effects were later found in the children of women who had eaten contaminated seafood during pregnancy: methylmercury had passed the transplacental barrier and was also notably present in breast milk, causing severe cognitive disruption and delays in the newborns.

Accordingly, several studies have examined the neurotoxicity and neurodevelopmental risk in children due to exposure to mercury, even at low levels (Karagas *et al.*, 2012).

The population of Menorca Island

This concise research focuses on the infant population of Menorca, an island of the Balearic Archipelago that is located in the Northwest part of the Mediterranean Sea, some 240 km east of Catalonia (Figure A.1).

Figure A.1: Geographical location of Menorca, in the Western Mediterranean Sea and 240 km east of Catalonia.

The island of Menorca has an extension of 700 km^2 and about 94,000 inhabitants.

The capital city is Maó which, together with Ciutadella, the ancient capital, are the major towns. Menorca is administratively divided into eight municipalities (Figure A.2).

Figure A.2: Geographical location of the municipal towns of Menorca.

Tourism is the most important economic sector of the island, which provides a visiting population that far exceeds the resident population.

Menorca also possesses a farming, cattle and fishing tradition, yet little industry. Due to its island condition, the inhabitants have easy access to, and regular consumption of fish and seafood.

In 1993, the island of Menorca was designated as a Biosphere Reserve by UNESCO to recognize its biodiversity, representing nearly all Mediterranean island ecosystems and in a fairly good state of conservation (UNESCO, 1993).

Objectives

The main objective of this concise research is to examine the levels and patterns of accumulation of mercury in an infant population against a set of socio-demographic factors, with a special focus on dietary intakes and taking into account the maternal-child interactions.

The specific objectives are as follows:

1. to assess the degree of accumulation of mercury in 4-year old children from Menorca island, on the basis of an analysis of 302 hair samples, comparing it with other infant populations worldwide;

2. to identify patterns and determinants of accumulation of mercury in children in relation to a set of socio-demographic factors, comprising sex, place of residence, a specific consideration of dietary habits (correlating with fish and seafood consumption) as well as the parental background (*e.g.* social class, educational level and smoking habits);

3. to examine the influence of maternal mercury transfer in 4-year old children through factors such as parity and breastfeeding; and

4. to assess the influence of mercury levels on neurodevelopmental outcomes, using the McCarthy Scales on Children's Abilities.

Methodology

INMA (*Infancia y Medio Ambiente*) [Childhood and Environment] is a research network that conducted a Spanish multi-center, mother-child cohort study to analyse the influence of environmental exposures on growth, development and health from early fetal life until childhood (Ribas-Fitó *et al.*, 2006).

The INMA project has followed about 4,000 pregnant women and their babies from gestation to 11 years of age in different geographic areas across Spain (Figure A.3). Each cohort presents different periods and types of recruitment. This research focuses on the Menorca island's cohort.

Figure A.3: Geographical location of the different INMA cohorts across Spain.

In the Menorca cohort, all the eligible women who went to a prenatal visit in any of the healthcare centres of the island (whether private or public) were invited to participate in the study. The recruitment lasted a year, starting in July

1997. A total of 530 pregnant women were included and at their children's four-year old visit, a total of 482 children were still enrolled. The study protocol was approved by the Ethics Committee of the reference hospital whereas informed consent was obtained for every participant.

Extensive information of pregnant women and their children were taken from questionnaires conducted by trained interviewers. These questionnaires provided information on socio-demographic factors and dietary habits, among others.

When aged 4 years, children provided a hair sample for the measurement of mercury levels and were also asked to perform a neuropsychological test, namely the McCarthy Scales on Children's Abilities (MSCA). The analysis of mercury in hair and the MSCA test are next described.

Analysis of mercury in hair

A lock of scalp hair (5 cm) was obtained from the children's nape. About 25-50 mg of each sample were treated with HNO_3 (1 ml; Baker Instra) and H_2O_2 (0.5 ml; Merck Suprapur) in a Teflon vessel (90°C overnight). The digested sample was diluted with deionized water (8.5 ml; Purelab Ultra).

The determination of total mercury (THg) was performed using inductively coupled plasma mass spectrometry (Agilent 7500 CE) operating under standard conditions and using rhodium as internal standard.

Certified Reference Materials (CRMs) from the International Atomic Energy Agency, IAEA-085 and IAEA-086, with 23.2 μg/g and 0.57 μg/g total mercury concentrations, respectively, were used for validation and verification of the accuracy of the analytical method (Heller-Zeisler *et al.*, 1998). Moreover, one procedural blank was included in each sample batch for possible contamination control. All samples were above the limit of detection.

MSCA neuropsychological test

The McCarthy Scales on Children's Abilities is an individually administered test that assesses the cognitive development and motor skills of children aged between 2 and 8 years (McCarthy, 1972). The length of the testing session is about 45 minutes for children under the age of 5. A wide range of puzzles, toys and game-like activities is used to evaluate each child according to five different parameters, as follows:

- `Verbal`: Consists of 5 sub-tests that assess comprehension and use of language.
- `Quantitative`: Consists of 3 sub-tests that measures mathematical ability.
- `Perceptual-Performance`: Consists of 7 sub-tests that evaluates a child's ability to conceptualise and reason without words.
- `Memory`: Consists of 4 sub-tests that tests short-term recall of words, numbers, pictures and tonal sequences.
- `Motor`: Consists of 5 sub-tests that assess both gross and fine motor coordination.

In addition to individual scores for each parameter, the Verbal, Quantitative, and Perceptual-Performance parameters are combined to yield a single General Cognitive index, which assesses the overall intellectual functioning.

An standardized Spanish adaptation of the MSCA test was administered and interpreted by trained neuropsychologists.

Data analysis

Data analysis and graphics were performed using the statistical software `R` (R Development Core Team, 2012).

The analysis included descriptive statistics (geometric means (GM) and 95% confidence intervals (CI)) and inference (multivariate regression analyses), as well as the use of statistical hypothesis testing (Chi-square, Student's t-test and ANOVA).

THg levels were not normally distributed and were transformed into natural logarithm (log THg) for the analyses.

Results: ARTICLE 5
Influence of socio-demographic and diet determinants in the accumulation of mercury in preschool children from a Mediterranean island

Mercè Garí, Joan O. Grimalt, Maties Torrent and Jordi Sunyer
Submitted to *Environmental Pollution*

Influence of socio-demographic and diet determinants in the accumulation of mercury in preschool children from a Mediterranean island

Submitted to *Environmental Pollution*
(under review)

Mercè Garí[*], Joan O. Grimalt[*], Maties Torrent[**], and Jordi Sunyer[***]

[*]Department of Environmental Chemistry. Institute of Environmental Assessment and Water Research (IDÆA-CSIC). Barcelona, Catalonia, Spain.
[**]Àrea de Salut de Menorca, IB-SALUT, Menorca, Spain.
[***]Centre for Research in Environmental Epidemiology (CREAL). Barcelona, Catalonia, Spain.

Abstract

Mercury levels measured in 302 hair samples of 4 year-old children from Menorca (western Mediterranean Sea) are reported. Their concentrations, geometric mean 1.4 μg/g, ranging between 0.040 μg/g and 10 μg/g, were higher than in other children inland populations but lower than in previously studied island cohorts, e.g. Faroe, Madeira and Seychelles. 20% of the samples were above the WHO recommended values. Higher concentrations in female than male were observed. Frequent consumption of fish and other seafood were significantly related to the observed mercury concentrations. Oily fish was the main source of this pollutant but shellfish and squid consumption were also associated to high mercury concentrations. Maternal smoking, occupational status or previous siblings were also found to influence on the accumulation of this pollutant. McCarthy Scales of Children's Abilities used to assess children's motor and cognitive abilities did not show association with mercury concentrations at 4 years of age.

Keywords: Mercury; Childhood and Environment research network; Sexual differences; Fish and shellfish consumption; Parity; Neurodevelopment

1 Introduction

Mercury is a recognized toxic pollutant of public health concern (WHO/IPCS.). It is distributed globally by both natural processes and anthropogenic activities. Inorganic mercury may be transformed into organic forms (mainly methylmercury, MeHg) which may then accumulate and biomagnify through the food chain.

The developing foetus is particularly vulnerable to acute and chronic mercury exposures. As MeHg crosses the placenta it may accumulate and damage the developing central nervous system because it may get across the permeable blood-brain bar-

rier (Davidson *et al.*, 2004). Contamination episodes in Japan showed irreversible neurological damage upon exposure to this compound (Harada, 1995). Several studies have focussed on neurotoxicity and neurodevelopmental risk among children exposed to low or moderate mercury levels (Karagas *et al.*, 2012) but the results are still inconclusive.

It is generally agreed that seafood consumption, either fish or mammals, is the main source of MeHg in humans (WHO/IPCS.; Centers for Disease Control and Prevention, 2005; US Environmental Protection Agency, 2007; McDowell *et al.*, 2004) Accordingly, infants from the populations of several islands have been studied for assessment of the accumulation patterns and neurodevelopmental effects, e.g. Faroe Islands (n= 917; Grandjean *et al.* (1997)), Madeira Islands (n= 149; Murata *et al.* (1999)), Seychelles Islands (n= 229; Myers *et al.* (2009)).

The Mediterranean basin is a global store of mercury due to important cinnabar deposits (Renzoni *et al.*, 1998). Marine organisms living in this basin accumulate higher mercury concentrations than those from other seas. To the best of our knowledge, mercury accumulation in Mediterranean Islands have only been considered in three sites, Eastern Aegean Islands (n=246, Gibičar *et al.* (2006)), Sardinia (n=22; Carta *et al.* (2003)) and Menorca (n=65; Montuori *et al.* (2006); Díez *et al.* (2009)) and the two former studies concerned adult populations. The present study is devoted to provide a description of the main socio-demographic and dietary factors that determine the accumulation of mercury in children from Menorca based on a large cohort (n=302).

Menorca is a Balearic Island located in the northwestern Mediterranean Sea. Its resident population is about 94 000 inhabitants and their livelihoods depend on extensive farming and tourism, with little industry. The inhabitants also have easy access to fish and seafood, a portion of them being fishermen. The fresh fish and shellfish consumed in the island are of local origin, e.g. Mediterranean. This island is therefore an interesting site for the assessment of the exposure levels of mercury in children from general population living in a Mediterranean environment.

Spain is a country with high fish and seafood consumption (Welch *et al.*, 2002). Studies in newborns and pre-school children in Catalonia, Valencia, Granada and Menorca showed elevated exposure to mercury contamination which was related to high fish intake (Montuori *et al.*, 2006; Ramón *et al.*, 2008; Díez *et al.*, 2009; Freire *et al.*, 2010)

In the present study detailed socio-demographic and diet analyses of the exposure to mercury contamination of a cohort of pre-schoolers from a typical Mediterranean population, e.g. Menorca has been performed. The body burden of mercury in four year-old children has been assessed by collection and analysis of hair samples. Significant associations between the concentrations of this metal and socio-demographic factors such as sex, residence site and other maternal and paternal characteristics have been investigated. The influence of fish and seafood consumption in the observed mercury concentrations has also been assessed. The study has also emcompassed an assessment of the effects of this contamination in neurodevelopment.

2 Materials and methods

2.1 Study population and sampling

The Menorca cohort was established within the Asthma Multicenter Infants Cohort Study (Polk *et al.*, 2004). 482 pregnant women were recruited from prenatal clinics over 12 months in 1997-1998 and their children were followed-up until 4 years of age, within the Childhood and Environment research network (INMA) (Ribas-Fitó *et al.*,

2006). Children at 4 years visit provided a hair specimen for total mercury (THg) analysis. Information on dietary intakes was obtained from their mothers. Preliminary analyses of mercury and methylmercury concentrations on a small number of cases (n=59 and 65, respectively) from the same infant population were carried out (Montuori *et al.*, 2006; Díez *et al.*, 2009). The present study provides mercury data for 302 children whose hair THg concentrations were analysed using an improved methodology (see next subsection below).

From 482 children enrolled at birth, 302 (63%) provided complete outcome data at 4 years and hair for mercury measurements. The characteristics of the participant population are described in Table 1. Except for sex, no differences were found between this subset and the children without mercury measurements. More female children were included due to the easier availability of hair samples. The subset with both THg measurements and MSCA neuropsychological test included 298 children (99%), and no differences between the subsets were found.

Written informed consent was obtained from the parents of each children before the study, which was approved by the Ethics Committee of the Institut Municipal d'Investigació Mèdica (Barcelona).

2.2 Hair sample analysis

A lock of scalp hair approximately 5 cm long was obtained, usually from the nape. The samples were coded and stored in sealed plastic bags until analysis. A minimum of 25 mg of hair was required. 25-50 mg of each sample were treated with HNO_3 (1 ml; Baker Instra) and H_2O_2 (0.5 ml; Merck Suprapur) in a Teflon vessel (90°C overnight). The digested sample was diluted with deionized water (8.5 ml; Purelab Ultra). One procedural blank was included in each sample batch for possible contamination control. THg determination was performed using inductively coupled plasma mass spectrometry (Agilent 7500 CE) operating under standard conditions and using rhodium as internal standard. All samples were above detection limit.

Certified reference materials from the International Atomic Energy Agency, IAEA-085 and IAEA-086, with 23.2 μg/g and 0.57 μg/g total mercury concentrations, respectively (Heller-Zeisler *et al.*, 1998), were used for validation and verification of the accuracy of the analytical method.

2.3 Covariates

Information on maternal and paternal socio-demographic characteristics, parity and maternal age were collected at recruitment. More data were obtained at birth (including time of gestation and smoking habits during pregnancy) and in follow-up surveys few months after delivery (such as type and duration of breastfeeding). Information on children's passive smoking at 4 years old, town of residence and dietary intake were gathered at the 4 years post-partum visit from food frequency questionnaires (FFQ).

The children's place of residence was classified into three categories: Ciutadella, Maó (the two main municipalities in the island) and other. Parity at child's birth was categorized as no siblings, one sibling and $\geqslant$2 siblings. Duration of breastfeeding was divided in 3 categories: <2 weeks (mainly formula-fed children), short-term (2-16 weeks) and long-term (>16 weeks). Time of gestation was divided into: 27-38 weeks, 39-40 weeks and 41-44 weeks. In some analyses, maternal age was divided in two categories: <30 years and >30 years.

According to the primary care register, all children followed the same vaccination program which precludes any potential role of Hg exposure through thimerosal-containing

Table 1: Characteristics of the study population according to participation in the mercury analysis (Menorca, Spain, 1997-2001).

	Children included[a] (n=302)	Children not included (n=180)	*p*-value [b]
Child			
Sex (%)			<0.01
Female	57	34	
Male	43	66	
Town of residence at 4 yr (%)			0.65
Ciutadella	45	48	
Maó	23	20	
Other municipalities	32	32	
Passive smoking at 4 yr (%)			0.90
No	49	48	
Yes	51	52	
Mother			
Age at delivery (%)			0.54
<30	55	52	
>30	45	48	
Parity at child's birth (%)			0.59
None	51	47	
One	36	41	
Two or more	13	12	
Breastfeeding (weeks) (%)			0.48
< 2	19	22	
2 – 16	25	28	
> 16	56	50	
Time of gestation (weeks) (%)			0.082
27-39	23	28	
39-40	56	45	
40-44	21	27	
Smoking during pregnancy (%)			0.92
No	79	79	
Yes	21	21	
Educational level (%)			0.53
Primary school or no education	60	56	
Secondary school or university	40	44	
Occupational status (%)			0.094
Unskilled, partially skilled or housewife	33	41	
Skilled	67	59	
Father			
Educational level (%)			0.70
Primary school or no education	68	66	
Secondary school or university	32	34	
Occupational status (%)			0.95
Unskilled or partially skilled	12	13	
Skilled	88	87	

[a] THg concentrations available.
[b] *p*-value from χ^2 (Chi-square) t-test.

vaccines in the variations in THg levels in this population.

2.4 Food frequency questionnaire

Children's dietary intake at 4 years old was assessed through a FFQ. The study design and FFQ methodology have been described elsewhere (Vioque and Gonzalez, 1991; Willett *et al.*, 1985). Fish intake was divided into 3 items: never or rarely consumers (<three times/month), 1-2 servings/week and >2 servings/week (frequent fish consumers). Information on the type of consumed fish was also available, differentiating between consumption of only white or mixed fish (both white and oily fish). Information on shellfish and squid intake was also available. These variables were divided into: never or rarely consumers (<1 month), 1-3 servings/month and ⩾1 servings/week. Total fish intake (fish, shellfish and squid) was divided into: < 2 servings/week, 2-3 servings/week and >3 servings/week.

2.5 Neuropsychological assessment

Cognitive and motor abilities of children at 4 years old were assessed using a standardized Spanish adaptation of the MSCA (McCarthy, 1972), which contained standardized test scores for five domains (quantitative, memory, verbal, perceptual-performance and motor). A general cognitive record for global intellectual function (Jacobson *et al.*, 1990) was calculated from the quantitative, verbal and perceptual-performance scores. Two neuropsychologists were trained to administer and interpret the MSCA tests.

2.6 Statistical analysis

THg levels were not normally distributed and were transformed into natural logarithms (log THg). For descriptive analysis, geometric mean (GM) of THg levels with 95% confidence intervals (CI) were used.

Statistical differences between covariates were tested for significance using the Chi-square test. Student's t-test or ANOVA were used to compare THg concentrations (in log scale) as a function of children's, maternal or paternal characteristics. P-values were considered at 95% and 99% thresholds.

Multivariate linear regression analyses were used to assess the association of covariates with THg concentrations according to this full model:

$$\begin{aligned} log_e(\text{THg}) = \alpha + \beta_1(\text{sex}) + \\ \beta_2(\text{municipality}) + \beta_3(\text{smoking pregnancy}) + \\ \beta_4(\text{passive smoking 4years}) + \beta_5(\text{maternal age}) + \\ \beta_6(\text{parity}) + \beta_7(\text{breastfeeding}) + \\ \beta_8(\text{gestational time}) + \beta_9(\text{maternal education}) + \\ \beta_{10}(\text{maternal social class}) + \beta_{11}(\text{paternal education}) + \\ \beta_{12}(\text{paternal social class}) + \beta_{13}(\text{fish intake}) + \\ \beta_{14}(\text{other seafood intake}) + \epsilon \end{aligned}$$

After applying a stepwise algorithm, the restricted model was defined by only including covariates from $\beta 1$ to β_6. Since all covariates were factors (dummy variables), the exponentials of the coefficients provided the odds ratios (OR) of the factor against the reference category.

MSCA neurodevelopment outcomes were standardized for the homogeneity of all scales, centring them at a mean of 100 (SD; 15). Multivariate regression models were also used to assess the association of each neurodevelopmental outcome and mercury concentrations. The models were adjusted by several other covariates, including total fish intake, breastfeeding, child's sex, parity, maternal and paternal educational levels, maternal and paternal social classes, place of residence, maternal age, psychologist and school term of evaluation.

Data analysis and graphics were conducted using the statistical program R (R Development Core Team, 2012).

3 Results

All studied four year-old children from Menorca showed detectable mercury concentrations in hair, ranging between 0.04 μg/g and 10 μg/g (Figure 1), with an arithmetical mean (AM) of 1.4 μg/g. The GM was 0.99 μg/g (95% CI between 0.90 μg/g and 1.1 μg/g; Table 2). Twenty percent of the samples were above the WHO recommended values (2 μg/g in human hair; Figure 1).

On univariate analysis THg concentrations were significantly higher among girls than boys ($p<0.01$; Table 2). Children living at Ciutadella (westernmost eastern end of the island) had significantly higher concentrations than those from other villages ($p<0.001$; Table 2). There were no differences between passive smokers and those not exposed to tobacco smoke.

Concerning maternal characteristics, GM values were significantly lower among children with two or more siblings than those with one or without siblings ($p<0.05$; Table 3). Mercury concentrations were significantly higher among children whose mothers had skilled occupational status ($p<0.01$; Table 3). Four year-old children whose mothers smoked during pregnancy showed significantly lower hair mercury concentrations ($p<0.05$; Table 3). Other maternal covariates such as age or educational level did not involve statistically significant THg concentration differences. Breastfeeding period or time of gestation were not significant for hair THg accumulation in these four year-old children. Paternal characteristics such as educational level or occupational status did not result into significant differences in THg concentrations (Table 3).

Study of the dependences between hair mercury concentrations and the FFQ food items only showed statistically significant associations with fish consumption. No other food group, e.g. chicken, vegetables, fruits, eggs, etc. was significantly associated with the accumulation of this pollutant (data not shown). GM THg levels in children consuming 1 or 2 servings of fish per week were two fold higher than in children who were not or rarely consumers (0.99 μg/g vs. 0.49 μg/g, respectively; Figure 2). Differences between fish type within these groups were not significant. However, among frequent fish consumers (more than two servings/week), children only eating white fish had significantly lower levels (GM 0.82 μg/g) than those eating mixed fish (both white and oily fish, GM 1.4 μg/g; Figure 3). Furthermore, children who were not consumers or rarely consumers of shellfish had significantly lower levels, 0.66 μg/g, than usual consumers, e.g. 1-3 servings/month (1.1 μg/g) or more than one serving per week (1.3 μg/g). The differences were also observed among squid consumers but were of lower magnitude. Children who were not consumers or rarely consumers had lower GMs, 0.81 μg/g, than those consuming 1-3 servings/month, 1.0 μg/g. The concentrations of those having one or more than one serving/week were 1.2 μg/g (Figure 2). In summary, total fish frequent consumers (3 or more servings/week of fish and other seafood) had significantly higher levels (1.4 μg/g) than children consuming 2-3 servings/week (0.96 μg/g) and more than twice higher concentrations than those consuming less than 2 servings/week (0.62 μg/g).

The results of the multivariate regression model are shown in Figure 2. In the general model (A), the characteristics remaining significant after simultaneous adjustment for total fish consumption and all other covariates were sex, place of residence, smoking exposure during gestation and maternal occupational status. After the stepwise algorithm, the variables that were selected for the final model were sex, parity, smoking during pregnancy and maternal occupational status (B). Place of residence lost its significance after

Table 2: Distribution of Total mercury (THg) concentrations in hair of four year-old children (μg/g) according to socio-demographic characteristics (Menorca, Spain, 1997-2001).

	THg (μg/g)		
Covariates	GM	95% CI	p-value
Total	0.99	0.90, 1.1	
Child's sex			<0.01
Female	1.1	0.99, 1.3	
Male	0.84	0.71, 0.99	**
Municipality of residence at 4 yr			<0.01
Ciutadella	1.2	1.0, 1.4	
Maó	0.80	0.67, 0.95	**
Other municipalities	0.91	0.76, 1.1	*
Passive smoking at 4 yr			0.94
No	0.99	0.85, 1.2	
Yes	0.99	0.87, 1.1	

* p-value<0.05; ** p-value<0.01 for t-test or ANOVA, with the first category as reference.
GM: geometric mean. CI: confidence intervals (95%).

stepwise algorithm and was therefore not included in the final model whereas parity at child's birth, which was not significant in the general model (A), was selected becoming significant at a 90% level (category '2 or more siblings' in relation to the category of 'children without siblings'). The variables related to fish and other seafood intakes were always significant in both general and final models (Figure 3).

The adjusted effect of total hair mercury concentrations on MSCA psychological outcomes is shown in Figure 4. No statistically significant associations were found between THg (in log scale) and child neurodevelopment.

4 Discussion

4.1 Mercury exposure

In general terms, the hair mercury levels of children from Menorca were lower than in many other previous studies on island populations, such as 7 year-old children from the Faroe Islands (GM 3.0 μg/g; Grandjean *et al.* (1997)), 5-6 year-old children from Seychelles Islands (MeHg AM 6.5 μg/g; Myers *et al.* (2000)) and 7 year-old children from the Madeira island (AM 4.1 μg/g; Murata *et al.* (2002)). The mercury concentrations from Menorca were also much lower than highly-exposed populations in the Amazon basin (Brazil; AM 10.2 μg/g; Malm *et al.* (2010)). However, the concentrations in Menorca were slightly higher than those found in many inland population studies, such as 6-16 year-old children from urban populations in Tarragona (Spain; GM 0.77 μg/g; Batista *et al.* (1996)) and 4 year-old children from Granada (Spain; GM 0.96 μg/g; Freire *et al.* (2010)), 8-10 year-old children from general population in Germany (GM 0.18 μg/g; Pesch *et al.* (2002)), 1-5 year-old children from the U.S. (GM 0.12 μg/g; McDowell *et al.* (2004)) and 3-5 year-old children from Canada (GM 0.66 μg/g; Tian *et al.* (2011)). An initial study on THg levels from the same infant population in Menorca but with a small number of cases (n=65) also

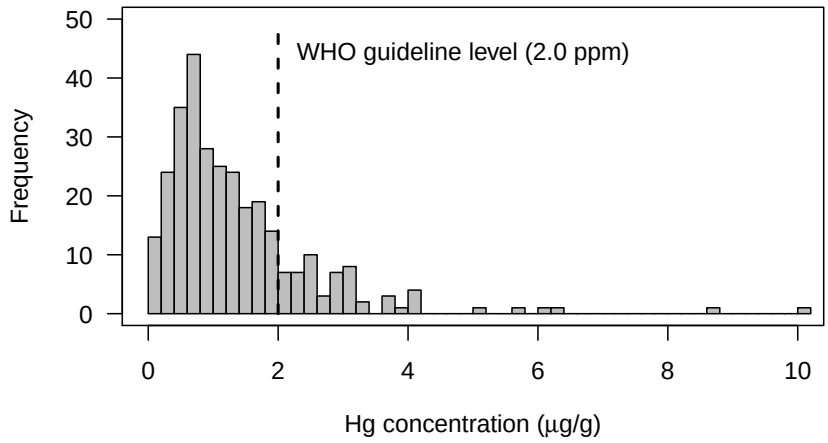

Figure 1: Distribution of mercury concentrations in hair in the infant population of Menorca (n=302). The WHO guideline level is indicated as reference.

showed lower levels than in the present study (AM 0.71 μg/g; Díez *et al.* (2009)).

20% of children from the Menorca cohort exhibited THg concentrations exceeding the WHO recommended level in human hair for exclusion of appreciable harm (2 μg/g; WHO/IPCS.). Similar proportions were found in preschool children from Canada ((Tian *et al.*, 2011)).

4.2 Mercury accumulation and fish consumption

A strong association between hair THg levels and total fish intake was found. This association is consistent with observations in other infant populations worldwide (Tian *et al.*, 2011; Myers *et al.*, 2009; Nadal *et al.*, 2005; Díez *et al.*, 2009; Freire *et al.*, 2010). Other reports have also shown positive correlations between maternal fish consumption and mercury cord blood levels (Grandjean *et al.*, 1997; Ramón *et al.*, 2008), including one on the risks of mercury-containing fish consumption from pregnant women and neurodevelopmental deficits in their children (Schoeman *et al.*, 2009).

In the present study differences in hair mercury levels as consequence of type of fish consumption were also established. Oily fish was found to be the main source for the higher mercury concentrations among frequent fish consumer children. These results are somewhat consistent with a study on 4 year-old children from Granada (n=72; Freire *et al.* (2010)) that also evaluated hair mercury levels by type of fish, finding a significant association between mercury levels and medium intake of oily fish (1-3 servings/month; $p<0.05$) but not with high intake ($\geqslant$ 1 serving/week). In contrast, in the present study the highest mercury concentrations were found among the most frequent fish consumer children (>2 servings/week) (Figure 2, left panel). The difference between the two cases may be due to the origin of oily fish. Granada is a main inland city with access to Atlantic and Mediterranean fish whereas in Menorca most fish is from

Table 3: Distributions of total mercury concentrations in hair of four year-old children (μg/g) according to maternal and paternal characteristics (Menorca, Spain, 1997-2001).

	THg (μg/g)		
Covariates	GM	95% CI	*p*-value
Maternal characteristics			
Age at delivery			0.79
*leqslant*30 years	0.98	0.89, 1.1	
>30 years	1.0	0.88, 1.1	
Number of siblings			<0.05
None	1.1	0.93, 1.2	
One	1.0	0.88, 1.2	
Two or more	0.67	0.50, 0.91	**
Breastfeeding (weeks)			0.44
<2	0.87	0.66, 1.1	
2-16	1.0	0.85, 1.2	
>16	1.0	0.90, 1.2	
Time of gestation (weeks)			0.28
27-38	1.0	0.86, 1.2	
39-40	0.98	0.85, 1.1	
41-44	0.93	0.75, 1.1	
Smoking during pregnancy			<0.05
No	1.1	0.94, 1.2	
Yes	0.79	0.65, 0.96	*
Educational level			0.16
Primary school or no education	0.94	0.82, 1.1	
Secondary school or university	1.1	0.93, 1.3	
Occupational status			%<0.01
Unskilled, partially skilled and housewife	0.79	0.68, 0.93	
Skilled	1.1	0.98, 1.3	**
Paternal characteristics			
Educational level			0.12
Primary school or no education	0.95	0.85, 1.1	
Secondary school or university	1.1	0.95, 1.3	
Occupational status			0.17
Unskilled or partially skilled	0.85	0.66, 1.1	
Skilled	1.0	0.92, 1.1	

*p-value<0.05; **p-value<0.01 for t-test or ANOVA, with the first category as reference.
GM: geometric mean. CI: confidence intervals (95%).

Mediterranean origin which has much higher mercury content than the former. The same strong dependence between total fish consumption and hair mercury levels has been observed in mothers from the Aegean Islands (Gibičar *et al.*, 2006) or in infants (cord blood concentrations) from Valencia, a coastal city on the Mediterranean (Ramón *et al.*, 2008). In these two sites, consumed fish is mainly of Mediterranean origin.

Hair mercury concentrations in the Menorca infants were also significantly associated with consumption of Mediterranean shellfish and squid (Figure 3). These results are consistent with those observed in Valencia, where most shellfish is of Mediterranean origin (Ramón *et al.*, 2008). Conversely, no associations between shellfish consumption and mercury levels have been observed in Flix (Catalonia, Spain), an inland village receiving food from diverse origins (Montuori *et al.*, 2006). As in the case of total fish consumption mentioned above, shellfish of Mediterranean origin appears to be an important vector for the introduction of mercury pollution into children.

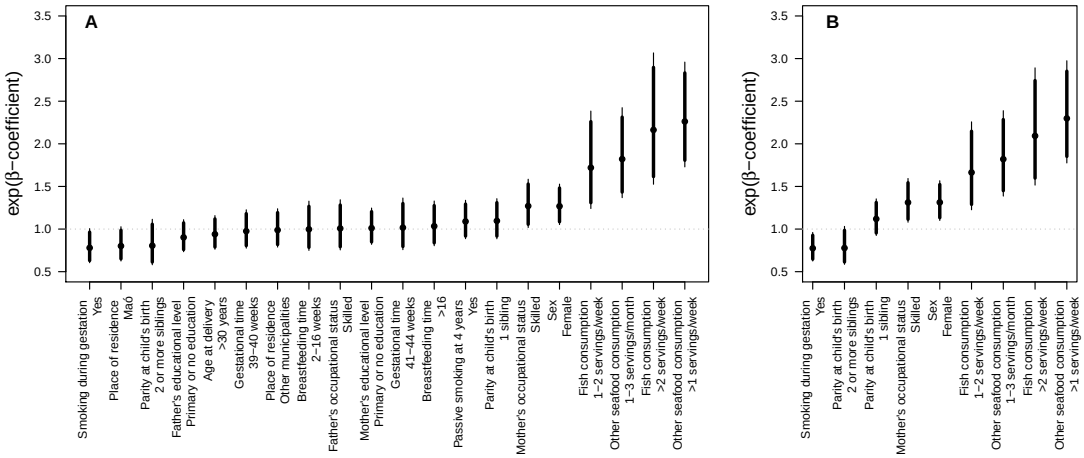

Figure 2: Mercury concentrations in hair of four year-old children (μg/g) by fish consumption. Total fish values are the sum of fish, shellfish and squid consumption values. Bars represent geometric means (GM) and 95% confidence intervals (CI). Sv: servings.

4.3 Parity and maternal mercury transfer

Parity was significantly associated with mercury concentrations ($p<0.05$), being lower in children with two or more siblings than in those with one or no siblings (Table 3). However, the opposite trend was reported in hair THg concentrations from preschool children from Granada in which children with two or more siblings showed higher THg concentrations than those without or less than two siblings (Freire *et al.*, 2010) but in this case the number of individuals was small (n = 72). In one study reporting maternal mercury concentrations in hair from French Guiana (Cordier *et al.*, 2002) decreases or increases of THg levels with higher parity were identified in different sites, again the number of individuals in each site was small (n=63-90). Dependence from parity has also been observed in the accumulation of other hydrophobic pollutants in newborns, e.g. DDTs in cord blood from Manhiça, in which number of siblings was the strongest determinant of pollution concentration in newborns (Manaca *et al.*, 2012).

Gestation and child delivery may represent an excretion way for women, decreasing their mercury burden. No associations were found between breastfeeding and hair mercury concentrations in the infant cohort of Menorca (Figure 3). Thus, mercury incorporated into newborns in their first months of life had no significant influence on the body burden at 4 years of age. Thereby, the transference of mercury from mother to foetus seems to have a higher influence than breastfeeding.

4.4 Parental socio-demographic characteristics and mercury accumulation

Paternal variables did not show significant associations with mercury accumulation in children (Figure 3). No statistically significant correlations with maternal or and gestational time were observed (Table 3, Figure 3) which is in agreement with the results

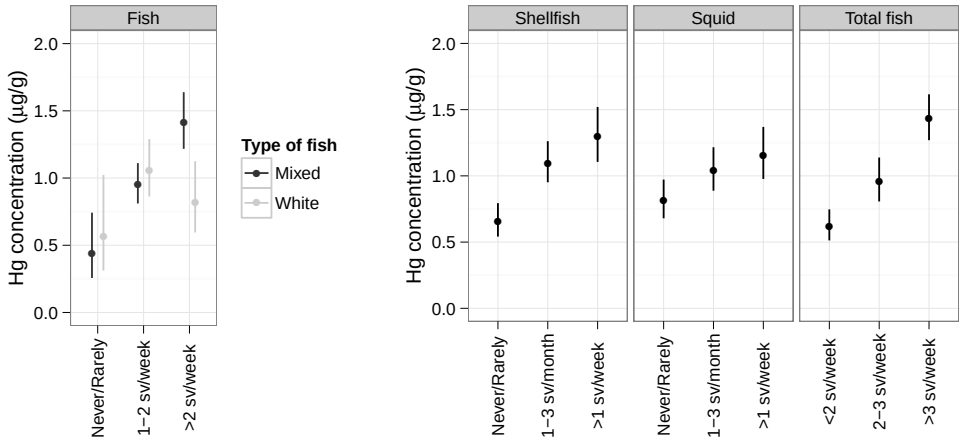

Figure 3: Associations between total mercury concentrations in hair of four year-old children and several covariates. Bars represent the 90% and 95% confidence intervals, shown in thick and thin lines, respectively. Panel A) Model adjusted for maternal age, smoking during pregnancy, gestational time, breastfeeding, place of residence, parity, passive smoking at 4 years, maternal and paternal occupation and educational level, child's sex, fish and other seafood consumption. Panel B) Model with the statistically significant covariates after application of the stepwise algorithm. The reference status represents a boy without siblings, which rarely consumes fish or other seafood, whose mother did not smoke during pregnancy and who has an unskilled or partially skilled occupation.

from other cohorts (Kim *et al.*, 2008; Freire *et al.*, 2010). However, an association between mercury concentrations in children's hair and maternal age has been reported in one case (Freire *et al.*, 2010).

Maternal educational level was not associated with mercury accumulation in children but the maternal occupational status was significantly related to THg levels. Children whose mothers had skilled abilities had significant higher levels than those whose mothers were unskilled, partially skilled or housewife (Table 3, Figure 3). Differences in dietary patterns may perhaps explain this difference. Higher mercury concentrations in children may be related with higher fish consumption, as people with higher economic level tend to incorporate a higher proportion of fish protein in their diet (Darmon and Drewnowski, 2008). In fact, the interaction between occupational status and total fish consumption was significant (data not shown).

Children exposed to tobacco during gestation showed lower mercury levels than in unexposed children (Figure 3). No difference in mercury concentrations was found between passive smokers and children unexposed to tobacco. The lower concentrations in tobacco-exposed children are consistent with the observations from another study on mothers from Valencia in which lower THg concentrations in cord blood from smoker mothers were found (Ramón *et al.*, 2008). In this case no data on children's hair was available. Another study conducted in older children (6-16 years of age) could not find significant correlations between maternal smoking habits and mercury levels (Batista *et al.*, 1996). Other studies have reported opposite results, since positive correlations between cigarette smoking and mercury content in hair have also been found (Freire *et al.*, 2010; Pesch *et al.*, 2002) being justified by the elemental mercury content of tobacco smoke (Rickert

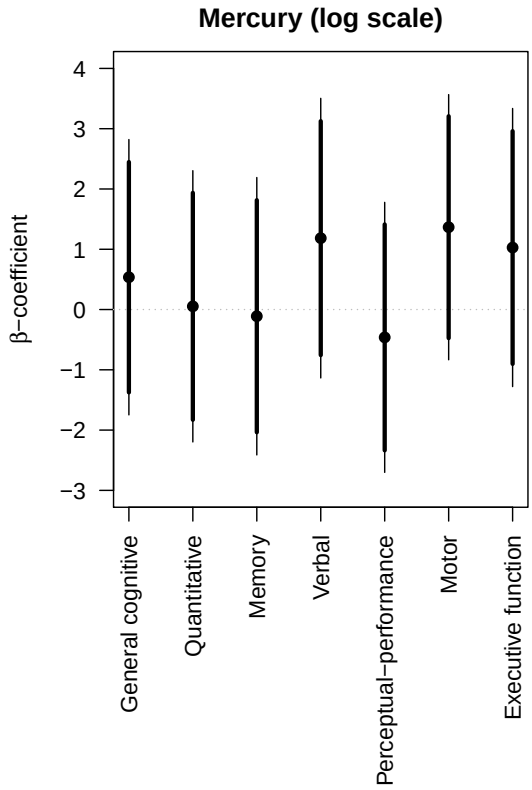

Figure 4: Graphical representation of the association between neurodevelopmental outcomes and mercury concentrations. β-coefficients from each outcome comes from a different multivariate model with THg levels on log-transformed scale. All the models have been adjusted for child's sex, parity, breastfeeding time, maternal age, maternal and paternal educational level and social classes, place of residence, total fish intake, school term of evaluation and psychologist. Bars represent the 90% and 95% confidence intervals, shown in thick and thin lines, respectively.

and Kaiserman, 1994).

4.5 Sexual differences in mercury accumulation

Higher mercury concentrations were found in girls. The difference was statistically significant after controlling for other covariates. Some previous studies have also reported higher mercury concentrations in female children (Batista *et al.*, 1996; Al-Saleh and Al-Sedairi, 2011), as well as in women from adult populations (Babi *et al.*, 2000). However, in other cases higher concentrations in male than female children have been found (Kim *et al.*, 2008; Díez *et al.*, 2008; Olivero *et al.*, 2002). A study on mice suggested that gender and genetic factors may contribute to mercury retention and whole-body accumulation (Ekstrand *et al.*, 2010).

A study on organochlorine compounds (OCs) in serum from the same infant population of Menorca found higher concentrations in female than male children (Grimalt *et al.*, 2010). Consistent results are therefore observed between mercury and OCs. In the OC study, female children were indicated to have higher retention capacity for the incorporation of these pollutants through breast-

feeding (Grimalt *et al.*, 2010). In the case of mercury, breastfeeding is not a significant ingestion route. However, the agreement in gender differences for the concentrations of both types of pollutants shows a consistent pattern. Significant gender differences in urinary mercury concentrations in dental amalgam treated children have been found, involving higher levels in girls than in boys (Woods *et al.*, 2007). This previous study is only quoted as one significant example. Obviously, it is unlike that dental amalgams can be potential sources of mercury contamination in four year-old children. In the present study, the observed results suggest higher retention capacity of female than male children for mercury ingestion as consequence of fish consumption.

4.6 Associations with neurodevelopment

No significant associations have been found between mercury exposure and measured neurodevelopmental outcomes irrespectively of adjusting or not adjusting for fish consumption (Figure 4). Significant associations between low neurodevelopment and high prenatal mercury exposure have been reported previously in studies on preschool children (Oken *et al.*, 2008; Lederman *et al.*, 2008) and 7 year-old children (Grandjean *et al.*, 1997). However, postnatal mercury exposure had not been associated with neurodevelopmental outcomes (Murata *et al.*, 2002; Palumbo *et al.*, 2000). Only one study which measured mercury levels in 4 year-old children found a significant association between lower scores in MSCA general cognitive, memory and verbal areas and this metal (Freire *et al.*, 2010). In this case, THg levels were divided in two categories with a threshold of $\geqslant 1$ μg/g. However, no association was found when the mercury concentrations were expressed as a continuous logarithmic variable. Moreover, this study was conducted in a relatively low sample size population (n=72) which is in contrast with the present study, which comprised 298 preschool children.

5 Conclusions

Mercury levels in four year-old children from the Mediterranean cohort of Menorca ranged within the high concentration interval when compared with other children populations worldwide, except for highly-exposed areas such as Amazonia (Brazil) and island populations, e.g. Faroe, Madeira or the Seychelles. The mercury distribution in Menorca was characterized by 20% of children with hair THg concentrations exceeding the WHO reference level for no effects. Fish and other seafood intake were observed to be the only diet component associated with mercury accumulation. Information collected on heavy fish consumers showed that oily fish was the main source of this pollutant but total fish consumption, shellfish and squid consumption were also associated to high THg concentrations in these children. Higher levels were found in female than male children which was consistent with gender differences previously observed in the accumulation of OCs in serum. No significant associations between children's cognitive abilities and mercury levels were observed.

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Discussion

Mercury is well known as a major persistent pollutant, yet research-based knowledge has not been meet with policy and regulatory action at the required level. The most recent international agreement on mercury, namely the *Minamata Convention on Mercury*, was just adopted in January 2013, signed by 140 countries (UNEP, INC5, 2013). It provides global–level control and reduction measures across a range of products, processes and industries where mercury is used, released or emitted.

This agreement came precisely few days after the release of a UNEP report on the matter, the *Global Mercury Assessment*, that showed concerning data about increasing mercury levels and emissions in ocean waters, related to highly polluting human activity (UNEP, 2013).

The present research has precisely confirmed that the greatest source of human body burdens of mercury is the fish and seafood consumption, thus endorsing the importance of the mentioned international agreement to prevent emissions and releases of this compound as well as the need to continue monitoring and regulating mercury emissions and exposure.

In particular, this research, as summarized in Figure A.4, shows that the overall fish consumption is the most relevant factor to explain the mercury levels among 4-year old children from Menorca (15% of total variability, far ahead from the second factor).

Although Spain is in general a highly fish-consumer country (Welch *et al.*, 2002), the population of the Menorca island has easy access to local fish and probably higher consumption level, making the levels of mercury in the Menorca population higher than in other Spanish locations, as shown in Table A.1 (Batista *et al.*, 1996; Freire *et al.*, 2010).

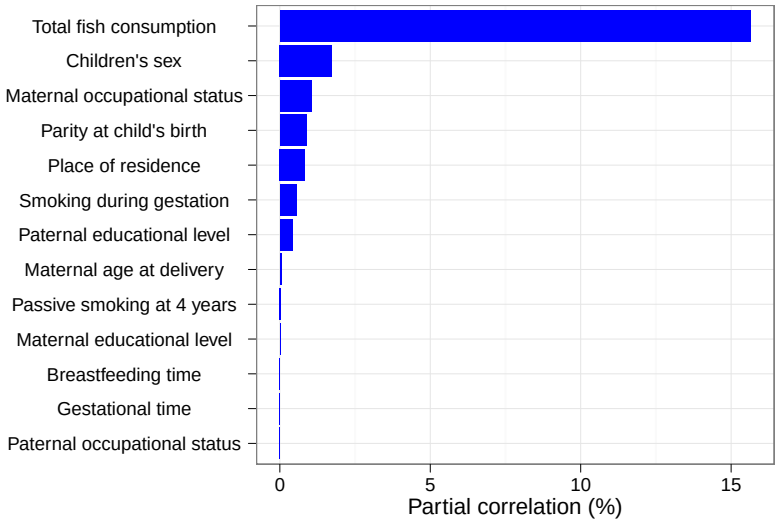

Figure A.4: Percentage contribution to total variability in log-transformed concentration of mercury in children, explained by several covariates in multivariate regression model.

When compared globally, the mercury levels in the studied infant population ranked high among the infant populations in the world so far analysed (Table A.1) (Pesch *et al.*, 2002; Al-Saleh and Al-Sedairi, 2011; McDowell *et al.*, 2004; Tian *et al.*, 2011). However, the levels are lower compared to those found in other island populations, which are prone to high fish consumption (*e.g.* Faroe, Madeira and the Seychelles islands; (Grandjean *et al.*, 1997; Murata *et al.*, 2002; Myers *et al.*, 2000a)), yet clearly surpassed by the levels found in areas highly exposed to mercury (such as Amazonia, in Brazil, due to widespread mercury use in mining; (Malm *et al.*, 2010)).

In addition to dietary habits (particularly fish and seafood consump-

Country	N	Age	Mean	GM	Reference
Menorca, Spain	302	4	1.4	0.99	Present study
Tarragona, Spain	233	6-16		0.77	Batista *et al.* (1996)
Granada, Spain	72	4		0.96	Freire *et al.* (2010)
Germany	245	8-10	0.23	0.18	Pesch *et al.* (2002)
Saudi Arabia	171	5-15	0.45		Al-Saleh and Al-Sedairi (2011)
US	838	1-5	0.22	0.12	McDowell *et al.* (2004)
Canada	388	3-5		0.66	Tian *et al.* (2011)
Brazil (Amazonia)	51	NR	12.4		Malm *et al.* (2010)
Faroe Islands	527	1		1.1	Grandjean *et al.* (1997)
	903	7		3.0	Grandjean *et al.* (1997)
Seychelles islands	708	5-6	6.5		Myers *et al.* (2000b)
Madeira island	113	7	4.1		Murata *et al.* (2002)

Table A.1: Comparison of mercury concentrations (μg/g) in children's hair from different populations worldwide.

tion), there are a number of other socio-demographic factors that explain the accumulation of mercury in this infant population: sex, place of residence, number of previous siblings and the maternal occupational status, among others (Figure A.4). Nevertheless, all those other factors, compiled together, contribute less than 5% to explain the total variability, in contrast with the fish consumption which, alone, explains the 15% of total variability.

When compared with other persistent pollutants, sexual differences in mercury levels in the Menorca population are consistent with those found in other persistent pollutants, particularly organochlorine compounds, in the same population, as already shown in the SUPPLEMENTARY RESEARCH ARTICLES (Annex III; Grimalt *et al.* (2010)).

This research seems unable to reveal the effects of moderate levels of mercury exposure in the infant neurodevelopment process, as already dis-

cussed in ARTICLE 5. Although infant neurological disorders are evident under extremely high levels of exposure to mercury (Harada, 1995), research has been unable to demonstrate neurodevelopmental impact when the levels of exposure to mercury are moderate (Murata *et al.*, 2002; Palumbo *et al.*, 2000; Freire *et al.*, 2010). This is equally the case of the present research, conducted on 300 children from a population that is moderately exposed to mercury, which has not been able to find associations between hair mercury concentrations and neurodevelopmental outcomes according to the MSCA test.

Conclusions

On the levels of mercury in the infant population of Menorca. In Menorca island, children show detectable levels of mercury (from hair samples). Their concentrations are rather high when compared with other populations, yet concur with higher levels in island populations, and certainly lower than in highly-exposed areas.

On the dietary habits and socio-demographic and maternal factors. The consumption of fish, which predominates in the diets of island populations, is clearly the leading factor explaining the degree of accumulation of mercury found. It is also the single most important factor: it explains 15% of all the variability, whereas the rest of the factors studied represent, all together, just 5%. Oily fish is the main source of this pollutant, yet intakes of other types of fish and seafood, including squid and shellfish, which are frequent resources in Menorca island, also contribute to the body burden of mercury.

On the relationships with neurodevelopment. The research was unable to show any association between the levels of mercury and adverse neurodevelopmental outcomes in the studied children. This anyway concurs with existing research, which only shows clear association among these two variables when the levels of exposure to mercury are severe, such as in heavily polluted locations.

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ANNEXES

Annex I

Supplementary Research Article

Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Catalonia

Porta M, Gasull M, Puigdomènech E, Garí M, Bosch de Basea M, Guillén M, López T, Bigas E, Pumarega J, Llebaria X, Grimalt JO, Tresserras R. Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Catalonia. Environ Int. 2010 Oct;36(7):655-64.

Annex II

Supplementary Research Article

Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Barcelona in 2006, and comparison with levels in 2002

Porta M1, López T, Gasull M, Rodríguez-Sanz M, Garí M, Pumarega J, Borrell C, Grimalt JO. Distribution of blood concentrations of persistent organic pollutants in a representative sample of the population of Barcelona in 2006, and comparison with levels in 2002. Sci Total Environ. 2012 Apr 15;423:151-61.

Annex III

Supplementary Research Article

An evaluation of the sexual differences in the accumulation of organochlorine compounds in children at birth and at the age of 4 years

Grimalt JO1, Carrizo D, Garí M, Font-Ribera L, Ribas-Fito N, Torrent M, Sunyer J. An evaluation of the sexual differences in the accumulation of organochlorine compounds in children at birth and at the age of 4 years. Environ Res. 2010 Apr;110(3):244-50.

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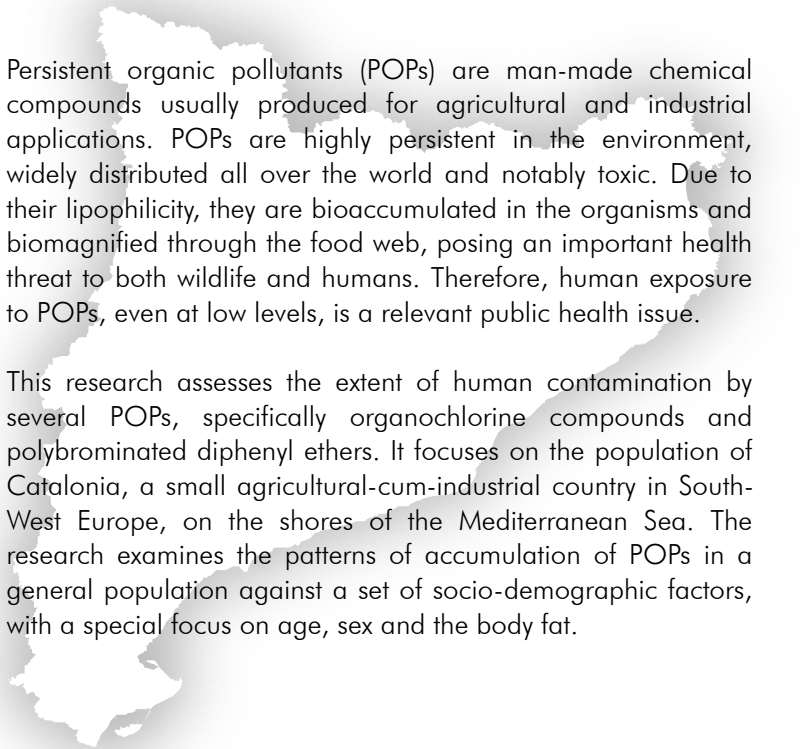

Persistent organic pollutants (POPs) are man-made chemical compounds usually produced for agricultural and industrial applications. POPs are highly persistent in the environment, widely distributed all over the world and notably toxic. Due to their lipophilicity, they are bioaccumulated in the organisms and biomagnified through the food web, posing an important health threat to both wildlife and humans. Therefore, human exposure to POPs, even at low levels, is a relevant public health issue.

This research assesses the extent of human contamination by several POPs, specifically organochlorine compounds and polybrominated diphenyl ethers. It focuses on the population of Catalonia, a small agricultural-cum-industrial country in South-West Europe, on the shores of the Mediterranean Sea. The research examines the patterns of accumulation of POPs in a general population against a set of socio-demographic factors, with a special focus on age, sex and the body fat.

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